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Capillary supercritical fluid chromatography combined with atmospheric pressure chemical ionisation mass spectrometry for the investigation of photoproduct formation in the sunscreen absorber 2-ethylhexyl-*p*-methoxycinnamate

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Abstract

The photoproducts formed on ultraviolet irradiation of the sunscreen absorber *trans*-2-ethylhexyl-*p*-methoxycinnamate, were separated and characterised using a new combined technique, capillary supercritical fluid chromatography–atmospheric pressure chemical ionisation mass spectrometry. Using this technique the commercially available *trans*-isomer was found to photoisomerise on irradiation at wavelengths greater than 300 nm. Photodimers were also separated and identified, and indicate that the sunscreen absorber can undergo [2+2] cycloaddition reactions with itself.

Keywords: Supercritical fluid chromatography–mass spectrometry; Photoisomerisation; 2-Ethylhexyl-*p*-methoxycinnamate

1. Introduction

Changing lifestyles and the increased pursuit of outdoor leisure activities have led to increased exposure of many people to ultraviolet radiation from the sun. In response to this, the cosmetic industry has developed a range of sunscreen products to protect the skin from the sun's harmful radiation. The purpose of sunscreen filters is to prevent the skin burning while permitting gradual tanning. This is achieved by the chemical sunscreen absorbing UV radiation via electron promotion from a molecular orbital of lower energy to one of higher energy. The energetically excited molecules have a short lifetime and return to their ground state by losing the excess energy in one or a combination of ways. Energy may be transferred to the neighbouring environment in the form of thermal energy or by fluorescence, phosphorescence or a combination of the two. Another possible way of losing energy is by chemical reaction.

The photochemistry of *trans*-2-ethylhexyl-*p*-methoxycinnamate (*trans*-EHMC) is of fundamental interest as this is currently the most commonly used UVB absorber and is incorporated into over 75% of sunscreen-containing cosmetic formulations [1]. The molecule has an electron-releasing methoxy group in the *para* position facilitating electron delocalisation,

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and this is responsible for its strong molar absorptivity (>23 000 $dm^3\ mol^{-1}\ cm^{-1}$ at 305 nm). *trans*-EHMC undergoes a decrease in UV absorbing ability as a result of exposure to light. The literature is vague concerning the origin of this apparent instability, with most publications attributing the loss of absorbing ability to either photodegradation or photoisomerisation of the EHMC molecule [2–4]. Kammeyer et al. [5] and Deflandre and Lang [6], however, suggest that photoisomerisation takes place initially and is followed by photodegradation after a stationary state has been established. Morliere et al. [7] have attributed the decrease in absorbance entirely to photoisomerism. According to their NMR data, the *cis*- and *trans*-isomers of EHMC were the only species present in irradiated solutions and no other photodegradative products were identified. Unfortunately, as the detection limit of 80-MHz NMR spectroscopy is of the order of milligrams, it is unlikely that their formation would be detected using such a technique unless photodegrative products were present in large amounts. Gonzenbach et al. [8] used high-performance liquid chromatography (HPLC) to analyse photoproducts. However, although they reported that photoisomerisation of *trans*-EHMC occurred and that no other products were detected, the chromatographic conditions and detection system employed were not stated.

The use of an efficient chromatographic technique coupled with a universal and selective detection system allowing both qualitative and quantitative analysis of irradiated samples would be the most suitable analytical method for revealing the predominant mechanism. Capillary supercritical fluid chromatography (SFC) has been demonstrated for the chromatographic analysis of a wide range of thermally labile, reactive, high-molecular-mass and moderately polar compounds [9,10]. Open tubular columns are particularly suited for the separation of thermally sensitive compounds in complex mixtures as these columns are well deactivated and separation can take place at low temperatures and in relatively inert carbon dioxide. Further, SFC with CO_2 mobile phase can be coupled with a variety of detectors, including the flame ionisation detector (FID), Fourier transform infrared (FTIR) and mass spectrometric (MS) detectors [11].

