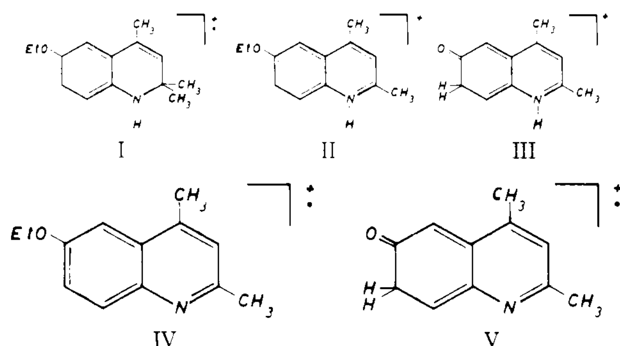
structure of the molecular ion is illustrated in formula I. (2) The principal peaks below the parent ion region of the spectrum are at masses 202, 174, 144, and 83. The formation of some of the fragment ions can be explained as follows: the base peak of the spectrum is accounted for by the loss of a methyl radical in β position to the nitrogen atom of the molecular ion producing the very stable quinolinium structure (formula II) with a corresponding m/e value of 202. The intense peak at base peak minus 28 is attributed to the loss of a C_2H_4 neutral fragment resulting from a McLafferty rearrangement (McLafferty, 1959) and a possible structure of this fragment is illustrated in formula III. The fragment of mass 144 is a characteristic ionic feature of a quinoline derivative.

Doing repetitive scanning of mass spectra, the GLC peak at the retention time of 5.8 min is found to contain several quinoline derivatives. One of the most abundant components was identified by mass spectrometry to be 6-ethoxy-2,4-dimethylquinoline. The molecular weight of this component can be seen to be 201 from the mass spectrum in Figure 2b. The structure of the molecular ion is illustrated in formula IV. The relative abundance of the molecular ion is 60% as compared to 12% for ethoxyquin. These relative abundances reflect the different stabilities of the two molecular ions. The base peak of this spectrum is attributed to the molecular ion undergoing fragmentation via the McLafferty rearrangement. A suggested structure of the base peak of m/e 173 is illustrated in formula V.



When scanning mass spectra along the GLC profile of the chloroform solution of ethoxyquin, a mass spectrum with a molecular ion of mass 216 and a fragmentation pattern comparable to that of ethoxyquin was seen. The structural formula of this compound may well be that of a radical which has been shown to be present in the solution by electron spin resonance spectroscopy (ESR) (Skaare and Henriksen, 1975). A radical with the quinoline structure intact has also been proposed as the intermediate for the formation of the dimer of ethoxyquin which is thought to be the main oxidative product when EMQ is acting as an antioxidant (Gordon and Maddy, 1958). However, using the above-mentioned GLC conditions we did not succeed in isolating or identifying any dimer of EMQ.

The stability of ethoxyquin in the hexane solutions as compared to the observations of the color change and decrease in concentration when dissolved in chloroform indicates that hexane should be used as solvent in analytical and extraction work with ethoxyquin. Likewise, gas–liquid chromatography using an SE-30 column seems to be applicable as a method of analysis when the quantitative determination of EMQ is required. This method, applied to the quantitative estimation of ethoxyquin in various biological materials, will be published (Dahle and Skaare, 1975).

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Gas Chromatographic Determination of Ethoxyquin in Feed and Food Products. II

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A procedure is described for the estimation of the antioxidant ethoxyquin in feed and food products. It involves homogenization of the samples, extraction with methanol, and extraction of the antioxidant from the extract using n-hexane. Gas–liquid chromatography (GLC) with a flame ionization detector is used for quantitative determination, and GLC combined with mass spectrometry (MS) is applied for the confirmation of identity. Only the oxidation inhibitor 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ), which has not been involved in the antioxidative processes, is determined gas chromatographically. Recovery studies adding ethoxyquin at levels of 100 ppm to fish meal, fish meat, and broiler meat showed that about 30% of the GLC measurable antioxidant was recovered, whereas approximately 70% was recovered from water.

