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# ANALYSIS OF BENZODIAZEPINES

## I. CHROMATOGRAPHIC IDENTIFICATION

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SUMMARY

The chromatographic mobilities of nineteen benzodiazepines were determined by thin-layer, gas–liquid, high-resolution gas–liquid and high-performance liquid chromatography. The results were correlated with literature data.

## INTRODUCTION

The number and frequency of requests made to toxicologists for analyses of blood and plasma samples for benzodiazepines are increasing dramatically, and therapeutic monitoring and screening of drug addicts are of interest in preventive medicine and forensic science[1,2]; these substances are also frequently found in judicial exhibits. Therefore, a reliable method for the identification of these compounds in biological samples and confiscated drugs is necessary.

Immunochemical techniques (EMIT and RIA)[3], generally employed as screening tests, are able to identify the presence of this class of compounds at microgram levels, but they do not discriminate between different commercial benzodiazepines; moreover quantitative analysis is difficult because the responses of different benzodiazepines and their metabolites towards the antigen-antibody reaction (EMIT-dau) are different[4]. The use of more specific techniques is thus necessary for identifying benzodiazepines and their metabolites accurately for correct pharmacological monitoring and for forensic purposes.

Thin-layer chromatography (TLC) has been proposed[5,6], but most workers prefer reliable methods based on gas–liquid chromatography (GLC)[7–11] and high-performance liquid chromatography (HPLC)[12–16]. The latter method is suitable for thermally labile substances such as some benzodiazepines. GLC techniques generally involve hydrolytic steps, owing to the formation of the same benzophenones from several different benzodiazepines[17,18], so that the individual parent drugs cannot be readily identified; however, some workers have separated intact benzodiazepines by GLC[19–21].

Published methods for benzodiazepine analysis have usually involved only two

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or three compounds and a few data on the chromatographic behaviour of several benzodiazepines and their benzophenones simultaneously with different techniques have been reported[15,22–24].

The purpose of this work was to examine the possibility of screening nineteen benzodiazepines currently available in Italy by different chromatographic techniques (TLC, GLC and HPLC) and to establish the sensitivity limits for each substance.

## EXPERIMENTAL

### *Standards and chemicals*

Pure chemical standards of chlordiazepoxide, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam and temazepam were obtained from Hoffman-La Roche (Nutley, NJ, U.S.A.). Bromazepam, camazepam, chlordesmethyldiazepam, clobazam, clorazepate, desmethyldiazepam, flurazepam, lormetazepam, medazepam, pinazepam and prazepam were obtained from commercial products by methanol extraction.

Benzophenones were obtained by acid hydrolysis of the parent benzodiazepines according to Berry and Grove[25].

Azinphos-methyl{phosphorodithioic acid O,O-dimethyl S-[4-oxo-1,2,3-benzotriazin-3(4*H*)-yl]methyl ester} was employed as an internal standard (I.S.) in HPLC.

Methylclonazepam, obtained by methylation of clonazepam with diazomethane[26] was used as the internal standard for high-resolution capillary gas chromatography (HRGC).

Other chemicals and reagents were of UV grade.

### *Thin-layer chromatography*

Pre-coated silica gel 60 $F_{254}$ fluorescent plates (20 cm × 20 cm) (Merck) were used.

Five eluents were tested for the analysis of benzodiazepines: (A) benzene–2-propanol–30% ammonia solution (85:15:1); (B) ethyl acetate–methanol–30% ammonia solution (85:10:5); (C) toluene–acetone–ethanol–30% ammonia solution (45:45:7:3); (D) chloroform–acetone (90:10); and (E) ethyl acetate–cyclohexane–30% ammonia solution (50:40:0.1).

$R_F$ values according to Moffat[27] were calculated only for eluents D and E, which gave better separations, using diazepam, clonazepam, pinazepam and oxazepam as reference compounds for eluent D and diazepam, lorazepam, pinazepam and bromazepam for eluent E.

Two eluents were also tested for the analysis of benzophenones: (D) Chloroform–acetone (90:10) and (F) benzene–acetic acid (97:3).

After development for 10 cm the plates were observed under UV light and sprayed with Dragendorff and Bratton–Marshall reagents[25].