Tyrefors et al. [12] have recently reported the development of an interface for the on-line combination of capillary SFC with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS). In this interface, the column effluent is expanded as neutral molecules into a moist nitrogen atmosphere where they are ionised by a corona discharge. Positive ionisation occurs through the creation of hydronium ion–water clusters $H_3O(H_2O)_n^+$, which act as the ionising agents [12]. The analyte ions are transferred by electrostatic forces through a small orifice and analysed in the evacuated quadrupole mass filter. There are several advantages in using APCI-MS detection. Firstly, this method of ionisation is very mild and usually results in the formation of protonated molecules $[M+H]^+$ with very little fragmentation (even when large or unstable molecules are analysed). Secondly, the method is sensitive, with detection limits in the total ion mode in the low nanogram range. Thirdly, in contrast to electron impact or chemical ionisation SFC–MS, the increasing flow of expanding mobile phase during pressure or density programming has little or no effect on the ionisation process. Other SFC–APCI-MS studies, employing packed columns, have been reported for the analysis of steroids [13], polycyclic aromatic hydrocarbons [14], polyoxyethylene glycol, polystyrene, and vitamins [15] and demonstrate the potential of this technique.

In this work, solutions of *trans*-EHMC were irradiated at wavelengths above 300 nm in order to simulate solar radiation reaching the earth's surface. These solutions were subsequently analysed by capillary SFC with flame ionisation and atmospheric pressure chemical ionisation mass spectrometric detection to determine whether any EHMC photoproducts do indeed form. Solutions of *trans*-EHMC and thymine were also irradiated and analysed by these techniques to investigate whether EHMC–thymine photoadducts form.

2. Experimental

The section is divided into photolytic and analytical methodologies.

2.1. Irradiation of dilute EHMC solution

Commercial *trans*-EHMC was obtained at 98% purity from Harmann and Reimer (Isando, Johannes-

burg, RSA). A solution of 1.46×10^{-2} *M* (approximately 0.5%) *trans*-EHMC and 6.74×10^{-3} *M* (approximately 0.2%) methyl stearate was prepared in dichloromethane. Aliquots of 400 μl of this solution were irradiated for varying lengths of time, ranging from 1 min to 19.5 h, with a Hanau St75 medium-pressure mercury lamp and a 313-nm narrow bandpass filter (Acton Research Corporation). All samples were protected from light both before and after irradiation and transferral of solutions to and from the irradiation cuvette were carried out in the dark, as were the actual irradiations. Dichloromethane was chosen as the solvent in these irradiations due to its low absorbance cutoff at 232 nm. Methyl stearate was chosen as the internal standard as it was considered unlikely to interact photochemically with EHMC and due to its low molar absorption coefficient (12.8 $dm^3\ mol^{-1}\ cm^{-1}$). In order to assess its photostability, a solution of methyl stearate was irradiated for 6 h with an intense light source; the HBO 500/W2 high-pressure mercury lamp (Osram) using a 313-nm narrow bandpass filter. SFC analysis of the irradiated sample showed no photodegradation.

2.2. Irradiation of neat EHMC

A sample of neat *trans*-EHMC was irradiated for 13.5 h with a HBO 500W/2 high-pressure mercury lamp combined with a 10-mm pyrex filter. This sample was diluted with dichloromethane and analysed by capillary SFC with FID and APCI-MS detection.

2.3. Irradiation of EHMC in the presence of thymine

A 1.00×10^{-2} *M* solution in both EHMC and thymine made up in 85% methanol and 15% water (by volume) was irradiated at wavelengths above 300 nm for 144 h in a photochemical reactor vessel with a Hanovia 200 W medium-pressure mercury lamp. The lamp was inserted into a quartz water cooling sleeve which in turn was placed into an external pyrex thimble which acted as a light filter, transmitting light of wavelengths greater than 300 nm. The photoproducts were concentrated for chromatographic analysis by blowing down the solvent with nitrogen.