Most of the methods described for the determination of ethoxyquin in feed and food products are based on fluorimetric principles built out for paper and thin-layer chromatography (Gordon and Maddy, 1958; Weilmann et al., 1972) or spectrophotometric equipment (Bickoff et al., 1956; Gordon et al., 1964; Witt et al., 1973). Color methods have also been used for routine work and some of these methods are reviewed in Fishing News International (1970). Gas–liquid chromatography (GLC) has previously not been reported as an analytical procedure for the determination of ethoxyquin. This could probably be due to the

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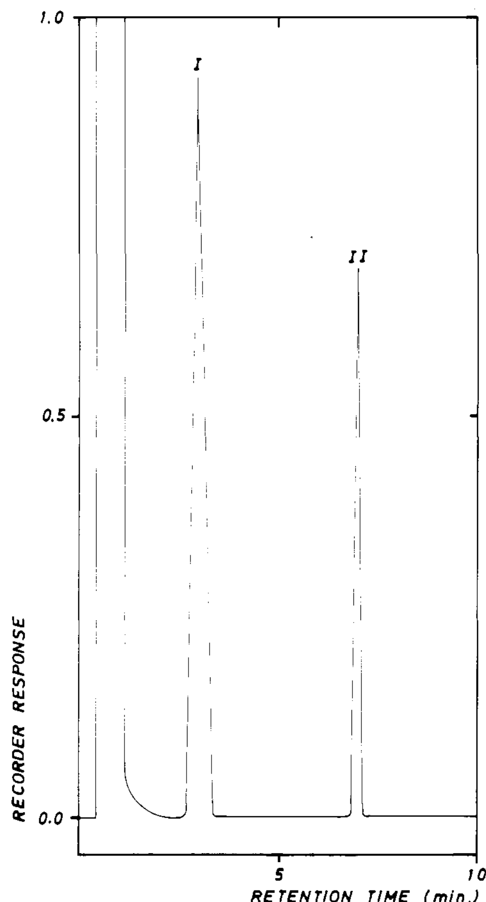

Figure 1. Gas chromatogram of 2 μl of an *n*-hexane solution containing 1.0 mg/ml of ethoxyquin and 1.5 mg/ml of the internal standard quinoline analyzed on a 3% SE-30 column; temperature programmed from 90 to 200° at 15°/min. A flame ionization detector was used.

continuing oxidative changes of the parent molecule 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) observed in some of the most commonly used solvents and the proposed formation of polymers when EMQ is working as an antioxidant (Gordon and Maddy, 1958). However, the sensitivity of a GLC method and the specificity which is obtained when GLC is combined with mass spectrometry (MS) still make gas chromatography applicable for determination of this oxidation inhibitor. Furthermore, the EMQ molecule has been shown to be sufficiently stable in hexane solutions, and the GLC parameters and the mass spectra of the EMQ itself and some of its derivatives have already been described (Skaare and Dahle, 1975). When GLC is to be used for quantitative estimation, a suitable internal standard has to be found. In this paper we present a GLC method for quantitative measurement of EMQ in different biological systems when quinoline (QI) is used as the internal standard.

MATERIALS AND METHODS

Feed and Food Products. The recovery studies were carried out on Norwegian fish meal based on *Mallotus villosus* (approximately 90% dry weight, 70% Kjeldahl protein, and 7% fat), fish meat of rainbow trout (*Salmo irideus*), and broiler meat.

Chemicals. Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) (EMQ) (pure) was obtained from Koch-Light Laboratories Ltd. Quinoline (QI), methanol, *n*-hexane, acetone, and sodium sulfate, all of analytical grade, were obtained from E. Merck, Darmstadt, Germany. The *n*-hexane had to be redistilled before use.

