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Note

Chromatographic studies of the autoxidation products of ethoxyquin and its photochemical conversion

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Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, EQ) which is used in food, animal feed and rubber as an antioxidant, is determined mostly by spectrofluorometry[1], high-performance liquid chromatography (HPLC) with a fluorometric detector[2,3] or gas–liquid chromatography (GC)[4].

Previously Skaare and Dahle[5] reported on the stability of EQ as determined by GC with mass spectrometry (GC–MS) and described the extreme sensitivity of the compound on exposure to light in chloroform solution noting a 35–70% loss of GC-measurable EQ. We have also studied the degradation of EQ and consequent changes in its analytical characteristics by LC and GC and reported[6] that EQ in methanol solution is easily converted into another substance upon exposure to daylight, resulting in the loss of its fluorescence characteristic. EQ is comparatively stable in a closed vessel in the dark, while in aged EQ many impurities can be detected by GC–MS[7]. The GC column temperature in these cases was 160 or 180°C and the peaks due to impurities were situated quite close to that of EQ. Nishikawa[7] described the chemical structure of the dimer produced by the oxidation of EQ, but neither its occurrence, nor its liquid and gas chromatographic behaviour have been described.

In this paper we report that EQ yields mainly its dimer upon autoxidation and the dimer is converted into many other compounds upon exposure to daylight.

EXPERIMENTAL

Apparatus

The LC equipment consisted of a Shimadzu LC-3A chromatograph, an UV detector UVD-2 operating at 254 nm, a spectrophotometric detector SPD-1 operating at 380 nm and a spectrofluorometric detector RF-530 (excitation wavelength, 360 nm; emission wavelength, 440 nm) (Shimadzu Seisakusho, Kyoto, Japan). The column used was an ODS-silica TSK-Gel LS-410 (Toyo Soda, Tokyo, Japan) packed in stainless steel (250 × 4.0 mm I.D.).

The GC equipment used was a Shimadzu GC-6A chromatograph with a flame ionization detector Model FID-6. The column was 3% OV-101 on Gas-Chrom Q (80–100 mesh) packed in Pyrex glass (100 × 3.0 mm I.D.). The carrier gas was nitrogen. For recording, integration and calculation of peak areas a Shimadzu Chromatopack C-R1A was used.

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The mass spectrometer was a JEOL Model 01SG-2 high resolution mass spectrometer (HRMS) (Japan Electron Optics, Akishima City, Tokyo, Japan) provided with an automatic data acquisition and management system by means of a NOVA01 minicomputer (Data General, Westboro, MA, U.S.A.). The software used was developed by the authors and perfluorokerosene was used as an internal standard. Mass spectra was measured under the following conditions: ionizing voltage, 75 eV; accelerating voltage, 10 kV; ionizing current, 200 mA. Solid samples were directly introduced into the ionization chamber.

A JEOL FX200 NMR spectrometer with a 4.7-T superconductive magnet was used. It was equipped with a floppy disk-based data-processing system supplied by the same manufacturer. The spectra of EQ and its derivatives in chloroform solution were measured.

Chemicals

Ethoxyquin (EQ) of 99.2% purity was obtained from Kohlin Chemical Co. (Higashi-Osaka-City, Japan) and no other substances were detected by LC and GC. The methanol and other reagents were of analytical grade. Toyo filter-paper No. 5A (11 cm diameter) was employed.

Procedure

Degradation of EQ on filter-paper. Five ml of a 0.1% methanol solution of EQ were pipetted onto a piece of filter-paper in the form of spherical spot of about 7.5 cm in diameter. Each spotted paper was covered with a petri dish in a dark room (A), a dimly litarea (B) or a light place (C) within the laboratory. After 4 months each filter-paper was extracted with 200 ml of methanol and the extracts were stored in a refrigerator.

LC analysis. A 5-μl volume of the extract was injected into the chromatograph equipped with UV (254 and 380 nm) detectors of sensitivity 16×10^{-2} a.u.f.s. and with a fluorometric detector of sensitivity L $\times$ 16 (arbitrary apparatus unit). The mobile phase was methanol–water (90:10) and the flow-rate 1.0 ml/min. The column temperature was ambient.

