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Mass spectrometric and gas and high-performance liquid chromatographic behaviour of an impurity in 2,5-hexanedione

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ABSTRACT

The analysis of 2,5-hexanedione, a metabolic compound of several industrial solvents, is normally carried out using gas chromatographic (GC) or GC–mass spectrometric (MS) techniques. This work, with the aim of verifying the possibility of determining the diketone by means of a high-performance liquid chromatographic (HPLC) method with UV detection, illustrates the importance of the choice of a 2,5-hexanedione standard for the determination of the diketone response factor. In some commercial diketone samples the presence of an impurity, which may interfere with the analysis of the target analyte, was ascertained. This impurity showed GC and HPLC behaviour similar to that of 2,5-hexanedione, but gave a very different UV response. This impurity was identified as 3-methylcyclopent-2-enone by means of MS, GC–MS, HPLC–photodiode-array detection, IR and UV spectrometry. The structure was confirmed by comparing the chromatographic, mass and ultraviolet data of the unknown compound with those of a synthesized reference sample. The well known difficulty in determining 2,5-hexanedione by HPLC with UV detection was reconfirmed owing to its low molar absorptivity.

INTRODUCTION

Occupational exposure to *n*-hexane and related compounds has been reported as causing peripheral neuropathies [1] and this disease in workers is due to 2,5-hexanedione (2,5-HD), the ultimate and most abundant metabolite resulting from the breakdown of this type of solvent [2]. The biological monitoring of exposure is normally done by measuring the diketone in hydrolysed urine using gas chromatography (GC) or GC–mass spectrometry (MS) [3,4]. The application of GC to biological samples is complicated owing to the need for extraction and concentration procedures involving the transfer of the target compound into an organic solvent.

Reversed-phase high-performance liquid chromatography (HPLC) may, however, be a valid alternative to GC owing to the sample characteristics and therefore many workers have tried to develop an HPLC method suitable for the detection of C_6 metabolites [5,6]. The main drawback to HPLC is the low molar absorptivity (ε) of 2,5-HD in UV detection; moreover, the presence in the commercial compound of an impurity with high ε and similar gas and liquid chromatographic behaviour may be a further source of error.

We and others [7] have tried, without success, to repeat the HPLC–UV method proposed by Marchiseppe *et al.* [6] for the detection of 2,5-HD in spiked samples. The proposed wavelength of 233 nm seems to be unsuitable for identifying this metabolite, because the absorbance in that UV range cannot be

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attributed with certainty to 2,5-HD. The need to obtain the correct UV spectrum and, consequently, the absorbance maximum of 2,5-HD led to the conviction that some commercial products should be studied.

EXPERIMENTAL

Chemicals

2,5-Hexanedione samples were purchased from Aldrich (Steinheim, Germany), Merck, (Darmstadt, Germany), Eastman Kodak (Rochester, NY, USA) and Carlo Erba (Milan, Italy). HPLC-grade water, methanol and 2,4-dinitrophenylhydrazine and all other analytical-reagent grade chemicals were obtained from Aldrich.

Synthesis of standards

3-Methylcyclopent-2-enone. According to the literature procedure [8], acetonylacetone (3.1 g) was added rapidly to a boiling solution of sodium hydroxide (0.25 g) in water (25 ml). After refluxing for 20 min, the brown solution was cooled to room temperature and extracted with diethyl ether (3 × 20 ml). The extracts were washed with water (2 × 1 ml), dried and evaporated. The crude reaction product, analysed by GC–MS, gave a mass spectrum corresponding to 3-methylcyclopent-2-enone.

2,4-Dinitrophenylhydrazone. According to the literature procedure [9], 2,4-dinitrophenylhydrazine was added to a sample of 2,5-HD enriched in 3-methylcyclopent-2-enone (3-MCP) by means of vacuum distillation (b.p. 76–80°C/25 mmHg), giving a yellow-orange precipitate. The filtered solid was washed with hot water–ethanol (3:2, v/v) and successively introduced into a cold-finger condenser in order to separate the 2,4-dinitrophenylhydrazone derivative of 3-MCP from that of 2,5-HD using their different fusion temperatures of 181 and 257°C, respectively [8,10]. The sample introduced directly into the ion source of the mass spectrometer gave a parent ion at *m/z* 276, corresponding to the calculated molecular weight of the derivative.

HPLC apparatus and conditions

All the measurements were performed with a Waters (Milford, MA, USA) high-performance liquid chromatographic system consisting of a Model 600 E MS pump, a Rheodyne manual loop injector with a 20-μl sample loop, a Model 990 plus MS photodiode-array (PDA) detector operating in the 200–330 nm range and a Model 990 plotter.

The acquisition and the elaboration of the data were carried out with an NEC (Boxborough, MA, USA) APC IV computer. The HPLC column employed was a Nova-Pak RP-18 stainless-steel column (150 mm × 3.9 mm I.D.) with spherical particles of 4 μm (Waters).

Separations were carried out by using methanol–water (40:60, v/v) as the mobile phase at a constant flow-rate of 0.6 ml min^{-1} and at room temperature. The HPLC solvents were degassed with helium. The injection volume for all the samples was 20 μl.

The PDA detector conditions were wavelength scan 200–330 nm, scan rate 1 s, detector sensitivity 1 a.u.f.s. and resolution 1.4 nm.