2.4. Supercritical fluid chromatography

Open tubular column SFC was performed using a Lee Scientific 600 Series SFC instrument (Dionex, Salt Lake City, UT, USA). SFE/SFC grade carbon dioxide (Air Products, Allentown, PA, USA) was used as the mobile phase. Separations were performed on a 10 m×50 μm I.D. fused-silica open tubular column coated with a 0.25-μm film of 30% biphenyl-methylpolysiloxane or 30% cyanobiphenyl-methylpolysiloxane (Dionex) using pressure programming. The column was housed in the oven at 100°C and the pump pressure was programmed from 120 atm (held 3 min) to 415 atm at 10 atm/min. Time split injections of 50 ms were made into the column. Flame ionisation detection (FID) was employed at 350°C. Post-column mobile phase flow restriction was performed using tapered restrictors in the FID [16] and integral restrictors [12] for SFC–APCI-MS. Both restrictors were made from 50 μm I.D. deactivated fused-silica capillary tubing. The tapered restrictors were made at one end of a 30 cm length of tubing, while integral restrictors were made using 80–100 cm lengths of tubing and extended from the oven through the heated transfer line and into the ion source. The column-to-restrictor connection was made inside the oven using a zero-dead-volume butt connector (SGE, Australia).

2.5. SFC–MS interface

The design, construction and operation of the SFC–MS interface has been previously described in the literature [12] and hence only the pertinent points will be discussed here. The interface consists of three parts: the gas manifold and temperature equilibration assembly placed inside the SFC oven, a transfer line at 150°C and an ion source assembly. The restrictor tubing was inserted into a 0.7 mm I.D. fused-silica transfer tube through which a make-up flow of water-rich synthetic air passed. The 0.7-mm tube was itself inserted inside a 1/4 in. O.D. Teflon tube through which compressed air passed. The compressed air was used solely for maintaining the interface at a constant temperature in order to retain

chromatographic integrity. This was achieved by using ceramic fibre fabric high-temperature insulation (Dalfratex stocking), a heating coil and a thermocouple. The restrictor tip was heated by a small heating coil to prevent analyte condensation as the fluid depressurised and to transfer the analytes effectively into the atmosphere of moist air for ionisation.

2.6. Mass spectrometry

Mass spectrometry was performed on a Sciex API III tandem quadrupole mass spectrometer (Sciex, Thornhill, Canada) equipped with an atmospheric pressure ionisation source with a corona discharge needle. Ionisation was achieved by a point-to-plane corona discharge. Cholesterol palmitate was used for tuning the source and lenses, and for mass calibration. Total ion chromatograms were recorded from full scan mass spectra in the range m/z 200–1200. In some cases single or selected ion acquisitions were also performed.

3. Results and discussion

3.1. Analysis of irradiated dilute EHMC solution.

On SFC analysis of the unirradiated stock solution, the internal standard peak eluted at 14.1 min and the *trans*-EHMC peak eluted at 17.30 min. Analysis of an EHMC solution that had been irradiated for 2 min clearly showed the formation of a photoproduct at 16.10 min which was suspected to be *cis*-EHMC as the peak increased in magnitude with irradiation time. This is clearly shown in the chromatograms in Fig. 1. As no standard *cis*-EHMC was available, confirmation of the identity of this peak was achieved using gas chromatography–mass spectrometry (GC–MS) and SFC with off-line Fourier transform infrared microspectrometry (SFC–FTIR) [9]. As expected, GC–MS analysis produced a similar total ion chromatogram to those obtained with SFC and the electron impact mass spectra of the two peaks were almost identical for *cis*- and *trans*-isomers. A molecular ion at m/z 290 and fragment ions at m/z 178 (base peak), 161 and 133 appeared in both mass spectra and on searching the mass spectra through the NIST library, both spectra were identified as EHMC with over 90% fit. The infrared spectra of the two isomer peaks obtained by SFC–FTIR were different, although there were strong similarities between the spectra. The characteristic C–H stretching bands of the 2-ethylhexyl group (2960–2850 cm^{-1}) were present in both spectra, as were the C=O stretching band of the ester (1705–1720 cm^{-1}) and absorbances due to the aromatic ring (1604, 1590 and 1512 cm^{-1}). Differences in the two spectra were observed in the regions where absorbance bands were associated with the C=C, for example at 1620–1633 cm^{-1} and 1430 cm^{-1}. The spectrum of the *trans*-isomer had a band at 980 cm^{-1} (due to deformation of the H on the C=C olefin group) which was absent in the spectrum of the *cis*-isomer.