GC analysis. Two μl of the extract were injected in the gas chromatograph at detector sensitivity of 0.5 (V) $\times$ 10^2 (MΩ). The column temperature was programmed at 160°C for 1 min and then increased at a rate of 10°/min to 280°C until all the peaks had emerged. The carrier gas flow-rate was 60 ml/min.

Preparation of pure dimer. A crystal of dimeric EQ was obtained by concentrating the combined extracts (200 ml) and left to stand in a refrigerator. It was recrystallized three times from methanol. The purified dimer was used for MS and NMR measurements and photochemical degradation experiments.

Photochemical degradation of EQ dimer. A 0.1% methanol solution of the EQ dimer in glass test-tubes (1.5 cm I.D.) was either exposed to daylight or placed in a light area within the laboratory.

RESULTS AND DISCUSSION

Degradation of EQ and production of the dimer

EQ was spread on a filter-paper, representing a matrix for feeds or foods, and

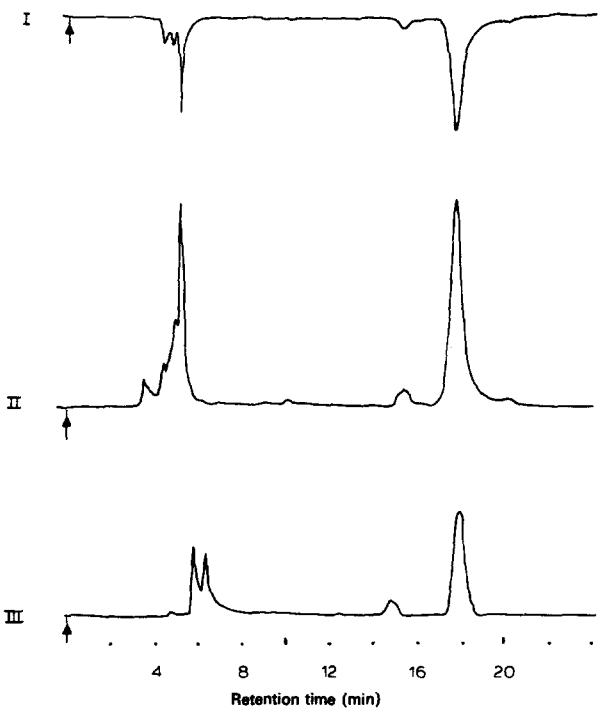


Fig. 1. HPLC chromatograms of the products of degradation of EQ on a filter-paper in dim light. Detections: I, UV (380 nm); II, UV (254 nm); III, fluorometric (excitation, 360 nm; emission, 440 nm).

left to stand in the air with or without light for 4 months. The results obtained under the dimly lit condition (B) are shown by the LC chromatogram (Fig. 1) with UV detection at 380 nm (I) or 254 nm (II) and with fluorometric detection (III), and the results obtained by GC are illustrated in Fig. 2.

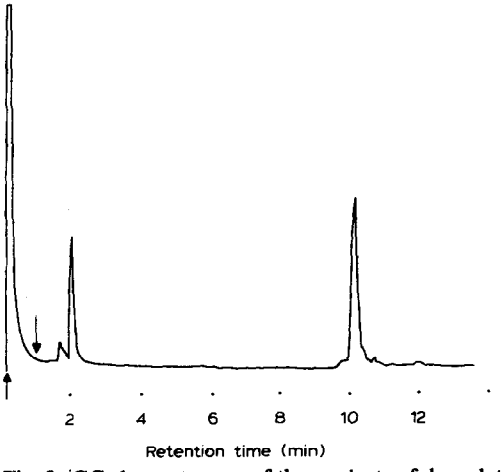


Fig. 2. GC chromatogram of the products of degradation of EQ on a filter-paper in dim light.

TABLE I

PERCENTAGE COMPOSITION OF DEGRADED ETHOXYQUIN ACCORDING TO THE PEAK AREAS DETERMINED BY TEMPERATURE PROGRAMMED GAS–LIQUID CHROMATOGRAPHY

Retention time (min)	*Sample*		
	A	*B*	*C*
1.47	0.6	0.9	0.62
1.8	5.92	6.33	5.69
2.18	31.29	34.0	51.02
9.8	1.74	2.35	1.29
10.13	58.05	56.41	41.38

Under the LC conditions the retention time of EQ was about 5 min and a large main peak was eluted at about 18 min. The results obtained from the samples in the absence of light (A) and in the presence of light (C) were similar to those obtained from sample B, but the main peak heights were smaller for sample C.