GC–MS apparatus and conditions

The measurements were performed using a Hewlett-Packard GC–MS system consisting of a Model 5890 gas chromatograph equipped with a 25 m × 0.31 mm I.D. fused-silica capillary column coated with Ultra-2 (cross-linked 5% phenylmethylsilicone; layer thickness 0.52 μm) (Hewlett-Packard, Palo Alto, CA, USA) and a Model 5971 A mass spectrometer. An HP 59970 C data system was used for data acquisition and editing.

For GC separations the column temperature was programmed from 50°C (isothermal for 3 min) at 10°C min^{-1} to 150°C (maintained for 3 min). The injector and transfer line temperatures were 260 and 280°C, respectively.

The MS conditions were electron energy 70 eV, emission current 300 μA and ion source temperature 176°C. Mass spectra were recorded by cyclically scanning from 40 to 250 mass units with a total cycle time of 0.49 s and a solvent delay of 3 min. The injection volume was 1 μl in splitless conditions (0.2 min).

UV apparatus

The UV spectra were recorded with a Hitachi (Tokyo, Japan) Model U-3200 spectrophotometer in the range 210–400 nm and 1 a.u.f.s. using methanol as solvent.

MS apparatus

The mass spectrum of the 3-MCP derivative was

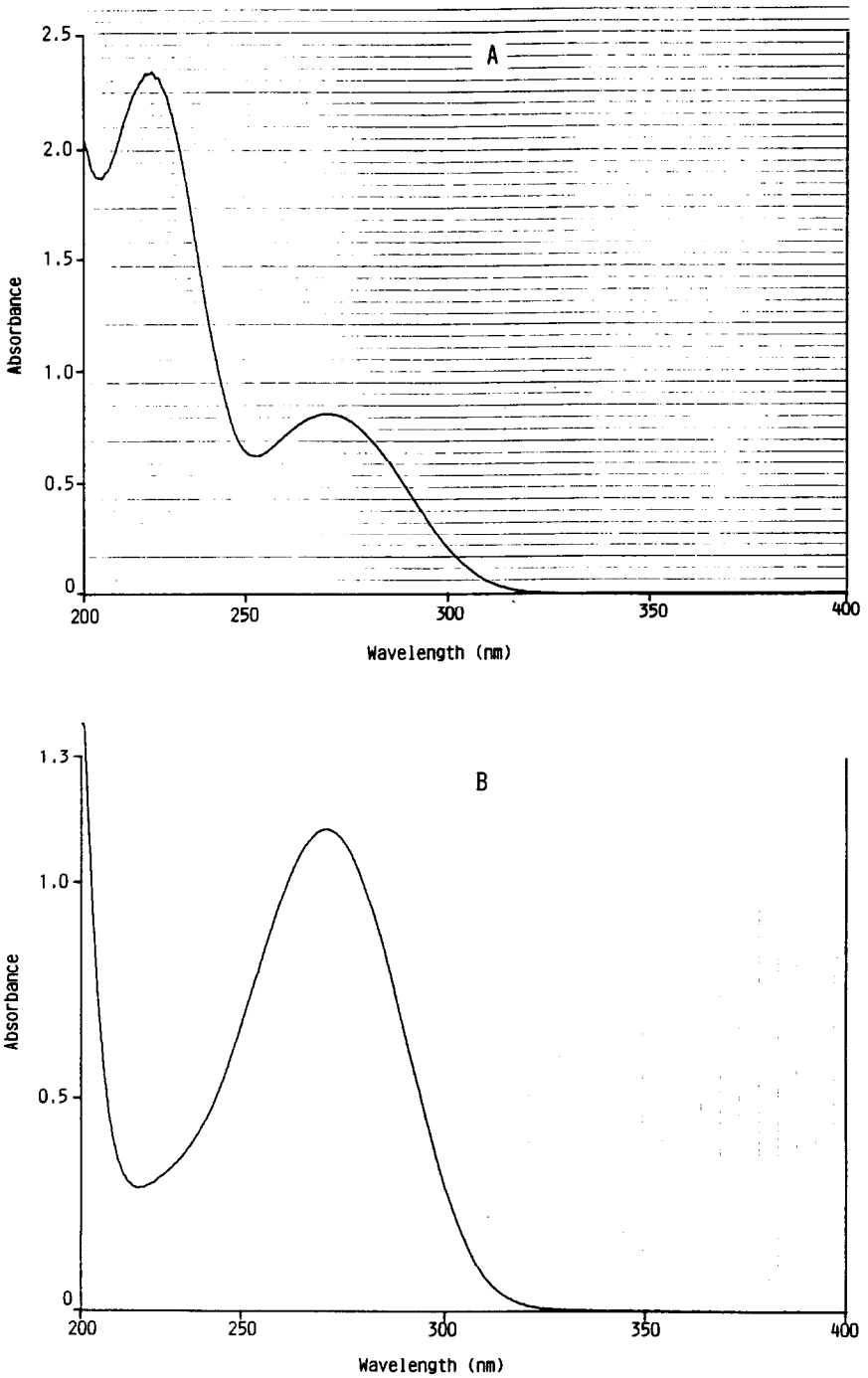


Fig. 1. UV spectra of 2,5-hexanedione from (A) Carlo Erba and (B) Eastman Kodak in methanol, recorded from 210 to 400 nm with a Hitachi U-3200 spectrophotometer.

obtained using a VG-ZAB2F mass spectrometer operating in the electron impact mode (70 eV, 200 μA, source temperature 280°C). The sample was introduced directly into the ion source.