As no other peaks appeared in the chromatogram, it was concluded that irradiation of dilute solutions of *trans*-EHMC (<1% v/v) with UV light at 313 nm gives rise to the formation of *cis*-EHMC only and that other photochemical reactions (apart from the reverse reaction) appear to be negligible. Application of these experimental constraints permitted investigation of the kinetics of the *trans–cis* isomerisation process in isolation [17]. The concentration of *trans*-EHMC was calculated using an internal standard method. Since the only reaction taking place was the conversion of *trans*-EHMC to *cis*-EHMC, the concentration of *cis*-EHMC was found by difference of the mass of *trans*-EHMC in the unirradiated solution and the mass of *trans*-EHMC in the irradiated solution. Concentrations were calculated using the molecular mass of EHMC (290.40 g mol^{-1}) and the volume of the original solution. From the graph of these results in Fig. 2, it is evident that isomerisation takes place rapidly within the first 4 h of irradiation. Thereafter, the rate of formation of *cis*-isomer drops off due to the backward reaction. The concentrations of both isomers become constant after approximately 8 h, with the development of a photostationary state. Obviously the rate of the isomerisation will be dependent on the intensity of the UV source, but once the photostationary state has been established, the less-efficient UV absorbing *cis*-EHMC is the isomer present in larger quantities.

It may well be argued that the above analysis

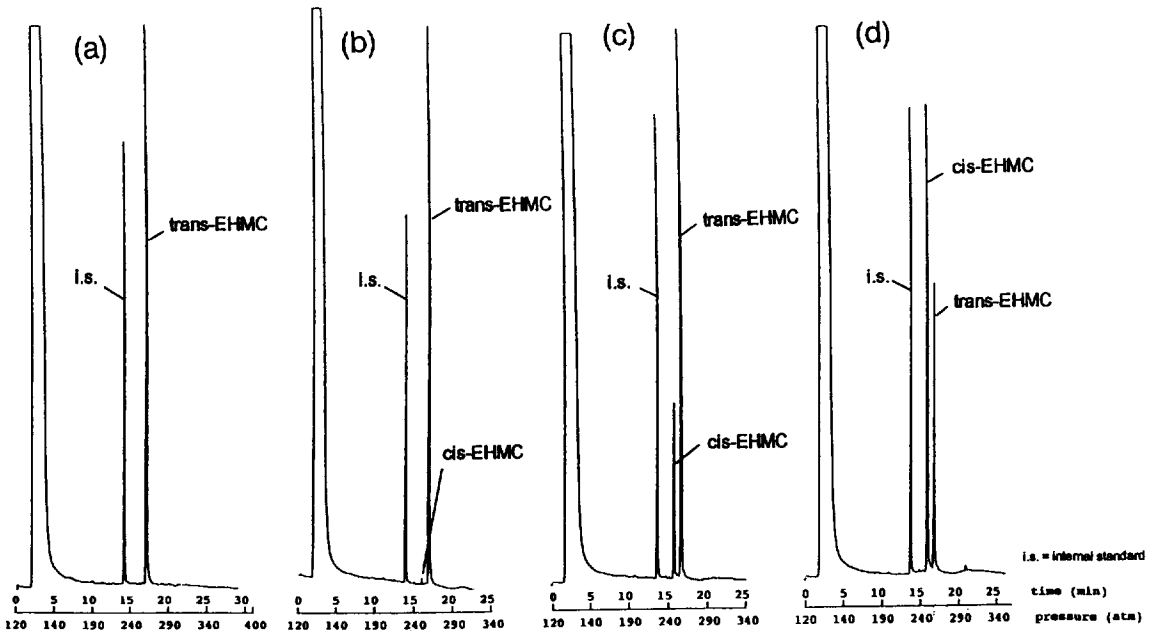

Fig. 1. Capillary SFC–FID chromatograms showing (a) unirradiated *trans*-EHMC, and the separation of *cis*- and *trans*-EHMC after (b) 2 min of irradiation, (c) 1 h of irradiation and (d) 5 h of irradiation.

could have been performed more efficiently by gas chromatography. This is true provided that the photoproducts are all thermally stable and volatile. It is however important to consider the possible formation of higher-molecular-mass and involatile compounds.