The GC retention time of the main LC peak fraction was about 10 min (column temperature: *ca.* 260°C). This fraction was identified as the dimer of EQ as described later. Under this programmed GC condition, EQ was eluted at about 2 min (column temperature: *ca.* 180°C). The GC chromatograms obtained from samples A–C were very similar and the percentage compositions according to peak area are shown in Table I. The peak area of the dimer from samples A, B and C occupied respectively 58.0, 56.5 and 41.4% of the total peak areas and there was a tendency for the peak area to decrease with increasing exposure to light.

The substances eluted within 3–6 min by LC and 1.4–2 min by GC were largely the degradation products of EQ and the amount of residual EQ found was small.

Identification of the dimer

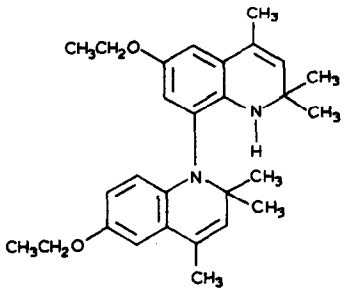
Analysis of the crystal, obtained from the LC fraction of retention time 10 min, by high-resolution MS, ^{13}C and H NMR spectrometry revealed that the compound was the dimer of EQ. The main characteristics of the spectra can be summarized as follows.

Common ions observed in the high resolution mass spectra included an ion cluster whose molecular formula was consistent with the dimer of EQ, $[M]^+$ (obs., 432.2758; calc., 432.2768), and its ^{13}C isotope (obs., 433.2818; calc., 433.2800), $[M-CH_3]^+$ (obs., 417.2676; calc., 417.2534), $[M-C_2H_5O]^+$ (obs., 387.2444; calc., 387.2429), $[M-CH(CH_3)_2-NH_2]^+$ (obs., 373.1912; calc., 373.2140) and the base peak of mass to charge ratio 201.1163, $[C_{13}H_{15}ON]^+$ (calc., 201.1153). The latter must be the fragment ion produced by monomerization and cleavage of a methyl function of the dimer.

The 50.1-MHz ^{13}C NMR spectrum shows resonance lines of CH_3(15.7, 19.3, 19.4, 28.2, 28.9, 30.7 and 31.3 ppm), $-CH_2-$(64.7 and 65.0 ppm), –CH–(none), quaternary sp^3 carbons (52.1 and 57.4 ppm) =CH– (110.8, 111.5, 114.7, 118.3, 131.1 and 131.6 ppm) and =C–(127.4, 128.7, 137.6 and 150.9 ppm). The 200-MHz 'H NMR spectrum shows signals at 0.96 (s,1H), 1.17 (s,3H), 1.22 (s,3H), 1.29 (s,3H), 1.37

(m,6H), 2.00 (s,3H), 2.06(s,3H), 3.97 (m,4H), 5.37 (s.1H), 5.45 (s,1H), 6.05 (d,1H), 6.48 (d,1H), 6.68 (s,1H) and 6.47 ppm (m,2H) respectively.

Comparison of ^{13}C NMR spectra of the parent and the derivative reveals that the frequency of the methyl resonance of the ethoxyl function appeared at the same position and one of the $-CH_2O$ carbons shows a slight (0.3 ppm) downfield shift, while the chemical shifts of the quaternary carbons adjacent to nitrogen and the neighbouring two methyls suffered downfield shifts of several ppm. The loss of one olefinic CH carbon resonance near 114.7 ppm and assignment of the ^1H NMR spectra of the aromatic resonance region and the protons attached to sp^3 carbons confirm that the product is formed by oxidative dimerization at the NH group of one EQ molecule and at the carbon at position 8 of the other. The chemical structure is thus as follows:



Ethoxyquin dimer : $C_{28}H_{36}O_2N_2$ (mw = 432.2768)

When the dimer was left to stand for a few weeks in methanol, the characteristic fluorescence of the dimer disappeared. LC chromatograms of a methanol solution (0.1%) of the EQ dimer left in the dark (a) or exposed to daylight (b) for 2 days are in Fig. 3a and b. Following exposure to daylight, the peak of the dimer was lost