IR apparatus

The IR spectrum of the neat crude reaction product of 3-MCP was recorded with a Perkin-Elmer (Norwalk, CT, USA) Model 682 infrared spectrophotometer in the range 2000–400 cm^{-1} on a potassium bromide disc.

RESULTS AND DISCUSSION

The UV absorption spectra (in methanol) of four commerical 2,5-HD compounds showed a considerable difference in the range 210–400 nm (Fig. 1). Fig. 1A shows two absorbance maxima at 270.4 and 226.6 nm, whereas Fig. 1B shows only one UV peak at 270.4 nm for two samples of the same compound, but of different brands.

The injection of 2,5-HD solution with the double absorption maximum (12 g/l) into the HPLC sys-

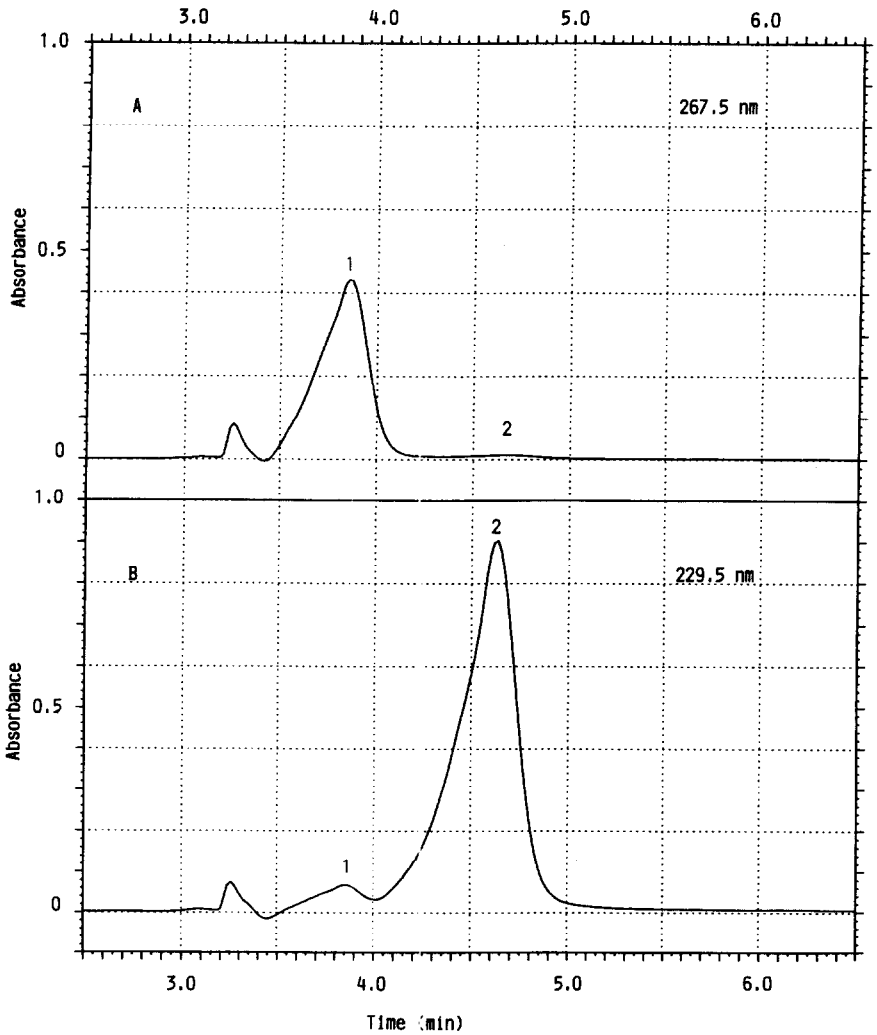


Fig. 2. Reversed-phase HPLC–PDA analysis of Aldrich 2,5-hexanedione sample (12 g/l) in water. Detection: (A) 267.5 nm; (B) 229.5 nm.