### *Gas–liquid chromatography*

A Carlo Erba Fractovap 4200 gas chromatograph was used. Retention indices (RI)[28] were calculated in the isothermal mode (240, 260 and 280°C) on three different packed columns (2 m × 4 mm I.D.) for benzodiazepine standards: (a) 3% GP 2100

DB on 100–120 mesh Supelcoport; (b) 1% Dexsil 300 on 100–120 mesh Chromosorb W; and (c) 3% SE-30 on 100–120 mesh Chromosorb W.

The injector and flame ionization detector temperatures were set 10°C above the oven temperature. Helium was used as the carrier gas at a flow-rate of 30 ml/min.

*High-resolution gas chromatography*

A Carlo Erba Fractovap HRGC gas chromatograph equipped with both split–splitless and on-column injectors, flame ionization and electron-capture detectors (10 mCi Ni-63, operated in the pulsed mode) and a temperature programmer was used.

The study of gas chromatographic mobility was performed on three different columns: (a) a 25-m glass capillary column with a 0.4 μm film of SE-54 stationary phase, using hydrogen as carrier gas at a flow-rate of 3 ml/min, temperature programming (from 100 to 220°C in the ballistic mode, hold for 1 min at 220°C, then heat to 270°C at 7°C/min; finally hold at 270°C for 15 min) and a detector temperature of 300°C; (b) a wide-bore SPB-5 glass capillary column (30 m × 0.75 mm I.D.) with bonded SE-54 stationary phase in the isothermal mode at 220°C using helium as the carrier gas at a flow-rate of 5 ml/min; and (c) wide-bore SPB-1 glass capillary column (30 m × 0.75 mm I.D.) with bonded SE-30 stationary phase, using helium at a flow-rate of 5 ml/min as the carrier gas, temperature programming (hold for 2 min at 200°C, heat to 285°C at 4°C/min, then 10 min in the isothermal mode) and a detector temperature of 300°C.

*High-performance liquid chromatography*

A Perkin-Elmer 3B liquid chromatograph equipped with LC 75 autocontrol set at 254 nm was employed.

The chromatographic separation of benzodiazepines and benzophenones was performed in the reversed-phase mode using a Perkin-Elmer 3-μm HS $C_{18}$ column (7 cm long) in the isocratic mode with two different elution systems:

(a) methanol–water (70:30) at a flow-rate of 0.5 ml/min and

(b) 5 m*M* phosphate buffer (pH 6)–methanol–acetonitrile (57:17:26) at a flow-rate of 1.5 ml/min.

## RESULTS AND DISCUSSION

*Thin-layer chromatography*

$R_F$ distributions (means of ten replicate analyses) of all benzodiazepines for the five eluents investigated are shown in Fig. 1.

Of the eluent systems tested, D and E gave the best chromatographic separations.

Tables I and II give mobility data for benzodiazepines (only for the chosen eluents) and for benzophenones (eluents D and F).

Table II gives $R_F$ values with two different detection techniques because some of the compounds show two or three spots detectable with UV light, whereas Bratton–Marshall reagent does not react with all of them (*e.g.*, flunitrazepam and desmethyldiazepam).

