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International Fishmeal and Oil Manufacturers Association

DETERMINATION OF ETHOXYQUIN AND TWO OF ITS OXIDATION PRODUCTS IN FISH MEAL BY GAS CHROMATOGRAPHY

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EXECUTIVE SUMMARY

In the mid-1980's the Association sponsored a PhD research project with the University of St Andrews and Torry Research Station, Scotland to fundamentally study the chemistry of the breakdown of ethoxyquin when used for stabilising fish meal. This study was considered of commercial importance because on occasions although fish meal had been satisfactorily treated with ethoxyquin it remained surprisingly unstable resulting in occasional commercial losses. On the other hand on many occasions it had been found that although ethoxyquin was virtually absent in fish meal following treatment with this product, the meal remained stable. It thus seemed that the breakdown products of ethoxyquin may affect the subsequent stability of the fish meal. The research project (published as IAFMM Research Report 1987-2) showed that the two main breakdown products of ethoxyquin were a quinolone and a dimeric oxidation product of ethoxyquin (dimer). The quinolone appeared to have reasonable antioxidant properties although not as good as the ethoxyquin, whereas the dimer was a poor antioxidant.

A research project was commissioned with the Fishing Industry Research Institute in South Africa to see whether normally accepted analytical techniques would distinguish between pure ethoxyquin, the quinolone and the dimer.

Research undertaken at FIRI showed that a standard method for measuring ethoxyquin yielded frequently incorrect values as a result of the poor separation of ethoxyquin from the dimer as well as other unidentified yellow substances in the meal.

A revised analytical method is detailed in this research report together with a proposal for calculating ethoxyquin equivalents in fish meal based on separately being able to identify the level of quinolone in the meal and the dimer. By calculating that the quinolone has approximately three-quarters and the dimer approximately one-third of the efficacy of ethoxyquin, it is possible to calculate the antioxidant content expressed as ethoxyquin equivalents. Additionally, it has been possible in a number of instances to determine the dimer in an aged meal when no ethoxyquin could be detected. This test therefore may serve as evidence that the meal had originally been treated with ethoxyquin.

This research work is still continuing with two antioxidant treated fish meals under commercial conditions being analysed over a period of time for ethoxyquin and its breakdown products.

Determination of Ethoxyquin and Two of Its Oxidation Products in Fish Meal by Gas Chromatography

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A gas chromatographic procedure for the determination of the antioxidant ethoxyquin (EQ) in fish meals using methoxyquin as internal standard is described. Recoveries of EQ from spiked meals ranged from 95 to 107%. Comparison of the results obtained with the proposed method and those given by a method applied on a routine basis using chromatography on alumina shows that the results obtained with the latter method should, on average, be corrected by multiplication by a factor of 1.1 when dealing with meals having EQ contents above approximately 30 mg kg^{-1}. Meals with EQ contents of less than 30 mg kg^{-1} yield values by chromatography on alumina which are too high. In addition, gas chromatographic methods for the determination of two oxidation products of EQ, *viz.*, a dimeric oxidative coupling product and a quinolone, are described. The progressive decrease of EQ and the simultaneous formation of the two oxidation products on storage of five fish meals is demonstrated.

Keywords: *Fish meal; ethoxyquin; methoxyquin; ethoxyquin dimer; quinolone*

1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline (**1**), commonly known as ethoxyquin (EQ), is used extensively as an antioxidant in fish meal to protect the highly unsaturated residual lipids in the meal.[1,2] In South African and Namibian fish meals EQ is normally added at a level of 400 mg kg^{-1}, whereas in South American meals it can exceed levels of 1000 mg kg^{-1}.