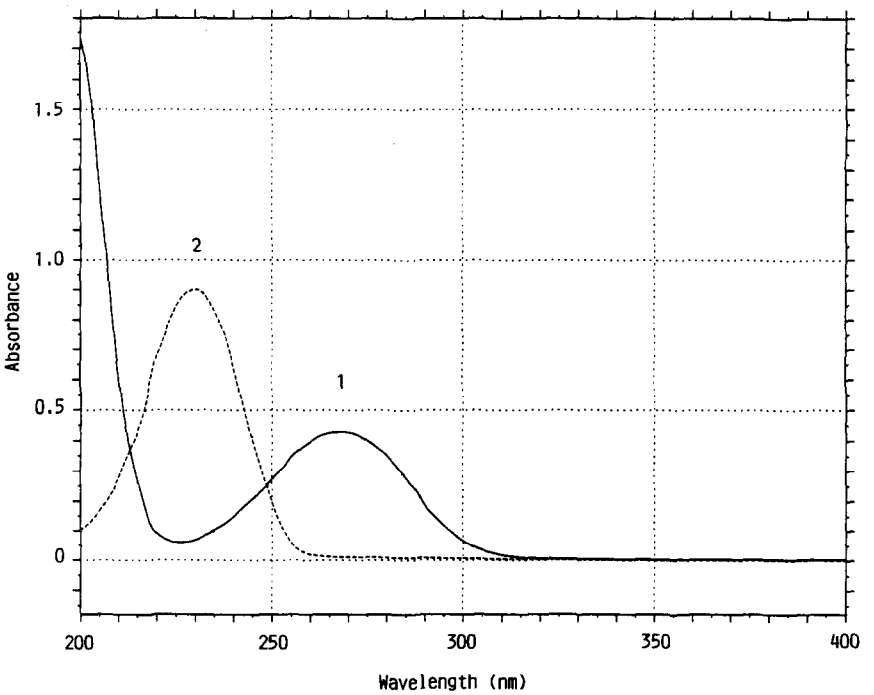


Fig. 3. UV spectra recorded with PDA detector of peaks 1 and 2 from Fig. 2.

tem gave a chromatogram with two separate chromatographic peaks (Fig. 2), to which the UV spectra reported in Fig. 3 corresponded. Comparison of the UV spectra indicated that the 267.5- and 229.5-nm absorbance maxima were independent of each other and seemed to belong to two different compounds. Assuming that one maximum must be assigned to 2,5-HD, the other could belong to another compound with a similar absorbance. To identify the interfering compound, four different commercial 2,5-HD samples were analysed without dilution using the GC–MS system, for which the signal intensity attributed to each peak is strictly dependent on the amount present. All the chromatograms obtained (Fig. 4) showed, close to a very intense peak which could be attributed unequivocally to 2,5-HD owing to its intensity and its mass spectrum, another small peak with reproducible retention times and different abundances.

The mass spectrum relating to the chromatographic peak (arrowed, Fig. 4) was attributed from a library search as having a high probability of being 3-MCP [11]. In fact, the mass spectra of the other reported isomers of the 3-MCP showed the following evident differences: 2-methylcyclopent-2-enone had the base peak at *m/z* 67 and the relative fragment ions at *m/z* 81 were not very abundant (10%); cyclohex-2-enone showed the base peak at *m/z* 68 and 2,5-dimethylfuran, with a very intense $[M-H]^+$ peak, had a retention time of about 2 min under the same GC conditions. Moreover, according to *Beilstein* [12], the 3-MCP compound was the only methylcyclopentenone isomer that is synthesized from 2,5-HD. In order to confirm the preliminary information obtained from the library search, the compound was synthesized in accordance with the literature procedure [8].

The crude reaction product, analysed by GC–MS, showed that 3-MCP was the more abundant product (Fig. 5) and had a mass spectrum identical with that reported in the literature [11], while the IR spectrum of the above sample gave the same absorption peaks with similar reciprocal intensities of those reported in the literature for technical 3-MCP [13]. The IR absorption maxima are shown in Fig. 6.