The results show that TLC allows the easy identification of benzodiazepines

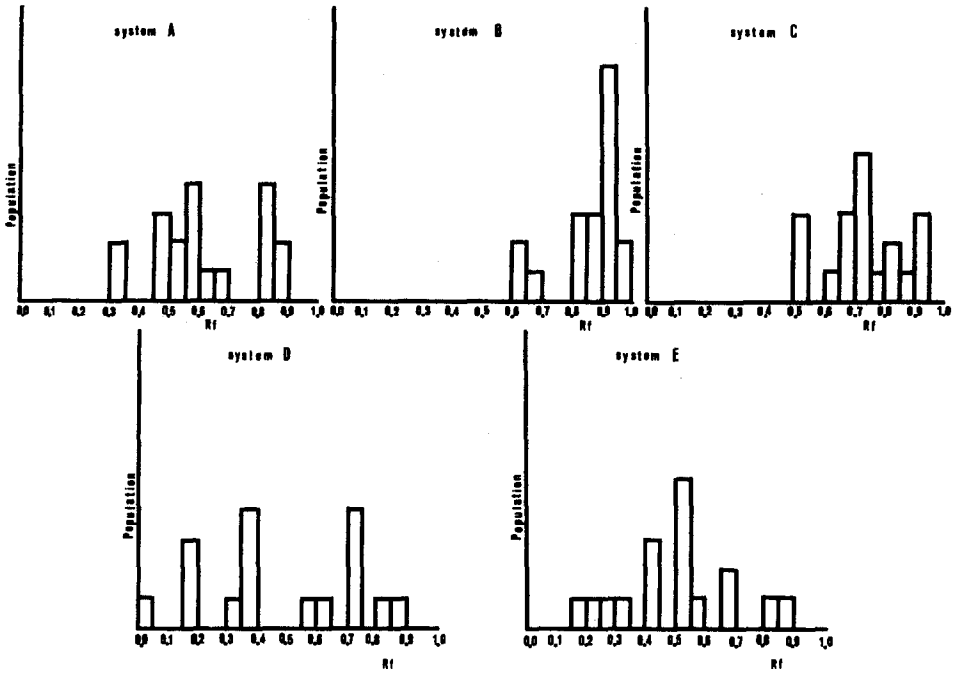


Fig. 1. Frequency distributions of $R_F$ values of nineteen benzodiazepines using eluent systems A, B, C, D and E (see text).

TABLE I

$R_F$ VALUES FOR BENZODIAZEPINES USING ELUENTS D AND E

Detection with Dragendorff reagent and UV light.

| *Compound* | $R_F$ | |
|---|---|---|
| | *D* | *E* |
| Bromazepam | 0.20 | 0.19 |
| Camazepam | 0.75 | 0.32 |
| Chlordesmethyldiazepam | 0.39 | 0.51 |
| Chlordiazepoxide | ND* | ND |
| Clobazam | 0.72 | 0.52 |
| Clonazepam | 0.40 | 0.42 |
| Clorazepate | 0.38 | 0.55 |
| Desmethyldiazepam | 0.37 | 0.56 |
| Diazepam | 0.75 | 0.70 |
| Flunitrazepam | 0.74 | 0.52 |
| Flurazepam | 0.00 | ND |
| Lorazepam | 0.20 | 0.25 |
| Lormetazepam | 0.60 | 0.45 |
| Medazepam | 0.76 | 0.68 |
| Nitrazepam | 0.35 | 0.45 |
| Oxazepam | 0.17 | 0.26 |
| Pinazepam | 0.86 | 0.89 |
| Prazepam | 0.82 | 0.83 |
| Temazepam | 0.62 | 0.53 |

* ND = not determined.

TABLE II

$R_F$ VALUES FOR BENZOPHENONES USING ELUENTS D AND F

| *Compound* | $R_F$ | | | |
|---|---|---|---|---|
| | *D* | | *F* | |
| | *UV light* | *Bratton–Marshall*[25]* | *UV light* | *Bratton–Marshall*[25]* |
| Bromazepam | | | | |
| Camazepam | 0.60 | | 0.06 | |
| | 0.96 | | 0.25 | |
| | | | 0.87 | |
| Chlordesmethyldiazepam | | | | |
| Chlordiazepoxide | 0.93 | 0.93 (v) | 0.24 | 0.55 (v) |
| | | | 0.55 | |
| Clobazam | 0.64 | | 0.07 | |
| Clonazepam | 0.88 | 0.88 (p) | 0.35 | 0.35 (p) |
| | 0.91 | | 0.73 | 0.73 (p) |
| | | | | 0.92 (p) |
| Clorazepate | 0.92 | 0.92 (v) | 0.53 | 0.53 (v) |
| Desmethyldiazepam | 0.93 | 0.93 (v) | 0.21 | 0.51 (v) |
| | | | 0.51 | |
| Diazepam | 0.97 | | 0.82 | 0.53 (v) |
| Flunitrazepam | 0.88 | 0.88 (p) | 0.36 | 0.36 (p) |
| | 0.95 | | 0.67 | |
| Flurazepam | 0.93 | 0.93 (v) | 0.61 | 0.61 (v) |
| Lorazepam | 0.90 | 0.94 (v) | 0.18 | 0.69 (v) |
| | 0.94 | 0.98 (v) | 0.69 | |
| Lormetazepam | 0.62 | | 0.05 | 0.05 (v) |
| | 0.96 | | 0.87 | |
| Medazepam | 0.88 | | 0.19 | 0.84 (v) |
| | 0.96 | | 0.24 | |
| | | | 0.84 | |
| | | | 0.97 | |
| Nitrazepam | 0.00 | 0.88 (p) | 0.00 | 0.30 (r) |
| | 0.88 | | 0.30 | 0.70 (p) |
| | 0.96 | | 0.70 | |
| Oxazepam | 0.93 | 0.93 (v) | 0.20 | 0.52 (v) |
| | 0.99 | | 0.24 | 0.90 (v) |
| | | | 0.52 | |
| | | | 0.90 | |
| Pinazepam | 0.96 | 0.96 (v) | 0.85 | 0.54 (v) |
| | | | | 0.85 (v) |
| Prazepam | 0.75 | | 0.10 | 0.54 (v) |
| | 0.97 | | 0.91 | |
| Temazepam | 0.65 | | 0.06 | 0.06 (v) |
| | 0.96 | | 0.25 | 0.53 (v) |
| | | | 0.85 | |