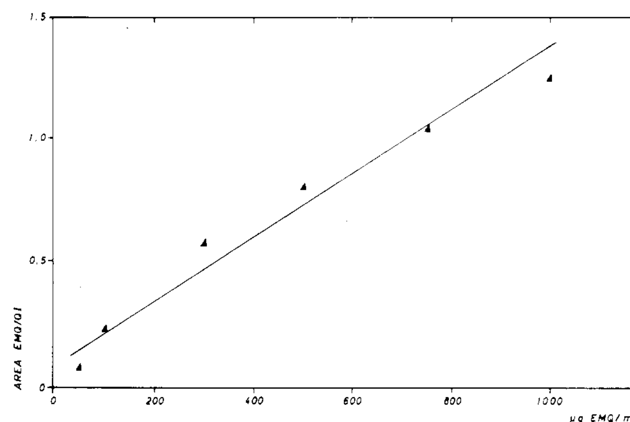

Figure 2. Relative area of ethoxyquin and the internal standard plotted vs. concentrations of ethoxyquin.

Instruments. A Varian Model 2100 gas chromatograph equipped with a flame ionization detector was used. The column was a 3 m × 3 mm i.d. glass tube packed with 3% SE-30 on acid-washed, DMCS-treated Chromosorb W, 80–100 mesh (Applied Science Lab., Inc., State College, Pa.). Operating conditions were as follows: temperatures, column 90–200° programmed at 15°/min, inlet at 220°, detector at 220°; flow rates (milliliters/minute), carrier gas (nitrogen) 30, hydrogen 30, air 300; range, 10^{-11}, 10^{-10}; attenuator setting, 8, 16; 0.1–2.0 μg of the hexane extract was injected. EMQ (10 ng) injected on the column could be determined utilizing the described operating parameters.

Experimental. *Preparation of Standard Curve.* The following standard solutions of ethoxyquin dissolved in *n*-hexane were prepared: 1.00, 0.75, 0.50, 0.25, 0.10, and 0.05 mg/ml. Quinoline was added to each of the standard solutions to a final concentration of 1.50 mg/ml. Two microliters was injected on the column and the ratio of the area of EMQ and QI was plotted against the concentrations of EMQ (Figure 2).

Recovery Studies. The isolation of ethoxyquin from feed and food products was carried out mostly as described by Witt et al. (1973). Ethoxyquin (1.0–0.1 mg) dissolved in methanol was added to 5-g samples of fish meal, fish meat, and broiler meat together with 3 mg of the internal standard (QI) also dissolved in methanol. Following the addition of 10 ml of water, the samples were homogenized for 5 min in a homogenizing machine. Water (50 ml) had to be used in the analysis of the fish meal. The water was added to facilitate the homogenization procedure and to favor the phasic distribution step with hexane.

Following the addition of 125 ml of methanol to the homogenates the samples were shaken for 30 min, and after centrifugation, the supernatants were adjusted to 150 ml with methanol. Two aliquots of 75 ml each were transferred to separatory funnels and extracted twice with 50-ml portions of *n*-hexane (3 min). The organic phases were dried over sodium sulfate and concentrated to the final volume of 2 ml using a Kuderna-Danish evaporator in a water bath held at 70°. Less than 5% of the antioxidant was lost in this step. On the column, 0.1–2.0 μg (2-μl aliquots) was injected.

RESULTS AND DISCUSSION

The GLC profile of ethoxyquin and the internal standard is shown in Figure 1, when dissolved in *n*-hexane. The identity of the main peak I is 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) and II is quinoline (QI) as confirmed by mass spectrometry (Skaare and Dahle, 1975).

Since EMQ is an unstable compound undergoing continuing oxidative changes in biological materials and in most solvents, *n*-hexane should be used as solvent for extraction and analytical work (Skaare and Dahle, 1975).