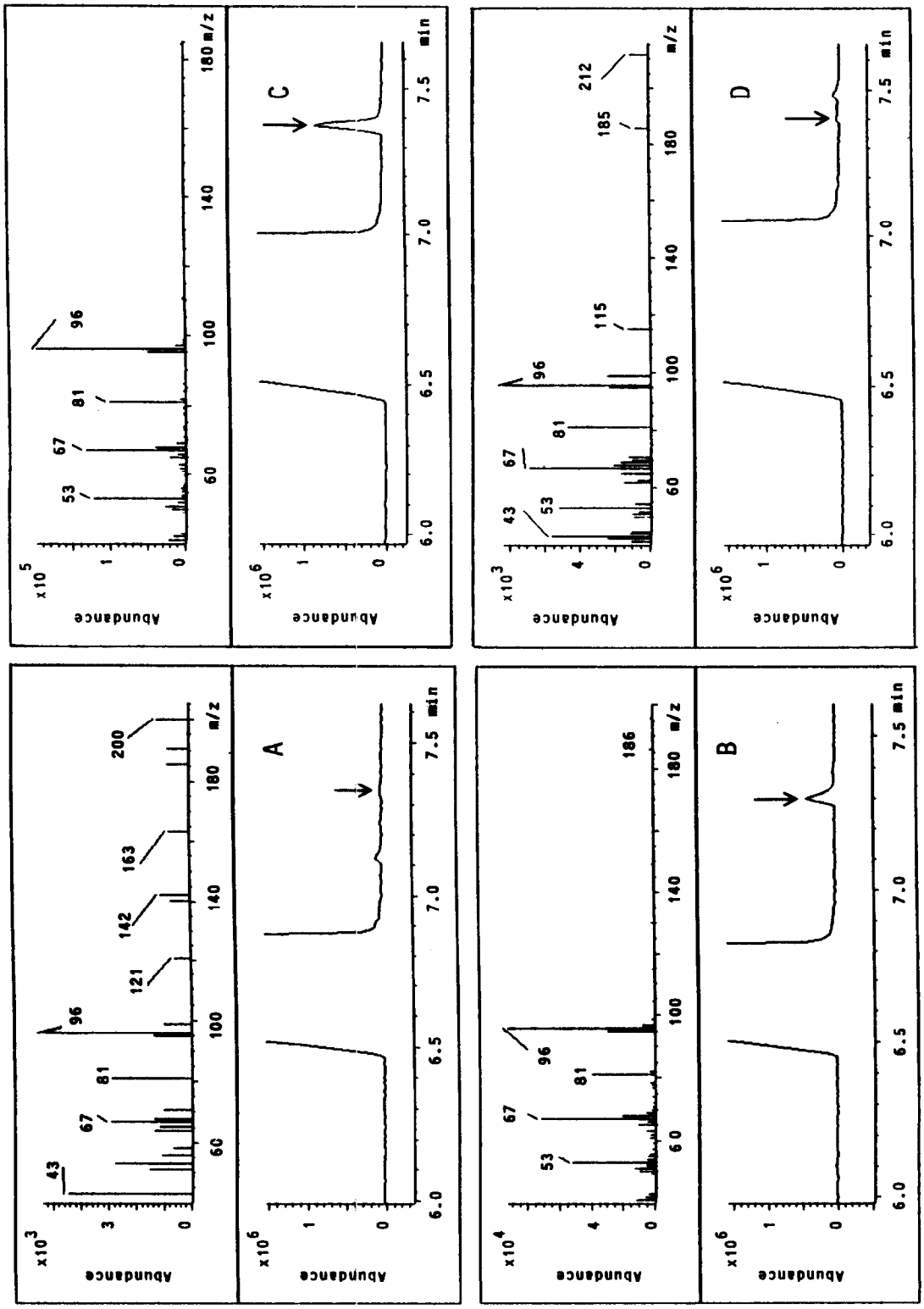


Fig. 4. GC–MS and relative mass spectra of impurity present in different 2,5-hexanedione samples: (A) Eastman Kodak; (B) Aldrich; (C) Carlo Erba, (D) Merck.

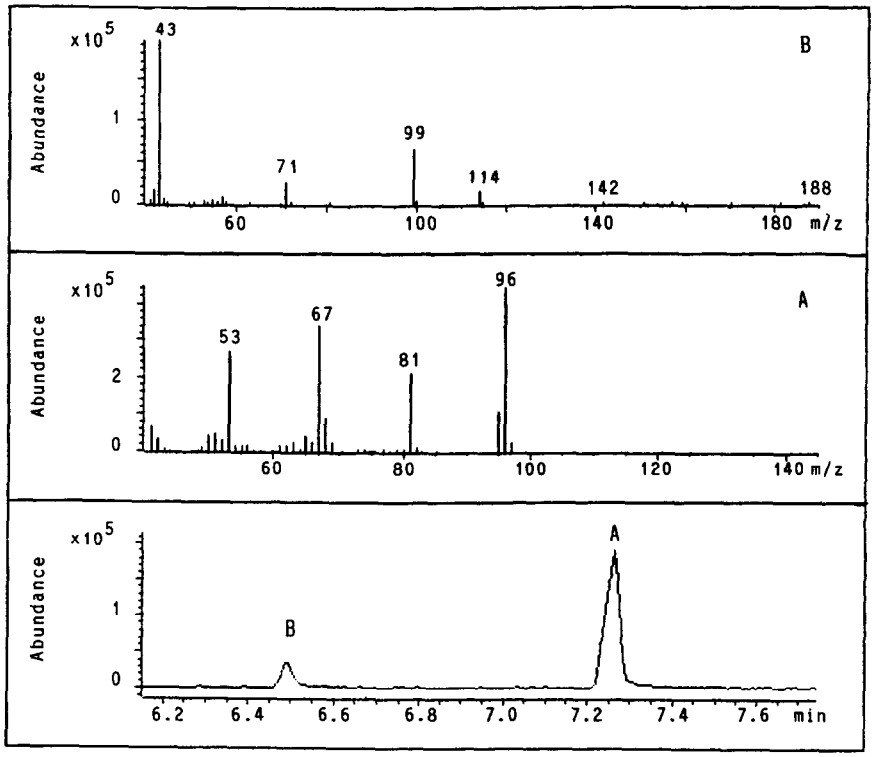


Fig. 5. GC–MS of crude reaction product and mass spectra of (A) synthesized 3-methylcyclopent-2-enone and (B) 2,5-hexanedione.