* p = pink; r = red; v = violet.

with the systems chosen when chromatography of benzophenones is carried out too, notwithstanding its low resolving power compared with GLC and HPLC. In this instance it is possible to discriminate between those groups of compounds not completely resolved with only one chromatographic system (eluent D or E). For example, flunitrazepam and clobazam migrate with similar $R_F$ values in both eluents D and

TABLE III

GC RETENTION INDICES

| *Compound* | *This work* | | | *Literature data* | |
|---|---|---|---|---|---|
| | *3% GP SP 2100 DB* | *Dexsil 300* | *3% SE-30* | *Ref. 29* | *Ref. 23* |
| Bromazepam | Neg.* | 2893 | Neg. | 2663 | 2670 |
| Camazepam | 3111 | 3191 | 2946 | ND | ND |
| Chlordesmethyldiazepam | ND** | ND | 2592 | ND | ND |
| Chlordiazepoxide | 2689 | 2636 | 2808 | 2530 | 2845 |
| Clobazam | 2773 | 2797 | 2559 | ND | 2660 |
| Clonazepam | Neg. | 3174 | Neg. | 2885 | 2965 |
| Clorazepate | 2701 | 2759 | Neg. | ND | 2655 |
| Desmethyldiazepam | 2794 | 2740 | 2502 | 2496 | 2555 |
| Diazepam | 2561 | 2641 | 2448 | 2425 | 2490 |
| Flunitrazepam | 2803 | 2883 | 2607 | 2645 | 2645 |
| Flurazepam | 2841 | 2960 | 2790 | 2785 | 2800 |
| Lorazepam | ND | 2601 | 2430 | 2402 | 2440 |
| Lormetazepam | ND | ND | 2491 | ND | ND |
| Medazepam | Neg. | 2871 | 2270 | 2226 | 2285 |
| Nitrazepam | Neg. | 3111 | 2746 | 2750 | 2830 |
| Oxazepam | 2503 | 2529 | 2354 | 2336 | 2380 |
| Pinazepam | 2683 | 2691 | 2529 | ND | ND |
| Prazepam | 2756 | 2868 | 2656 | 2641 | 2715 |
| Temazepam | ND | ND | ND | ND | 2630 |

* Neg. = no peak between 1200 and 3200.
** ND = not determined.

TABLE IV

HRGC RETENTION INDICES

| *Compound* | *Stationary phase* | | |
|---|---|---|---|
| | *SPB-1* | *SPB-5* | *SE 54* |
| Bromazepam | 2510 | 2662 | 2658 |
| Camazepam | 2935 | 3036 | 3059 |
| Chlordesmethyldiazepam | 2552 | 2647 | 2700 |
| Chlordiazepoxide | 2703 | 2882 | 2855 |
| Clobazam | 2557 | 2605 | 2650 |
| Clonazepam | 2814 | 2879 | 2945 |
| Clorazepate | ND* | ND | 2588 |
| Desmethyldiazepam | 2426 | 2549 | 2591 |
| Diazepam | 2434 | 2492 | 2531 |
| Flunitrazepam | 2577 | 2661 | 2707 |
| Flurazepam | 2703 | 2809 | 2926 |
| Lorazepam | 2391 | 2466 | 2484 |
| Lormetazepam | 2603 | 2717 | 2753 |
| Medazepam | ND | 2674 | ND |
| Nitrazepam | 2718 | 2820 | 2865 |
| Oxazepam | 2314 | 2386 | 2436 |
| Pinazepam | 2505 | 2572 | 2604 |
| Prazepam | 2619 | 2685 | 2712 |
| Temazepam | 2517 | 2637 | 2684 |

* ND = not determined.