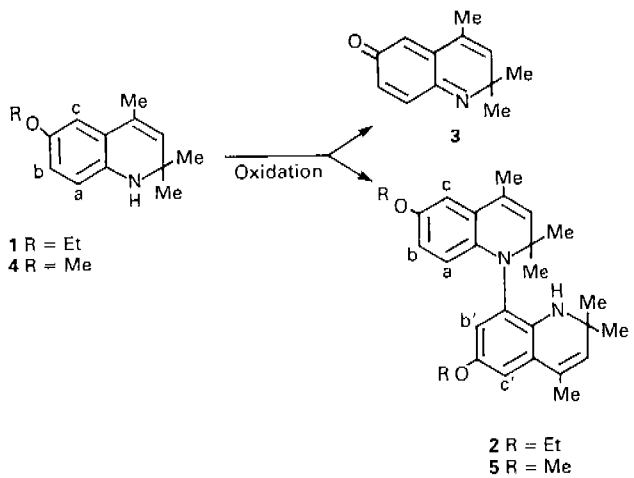
Ethoxyquin is determined on a routine basis in this Institute by separating it in a hexane extract of the meal from other hexane-soluble compounds by chromatography on alumina. The progress of the separation is monitored with an ultraviolet (UV) lamp as EQ is strongly fluorescent. After dilution, the amount of EQ is determined spectrophotometrically at 362 nm.[1]

The fate of EQ in fish meals was not known until Thorisson[3] showed that oxidation of EQ with *tert*-butylhydroperoxide leads to the formation of two compounds, *viz.*, a dimeric oxidative coupling product, 1,8′-di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) (**2**, EQ-dimer), and a quinolone, 2,6-dihydro-2,2,4-trimethyl-6-quinolone (**3**).

It was claimed by Thorisson[3] that the EQ-dimer (**2**) showed no antioxidant activity, whereas the quinolone (**3**) was only slightly less active than EQ. It was also found that both the EQ-dimer and the quinolone were present in a fish meal treated with EQ after storage for 1 week at ambient temperature.

In view of these results it was questionable whether the above procedure for the determination of EQ was sufficiently specific to distinguish it from the EQ-dimer and the quinolone. Hence, a gas chromatographic method using methoxyquin, 1,2-dihydro-6-methoxy-2,2,4-trimethylquinoline (**4**, MQ), as internal standard was developed and the results obtained by this method were compared with those of the routine procedure. In addition, the decrease in EQ during storage of a number of fish meals and the simultaneous formation of the EQ-dimer and the quinolone was demonstrated.

Gas chromatographic methods for the determination of EQ have been described[4–6] but these methods do not use MQ as internal standard and no analysis for the oxidation products of EQ has been carried out.



Experimental

Apparatus and Reagents

Ultraviolet spectra in hexane were recorded with a Varian Superscan 3 spectrophotometer and the molar absorptivity (ε) was calculated at the maximum absorption.

Elemental analyses were carried out with a Heraeus universal composition analyser.

Gas chromatography was carried out on a Hewlett-Packard 5710A gas chromatograph using a 15 m fused silica capillary column with an i.d. of 0.25 mm having a DB-1 liquid phase coating and using hydrogen as carrier gas.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded in $CDCl_3$ solution on a Varian VXR 200 superconducting spectrometer with tetramethylsilane (TMS) as internal standard.

All chemicals used were of analytical-reagent grade.

Fish Meals

Meals treated or not treated with EQ were obtained from a local fish meal factory. They were transported to the Institute in polypropene buckets where they were stored, after pure EQ had been added to untreated meals (see ref. 1), at 25 or 70 °C.

Preparation of Compounds

Ethoxyquin (**1**)

Pure EQ was obtained by distillation of a commercial product under high vacuum as described elsewhere.[7] It was obtained as a yellow fluorescent oil, $n_D^{20} = 1.5734$.

Methoxyquin (**4**)

Methoxyquin was synthesized from 4-methoxyaniline and acetone in the presence of a catalytic amount of iodine.[8] It was obtained as a yellow fluorescent oil, $n_D^{20} = 1.5843$.

1,8′-Di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) (**2**) *and 2,6-dihydro-2,2,4-trimethyl-6-quinolone* (**3**) *from EQ*

The EQ-dimer (**2**) and the quinolone (**3**) were prepared by oxidation of EQ with *tert*-butylhydroperoxide essentially as described by Thorisson.[3] The EQ-dimer was obtained in 36% yield as khaki fluorescent crystals, m.p. 108–109 °C (literature value[3] 107–109 °C). Ultraviolet in hexane: $\varepsilon_{375\ nm}$ = 8263 dm^3 mol^{-1} cm^{-1} [literature value[3] in light petroleum (40–60 °C boiling range): $\varepsilon_{380\ nm}$ = 5370 dm^3 mol^{-1} cm^{-1}]. The quinolone was obtained in 14% yield as a brown non-fluorescent material, m.p. 54–56 °C (literature value[3] 52–53.5 °C). Ultraviolet in hexane: $\varepsilon_{345\ nm}$ = 5386 dm^3 mol^{-1} cm^{-1} (literature value[3] in ethanol: $\varepsilon_{364\ nm}$ = 6610 dm^3 mol^{-1} cm^{-1}).