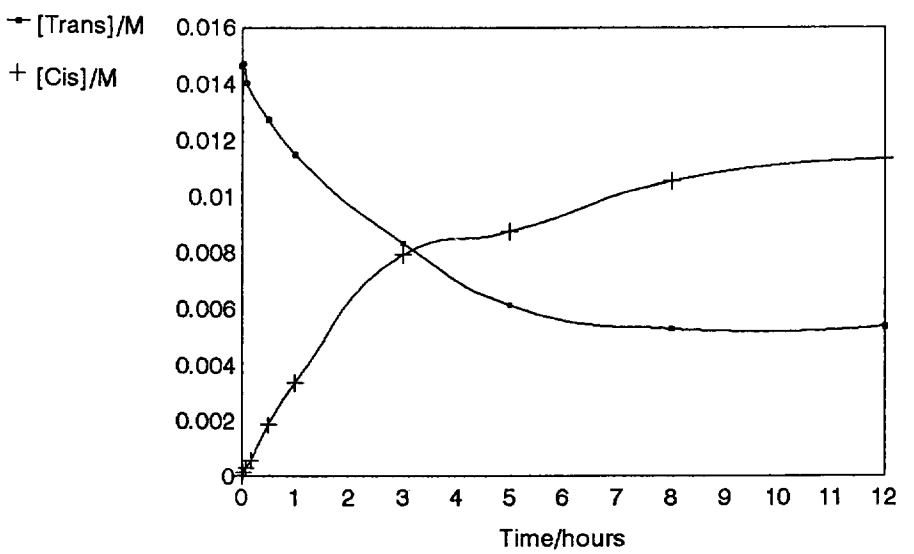

Fig. 2. Graph of concentration of *trans*-EHMC (□) and *cis*-EHMC (+) versus irradiation time.

3.2. Analysis of irradiated neat EHMC

SFC analysis of *trans*-EHMC irradiated neat at wavelengths above 300 nm, but with a more intense source, showed the appearance of a number of peaks eluting at higher pressures than the EHMC isomers. These were suspected to be EHMC cyclobutane dimers formed via a [2+2] cycloaddition reaction. The formation of these dimers has not previously been reported, although the literature concerning similar molecules suggests that EHMC photodimerisation is likely to occur; for example *trans*-*p*-hydroxycinnamic acid and *trans*-ferulic acid have been reported to photodimerise [18]. These dimers would be expected to be involatile due to their high molar mass (580.8 g mol^{-1}) and to have significantly reduced chromophores when compared to the monomers due to the conversion of the ethylenic double bonds to single bonds in the cyclobutane ring. It is therefore not surprising that these compounds were first detected not by HPLC with UV detection or GC with FID, but by SFC with FID as shown in Fig. 3a. The other small peaks present in the chromatogram are due to contaminants originally present in the neat EHMC which was only available at 98% purity.

SFC with APCIMS detection was used to confirm the identity of the *cis*-isomer and EHMC dimers on irradiation of neat *trans*-EHMC. The total ion chromatogram obtained (Fig. 3b) is very similar to that obtained by SFC–FID (Fig. 3a), with peaks 1 and 2