Table I. Recoveries of Ethoxyquin following Addition of the Antioxidant to Broiler Meat, Fish Meat, Fish Meal, and Water

Sample, 10 g	No.	Amount added, mg	Recovery ($\bar{x}$), %	Std dev (S_x), %
Broiler meat	10	1	36	1.6
Fish meat	10	1	34	1.4
Fish meal	8	1	28	1.1
Water	6	1	72	1.2

Only EMQ (mol wt 217) is determined in the GLC analysis. Therefore, the choice of solvent is important for the loss of GLC measurable antioxidant caused by oxidation in the analytical procedure to be kept at a minimum.

For quantitization of the antioxidant, a suitable internal standard is required. Quinoline (QI) is found acceptable in these systems. Figure 2 shows the ratio of the area of EMQ and QI as plotted against different concentrations of EMQ. The linearity is observed in the range from 0.1 to 1.0 mg/ml corresponding to 0.2 to 2.0 μg of EMQ injected. The lower limit of detection is 10 ng.

Table I gives the results of the recovery studies. Only about one-third of the antioxidant added to the three different biological materials is found as GLC measurable EMQ following the homogenization and clean-up procedure. Recoveries of approximately 70% of the ethoxyquin added to water are found, which indicates a loss in the analysis of only 25–30%.

Even though the experimental conditions varied with respect to time (2–20 days) due to the practical limitations of the laboratory capacity, it is seen in Table I that the standard deviations of the recoveries are all acceptable. The observed difference of the recoveries from water and biological systems is probably explained by EMQ working as an antioxidant in the biological redox system. Earlier experiments carried out by Monsanto Chemical Co. (Gordon and Maddy, 1958) showed that the primary oxidation products of EMQ, in addition to EMQ itself, have antioxidative effects. Therefore, the recovery of 30% observed, regardless of the biological system to which the antioxidant is added and the variations of time between homogenization and analyses of the homogenate, may indicate that a spontaneous consumption of the GLC measurable antioxidant is taking place until an intermediate equilibrium state is reached. The general oxidation inhibition is then proceeding.

As can be seen antioxidants pose a special analytical problem since a static image has to be established of a dynamic system. This study presents a method of determination of unchanged or unreacted antioxidant 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (EMQ) in feed and food products.

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Characterization of Bound Residues of Nitrofen in Cereal Grains

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Starch isolated from the grain of mature rice and wheat which contained radioactive residues resulting from the preemergence use of ^{14}C-labeled nitrofen was found to be radioactive. The starch was hydrolyzed to glucose and derivatized to the osazone with phenylhydrazine. The osazone was isolated and recrystallized several times. From 64 to 90% of the radioactivity in the rice and wheat grain was found to be in the starch, leading to the conclusion that the ^{14}C from the nitrofen had been reincorporated into glucose and subsequently into starch.

Nitrofen (I), 2,4-dichloro-1-(4-nitrophenoxy)benzene, is a selective herbicide which has been used under the trade name of TOK to control annual grasses and many broadleaved weeds. This herbicide is currently registered for use on many crops in the United States.

Nitrofen can be metabolized in vivo to the corresponding amine (II) and to the acetamide (III) [Gutenman and Lisk, 1967; Adler et al., 1971]. It has also been shown that ^{14}C from nitrofen-^{14}C can be found in the lignin from ^{14}C-treated rice and wheat straw (Honeycutt and Adler, 1975). Even though diphenyl ether cleavage of this herbicide has not been reported in plants, Frear and Swanson (1973) have shown that the diphenyl ether herbicide fluorodifen is readily metabolized in peas at the diphenyl ether linkage.

In the course of work with the use of nitrofen-^{14}C as a preemergence herbicide in rice and wheat plots, we obtained grain from these two crops which contained ^{14}C. Very little of the radioactive residue, however, could be removed by conventional solvent extraction techniques. These residues are commonly referred to as bound residues.

A high percentage of grain is composed of starch ("Food and Life", 1959). The studies reported here were undertaken to determine if starch isolated from grain of wheat and rice crops treated with nitrofen-^{14}C contained radioactivity and whether the radioactivity was incorporated into the natural metabolic pool.

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