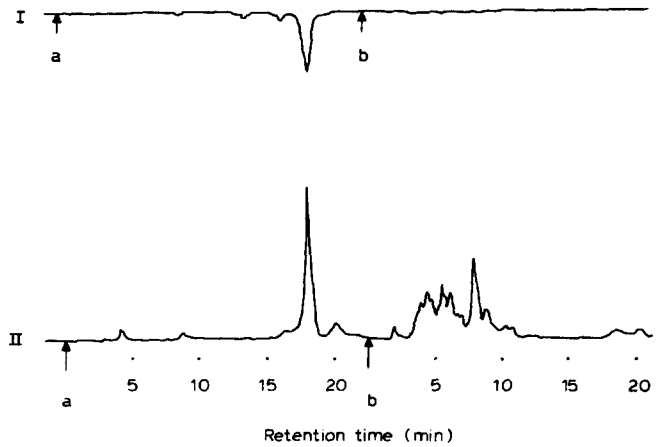


Fig. 3. HPLC chromatograms of the EQ dimer in methanol solution (0.1%) without light (a) and exposed to daylight (b). Detections: I, fluorometric (excitation 360 nm; emission, 440 nm); II, UV (254 nm).

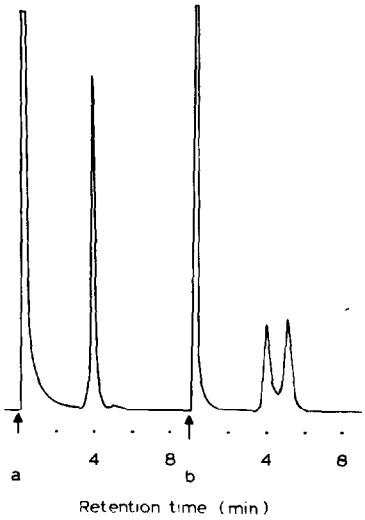


Fig. 4. GC chromatograms of the EQ dimer in methanol solution (0.1%) stored without light (a) and exposed to daylight (b). Column temperature: 260°C.

and many new peaks were observed at retention times of 1–8 min. Corresponding GC Chromatograms for experiments a and b are in Fig. 4a and b.

To elicit intermediate degradation, the same methanolic solution was exposed to daylight for 1 h (a), 2 (b) or 6 h (c) and subsequently examined by LC. The respective chromatograms are in Fig. 5a, b and c. The series of peaks suggested that there are many steps in the photochemical conversion of the dimer. Some of the LC peaks were fractionated and examined by GC at a column temperature of 260°C. The substances eluted from 9.5 to 13 min by LC (Fig. 5) produced peaks having GC retention times nearly the same as for the dimer fraction. It is suggested thar these fractions comprised derivatives of the dimer. Some of the substances that underwent greater conversion as shown in Fig. 3b were in part eluted at nearly the same GC retention time as the dimer, and the other derivatives seem to have longer retention times as illustrated in Fig. 4b.

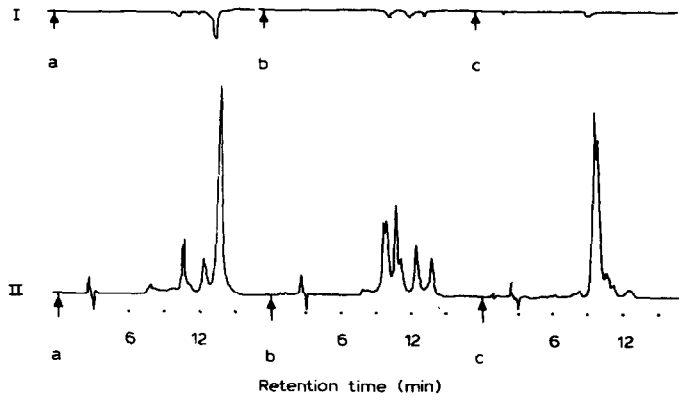


Fig. 5. HPLC chromatograms of the EQ dimer in methanol solution (0.1%) exposed to daylight for 1 (a), 2 (b) and 6 h (c). Detectors: I, UV (380 nm); UV (254 nm). Flow-rate: 1.5 ml/min.

These LC and GC data suggest that more polar compounds are derived from the dimer by successive exposure to daylight.

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