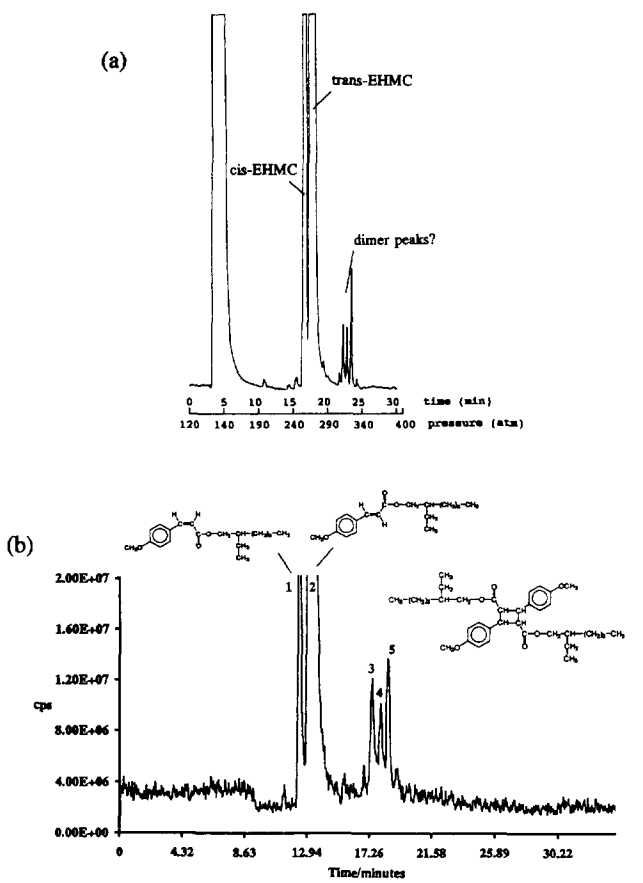

Fig. 3. (a) SFC–FID analysis of neat *trans*-EHMC which had been irradiated for 13.5 h at wavelengths above 300 nm. The sample was diluted in dichloromethane for injection and separated under conditions given in the text. (b) SFC–APCI-MS analysis of the same sample as analysed in (a). Total ion chromatogram of the mass range *m/z* 200–1200. The symbol cps indicates counts per second. SFC conditions are given in the text.

being *cis*- and *trans*-EHMC, respectively, and peaks 3–5 being the peaks of interest. The mass spectra of peaks 1 and 2 showed two main ions at m/z 291 and m/z 581.5. The ion at m/z 291 corresponded to the protonated molecules $[M+H]^+$ of EHMC and confirmed the GC–MS and SFC–FTIR results showing that peak 1 was *cis*-EHMC. However, there was also an appreciable amount of $[2M+H]^+$ ion at m/z 581.5 in the mass spectrum, which indicated that dimerisation had also occurred within the interface. Although the restrictor tip was heated in the experiment, the dimerisation is unlikely to have been thermally induced as [2+2] cycloaddition is symmetry-forbidden at ground state. Further, on examining different mass spectra measured during the elution of the *cis*-EHMC peak, the ratio of dimer-ion intensity to monomer-ion intensity increased as the gas-phase concentration of monomer in the source increased towards the peak apex. The $[2M+H]^+$ ions observed were thought to be non-covalent proton-bound dimer ions formed by ion–molecule reactions in the APCI source.

In order to study the dimerisation in more detail, the irradiated sample of neat EHMC was analysed by SFC–MS at two different orifice potentials. Not surprisingly, it was found that the dimerisation of the *cis*- and *trans*-isomers that had occurred in the ion source could easily be broken by increasing the orifice potential from 35 to 70 V (Fig. 4). In contrast, the mass spectra of peaks 3, 4 and 5 confirmed that these peaks were covalently bonded EHMC photodimers due to the presence of stable protonated dimer molecules at m/z 581.5 at both low and high orifice potential. As can be seen from Fig. 5,

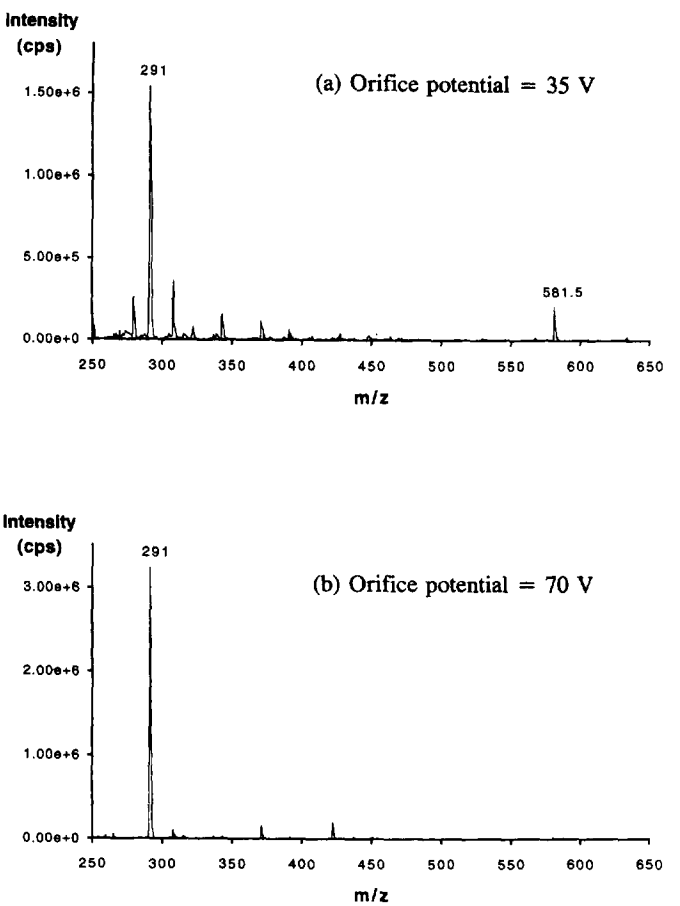

Fig. 4. APCI mass spectrum of peak 1 (Fig. 3b) obtained using an orifice potential of (a) 35 V and (b) 70 V.