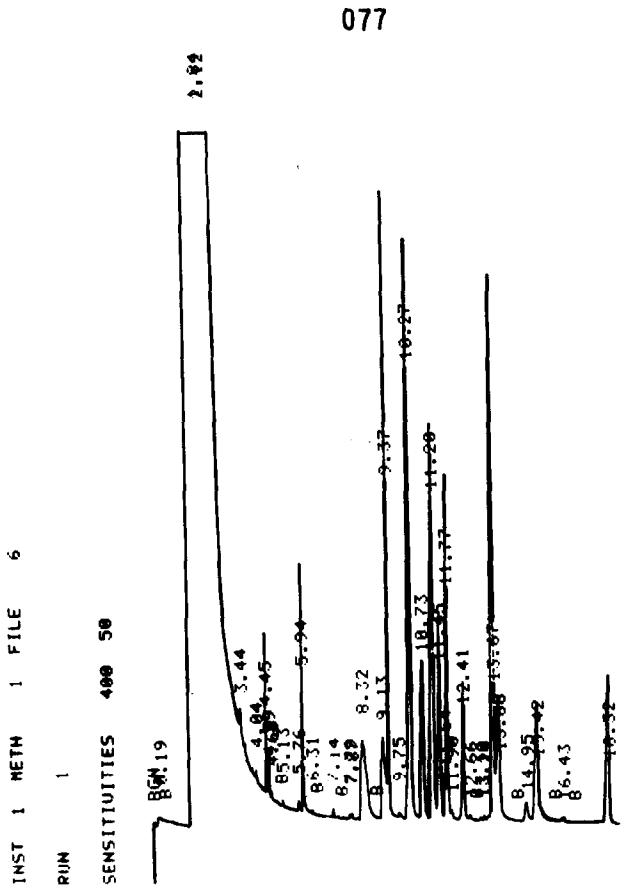


Fig. 2. Gas chromatographic separation of sixteen benzodiazepines on an SE-54 capillary column. Retention times: oxazepam 4.45, lorazepam 5.94, clorazepate 8.32, pinazepam 9.37, clobazam 10.27, bromazepam 10.73, temazepam 11.20, chlordesmethyldiazepam 11.45, flunitrazepam and prazepam 11.77, lormetazepam 12.41, chlordiazepoxide 13.67, nitrazepam 13.88, flurazepam 14.95, clonazepam 15.42 and camazepam 18.32 min.

E, but they give different benzophenones and in this way they can be identified after acid hydrolysis with system D or F.

Moreover, Dragendorff reagent is able to detect all benzodiazepines except lorazepam and clorazepate. Bratton–Marshall reagent gives different coloured spots with all benzophenones except camazepam and clobazam.

TLC analysis can also be used as in inexpensive screening technique when acute intoxication is suspected[25].

*Gas–liquid and high-resolution gas chromatography*

Retention indices (RI) obtained on packed and capillary columns are reported in Tables III and IV.

Table III shows our results and values obtained by Ardrey and Moffat[29] and Schuetz and Westenberger[23] using SE-30.