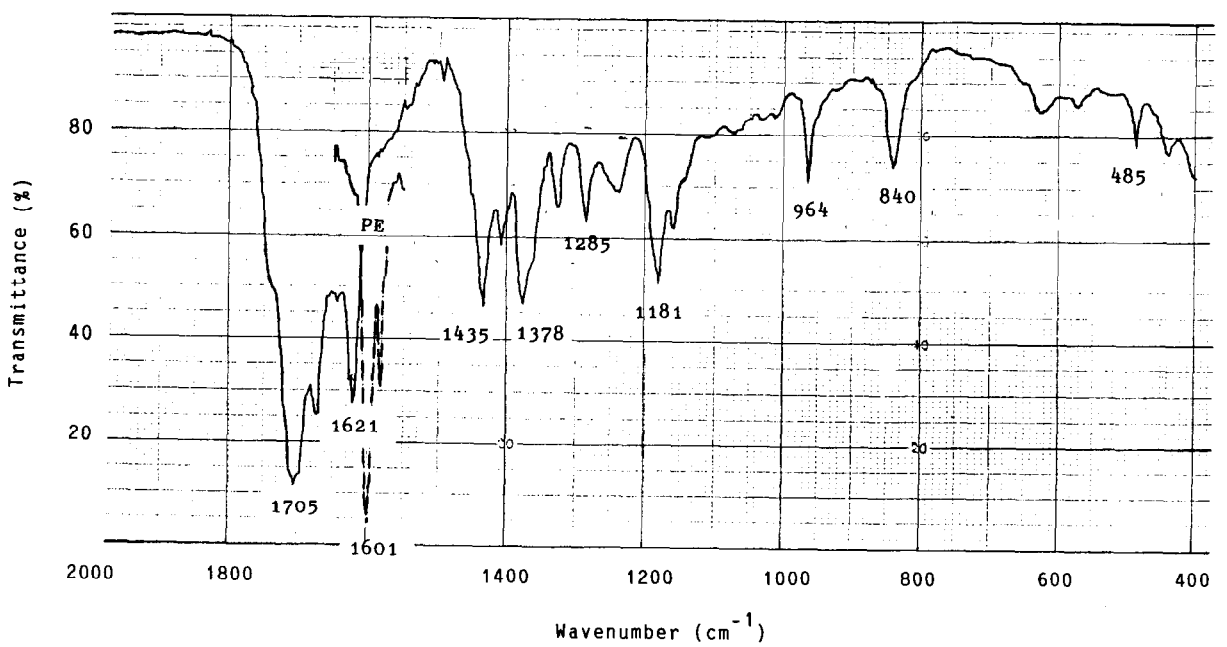


Fig. 6. IR spectrum of neat crude reaction product obtained from 3-methylcyclopent-2-enone synthesis.

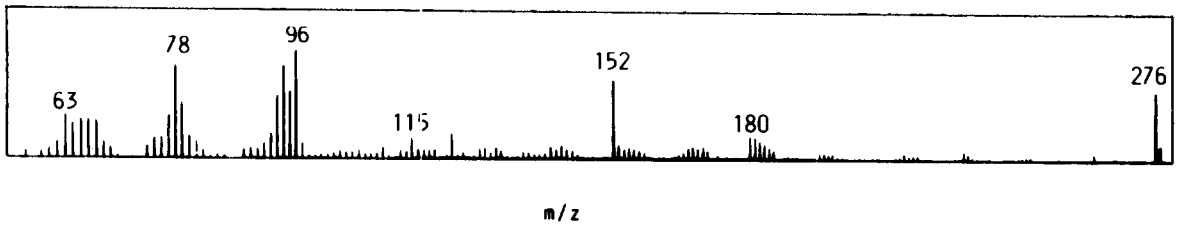
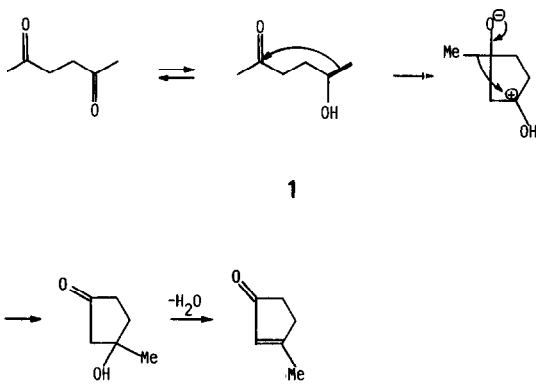


Fig. 7. Electron impact mass spectrum of 2,4-dinitrophenylhydrazone derivative of 3-methylcyclopent-2-enone (*m/z* 276) contained in the commercial 2,5-hexanedione sample.

On the basis of the MS and GC data, it was concluded that 3-MCP is generally present in commercial 2,5-HD samples. For further confirmation that the impurity was 3-MCP, the 2,4-dinitrophenylhydrazone derivative was prepared, as described under Experimental, and then analysed using a VG-ZAB 2F mass spectrometer. The resulting spectrum gave a value of *m/z* 276 for the parent ions, as reported in Fig. 7.

The presence of this compound in 2,5-HD can be explained by considering the following decomposition scheme (Me = methyl):



The enol form **1** is the driving force for the subsequent rearrangements, and the cyclization and the successive loss of water lead to the formation of 3-MCP. The different 3-MCP concentrations in the four different 2,5-HD samples seem to be independent of the declared purity (97–98%), but they appear to be closely linked to the specific synthesis process. In fact, the other unreported impurities present in the 2,5-HD samples analysed were all different compounds, while 3-MCP was common to all four samples. Fig. 4 demonstrates that this impurity, in the concentrations found in the four commercial samples, does not affect the GC–MS detection of the principal compound, but plays a very important role in the analytical technique when UV detectors are used. In order to verify the possibility of carrying out a correct determination of 2,5-HD by means of HPLC–UV detection, it appears to be of great importance not only to assign the correct UV spectrum to 2,5-HD, but also to know the exact HPLC and UV behaviour of 3-MCP.