depicting one of these dimers at orifice potentials of 35 and 70 V, respectively, the fragmentation pattern is also different from the non-covalently bonded dimers shown in Fig. 4, and a fragment at m/z 451 dominates the spectrum. The fragmentation observed involves the loss of 130 amu and is due to the loss of 2-ethylhexanol from the protonated dimer molecules.

APCI is a useful ionisation mode for these molecules because it is a soft ionisation technique and gives clear molecular mass information. Further, as illustrated in Fig. 5 and Fig. 6, collision induced dissociation can be used as an aid to structural elucidation. Unfortunately, however, APCIMS gives little indication of the structural orientation of the EHMC photodimers. This is compounded by the fact that there are thirteen possible orientations of EHMC dimer, as shown in Fig. 6, not all of which can be separated and some of which may not form due to steric interference. A more polar stationary phase (cyanobiphenyl-methylpolysiloxane) was therefore investigated. Unfortunately, although the dimers were retained longer on the column, they could not be resolved to any greater extent on this phase.

3.3. Analysis of EHMC irradiated in the presence of thymine

The confirmed formation of photodimers at high concentrations of EHMC indicates that similar [2+2] cycloaddition reactions might occur with other moieties present in the skin on irradiation. This is of concern because a photoreaction of EHMC with thymine or other nucleic acid base in the DNA could lead to the formation of an EHMC–thymine (or other

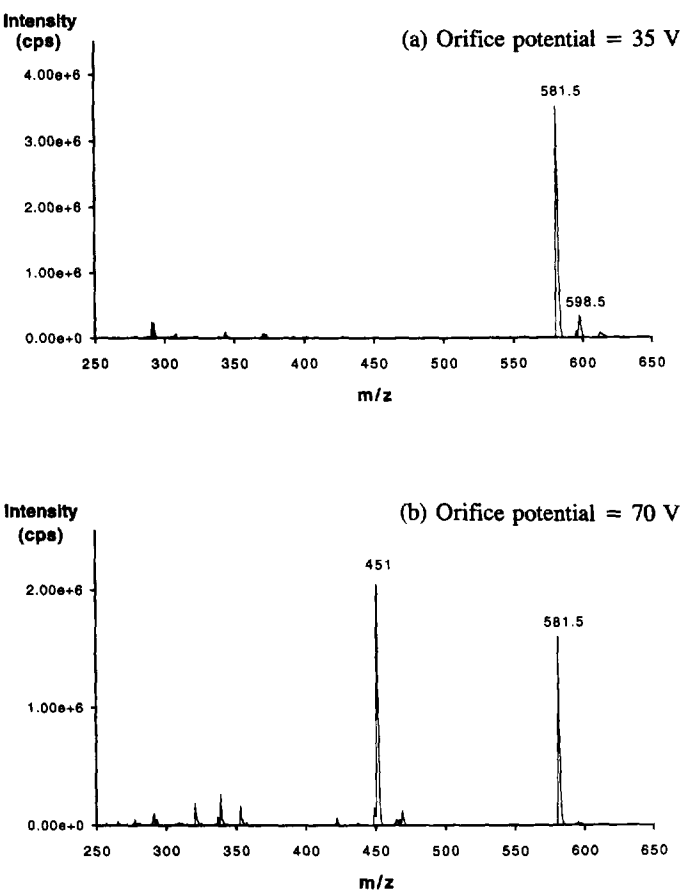

Fig. 5. APCI mass spectrum of peak 5 (Fig. 3b) obtained using an orifice potential of (a) 35 V and (b) 70 V.