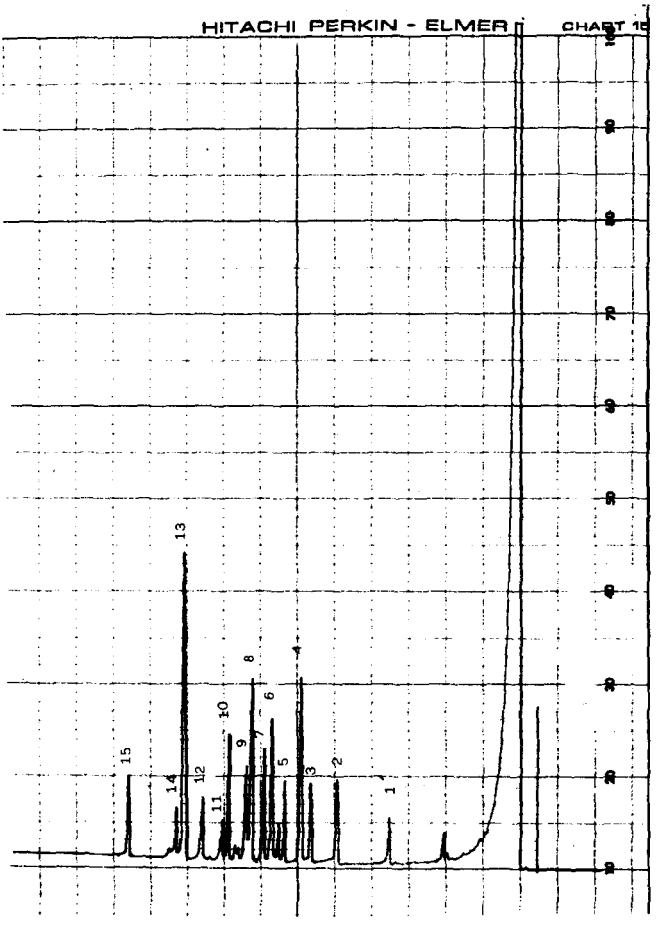


Fig. 3. Gas chromatographic separation of fifteen benzodiazepines on an SPB-1 capillary column. 1 = Oxazepam; 2 = lorazepam; 3 = desmethyldiazepam; 4 = diazepam; 5 = pinazepam; 6 = bromazepam; 7 = temazepam; 8 = chlordesmethyldiazepam; 9 = clobazam; 10 = flunitrazepam; 11 = lormetazepam; 12 = prazepam; 13 = flurazepam; 14 = nitrazepam; 15 = clonazepam.

For several benzodiazepines our results agree with the literature data; however, in some instances a variation more than 50 retention index units between the three sets of data was observed.

Retention indices obtained on the capillary column (SPB-1) were in agreement with those obtained on SE-30 under our analytical conditions. However, the packed column cannot allow the complete separation of all of the standards tested, particularly when using 3% GP and SE-30. The use of Dexsil 300 permits lower analytical temperatures with less pyrolytic destruction of the compounds.

In contrast, using a capillary column a complete separation is obtained and especially with SPB-5 in the isothermal mode the rapid identification of benzodiazepines is possible. The use of temperature programming and the high resolving power of SE-54 and SPB-1 (Figs. 2 and 3) allow a complete separation even when the

TABLE V

HPLC RESULTS OBTAINED USING ELUENTS a AND b

| *Compound* | *Relative retention time** | |
|---|---|---|
| | *a* | *b* |
| Bromazepam | 0.717 | 0.277 |
| Camazepam | 1.403 | 1.085 |
| Chlordesmethyldiazepam | ND** | ND |
| Chlordiazepoxide | 1.008 | 0.462 |
| Clobazam | 0.755 | 0.582 |
| Clonazepam | 0.684 | 0.406 |
| Clorazepate | 1.032 | 0.598 |
| Desmethyldiazepam | 1.040 | 0.589 |
| Diazepam | 1.198 | 0.879 |
| Flunitrazepam | 0.725 | 0.510 |
| Flurazepam | 2.050 | 1.329 |
| Lorazepam | 0.772 | 0.417 |
| Lormetazepam | 0.902 | 0.644 |
| Medazepam | 3.104 | 0.393 |
| Nitrazepam | 0.703 | 0.380 |
| Oxazepam | 0.802 | 0.390 |
| Pinazepam | 1.168 | 1.308 |
| Prazepam | 1.979 | 2.130 |
| Temazepam | 0.914 | 0.538 |

* Relative to azinphos-methyl.
** ND = not determined.

retention indices differ by less than 5 units. Moreover, SPB-1 permits complete resolution without tailing peaks for more polar compounds.

Using a capillary column, the detection limit with the flame-ionization detector was about 1–2 ng, whereas with the electron-capture detector 1 ng of each benzodiazepine gave a nearly full-scale response (>95%) on the 1.0-mV recorder.

Our data confirm the reliability of the use of retention indices especially on a capillary column with temperature programming, because of the good reproducibility (standard deviation) <2% (five replicate analyses).

*High-performance liquid chromatography*

Retention time relative to azinphos-methyl are reported in Table V for eluent systems a and b.

Complete separation of the compounds was achieved with eluent a. Nevertheless, with eluent b it is possible to carry out screening using a mixture that is easy to prepare and commonly employed for washing and preserving reversed-phase columns. UV detection allows a detection limit as low as 10 ng, which can be decreased further by shifting the wavelength to 220 nm, but then the background increases when analysing biological extracts.

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