The three-dimensional chromatogram of the synthesized compound (Fig. 8) showed the presence of only one peak with a retention time and UV spectrum that could be superimposed over the second peak in the commercial 2,5-HD chromatogram (Fig. 9), whereas the mixture of two samples (1:1, v/v) gave rise to a chromatogram (Fig. 10) in which

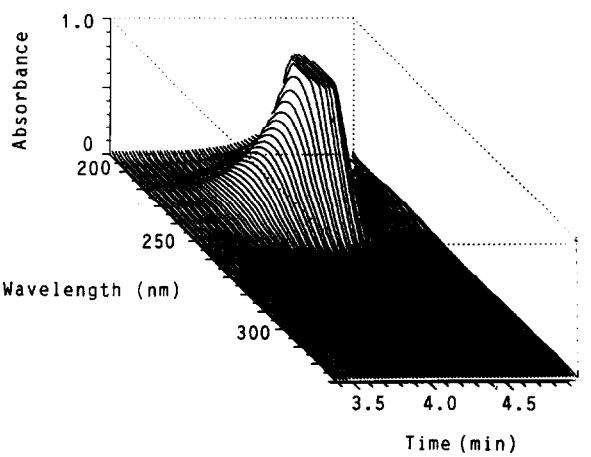


Fig. 8. Three-dimensional HPLC–PDA of synthesized 3-methylcyclopent-2-enone sample.

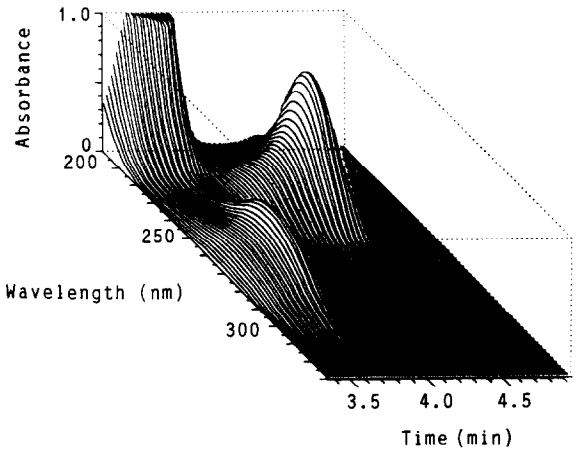


Fig. 9. Three-dimensional HPLC–PDA of Aldrich 2,5-hexanedione sample (12 g/l).

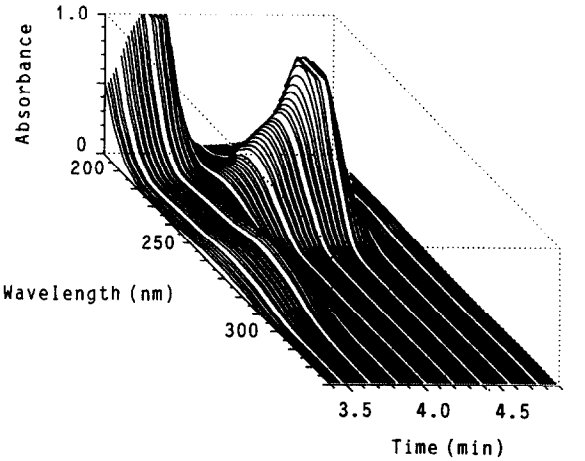


Fig. 10. Three-dimensional HPLC–PDA of synthesized 3-methylcyclopent-2-enone and Aldrich 2,5-hexanedione (12 g/l) mixture (1:1, v/v).

a decrease in the first peak and an increase in the second peak with respect to the above two chromatograms were evident.

Comparison of the HPLC–PDA and GC–MS results for the same sample showed the different instrumental responses for the two compounds (Figs. 2 and 11).

In the HPLC–UV system, the molar absorptivity greatly influenced the response factor of the analyte and therefore a strong signal was associated with a small concentration of 3-MCP owing to its high ε, unlike 2,5-HD. The two values of ε are 18 150 and 123 for 3-MCP and 2,5-HD, respectively [8,14], emphasizing that a 2,5-HD concentration 147 times

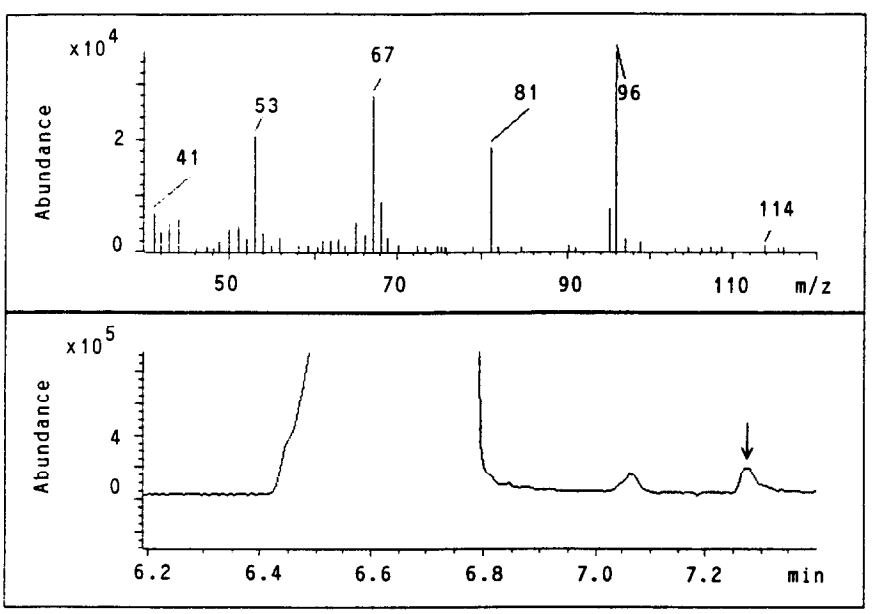


Fig. 11. GC–MS of Aldrich 2,5-hexanedione sample (12 g/l).