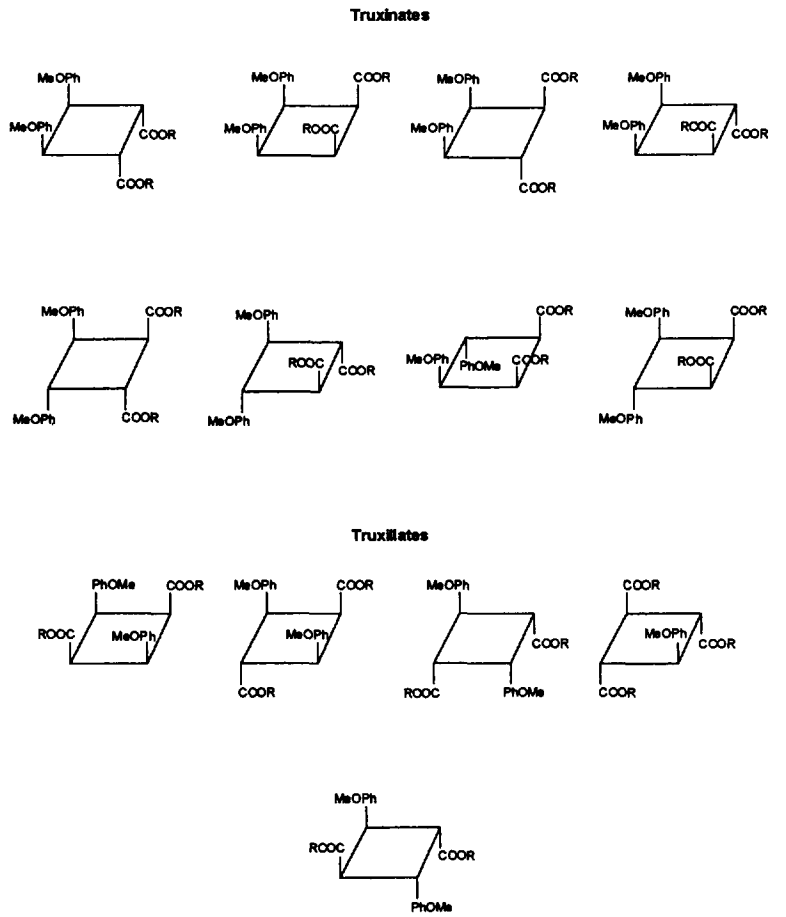

Fig. 6. Molecular structures of the thirteen possible EHMC dimers which could be formed on UV irradiation at wavelengths above 300 nm.

nucleic acid base) photoadduct. These adducts could be potentially carcinogenic as they would block the reading of the genetic code, thus interfering with the replication process. To investigate possible photoadduct formation, a solution of 0.01 *M* *trans*-EHMC and 0.01 *M* thymine in 85% methanol–15% water was irradiated at wavelengths greater than 300 nm using a Hanovia medium-pressure mercury lamp. Since the intensity of this lamp was considerably less than that of the HBO high-pressure mercury lamp, longer irradiation times were employed (>72 h).

On SFC–APCI-MS analysis, no adducts were detected during selected ion monitoring of *m/z* 416.5 to 417.5. Work is continuing to improve the analytical procedure and to investigate photoadduct formation on irradiation of solutions of thymine and thymidine, respectively, with *trans*-EHMC at different wavelengths.

Whether, EHMC in the presence of UV light is potentially harmful to the skin remains to be confirmed. There has been some controversy regarding the potential phototoxicity of EHMC, with an early report of tumour induction [19], contradicted by later investigations [20]. More recently, Mohammad et al. [21] reported that on UV irradiation, radiolabelled *p*-methoxycinnamic acid becomes incorporated into calf thymus DNA, and they suggested that at long wavelengths a [2+2] cycloaddition mechanism is favoured. Our work shows that on UV irradiation, EHMC undergoes photoisomerisation and that [2+2] cycloaddition reactions with itself can also occur. From an analytical point of view, this research

highlights the need for sensitive and selective analytical methods in order to understand the processes involved in photoproduct formation. SFC with AP-CIMS detection not only allows efficient separation at low temperatures but also provides important molecular mass data for structural identification of photoproducts varying in polarity and molecular mass.

Acknowledgments

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