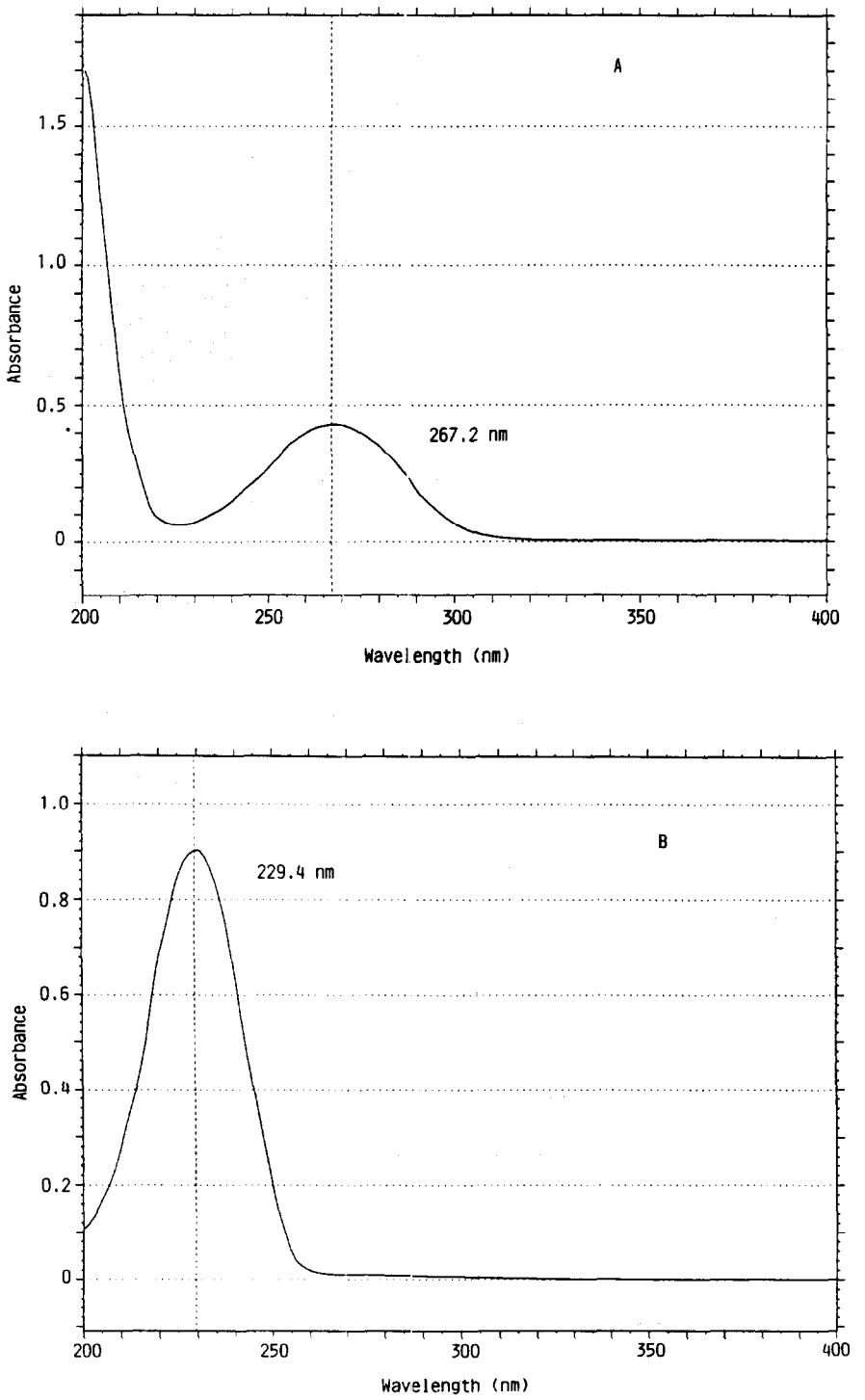


Fig. 12. UV spectra recorded with PDA detector of pure (A) 2,5-hexanedione and (B) 3-methylcyclopent-2-enone and respective wavelengths of maximum absorption.

higher than that of 3-MCP would give rise to two UV chromatographic peaks of similar intensity if measured at the respective absorption maxima.

All the above data allowed the UV spectra shown in Fig. 12A and B to be attributed unequivocally to 2,5-HD and 3-MCP and consequently to establish the correct wavelength for the UV detection of the metabolite, which was found to be 267 nm.

CONCLUSIONS

The use of independent chromatographic and detection techniques allowed the identification of the 3-MCP compound present as an impurity in four different commercial 2,5-HD samples. This compound of high ε may constitute a misleading interference in the UV detection of 2,5-HD in standard solutions and therefore a wavelength of 233 nm does not seem suitable for HPLC–UV analysis because at this value the 2,5-HD is undetectable.

It is the opinion of the authors that any HPLC–UV procedure used to determine directly metabolic 2,5-HD in urine is not a particularly accurate, specific or sensitive method owing to the low molar absorptivity and low concentration levels of the diketone. The HPLC method with UV detection would show a better performance if 2,5-HD underwent a pre- or post-column derivatization by reaction with a specific chromophore so as to increase the molar absorptivity and shift the UV detection window to a range less troubled by the many compounds present in the urine matrix.

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