1,8′-Di-(1,2-dihydro-6-methoxy-2,2,4-trimethylquinoline) (**5**) *and 2,6-dihydro-2,2,4-trimethyl-6-quinolone* (**3**) *from MQ*

The MQ-dimer (**5**) was prepared by oxidation of MQ in a similar way to the preparation of the EQ-dimer. The MQ-dimer was isolated in 23% yield as yellow fluorescent crystals, m.p. 108–109 °C. Proton NMR ($CDCl_3$, 200 MHz): δ 0.96 (3 H, s), 1.16 (3 H, s), 1.21 (3 H, s), 1.29 (3 H, s), 1.99 (3 H, d, J = 1.40 Hz), 2.06 (3 H, d, J = 1.40 Hz), 3.73 (3 H, s), 3.75 (3 H, s), 4.03 (1 H, s, NH), 5.37 (1 H, d, J = 1.40 Hz), 5.45 (1 H, d, J = 1.30 Hz), 6.07 (1 H, d, J = 8.85 Hz, H^a), 6.50 (1 H, d of d, J = 8.85 and 2.93 Hz, H^b), 6.67 (1 H, d, J = 2.76 Hz, H^c), 6.71 (1H, d, J = 2.73 Hz, H$^{c'}$) 6.75 (1 H, d, J = 2.90 Hz, H$^{b'}$). Ultraviolet in hexane: $\varepsilon_{375\ nm}$ = 8349 dm^3 mol^{-1} cm^{-1}. [Found: C, 77.1; H, 7.6; N, 7.0. Calc. for $C_{26}H_{32}N_2O_2$ (404.56): C, 77.19; H, 7.97; N, 6.93%.]

The quinolone (**3**) was isolated in 17% yield.

Antioxidant Properties of the EQ-dimer (2) and the Quinolone (3)

In order to establish whether the EQ-dimer and the quinolone possess antixodant properties they were tested together with EQ in fish oil by the active oxygen method as described previously.[8]

Determination of EQ in Fish Meal

Ethoxyquin was determined in fish meals by two procedures.

Column chromatography on aluminium oxide followed by spectrophotometry

A column of alumina (Grade IV, Brockmann; 10 × 2 cm) was prepared in hexane in a glass tube (30 × 2 cm) with a sintered glass disc and a tap. Anhydrous sodium sulfate was added to a depth of 2 cm above the alumina and the glass tube was filled with hexane to about 8 cm from the top.

A 5.0 g amount of fish meal was poured onto the top of the column, the tap was opened and the hexane allowed to run until it was about 2 mm from the top of the meal. A total of 50 cm^3 of hexane was used to elute the EQ from the meal onto the alumina and the collected hexane was discarded. Ethoxyquin was then eluted with hexane–diethyl ether (9 + 1 v/v). The progress of the elution was followed with a UV lamp, and just before the fluorescent band reached the end of the column the eluate was collected in a 100 cm^3 calibrated flask. All the EQ was collected, the volume made up to 100 cm^3 with hexane and the absorbance read at 362 nm. The amount of EQ in mg cm^{-3} was found by multiplying the absorbance at 362 nm by 0.067[7] (and not 0.074[1]). With older meals it was frequently possible to distinguish a light blue fluorescent band below EQ. This band, which consists of the EQ-dimer (**2**) (see above), was always separated as effectively as possible from the EQ.

Gas chromatography using MQ as internal standard

To 5.0 g of fish meal was added a small amount (1–3 mg) of accurately weighed MQ. This was performed conveniently by using a small finely drawn glass capillary and weighing on a five decimal place balance. The meal was treated with 50 cm^3 of hexane and the mixture warmed on a water-bath until it just boiled, whereupon the hexane was filtered into a 250 cm^3 separating funnel. The extraction was repeated twice and 50 cm^3 of 1 mol dm^{-3} hydrochloric acid were added to the combined hexane extracts. After vigorous shaking, the lower layer was removed and the extraction repeated twice. The combined aqueous layers were made alkaline by the addition of about 15 g of sodium hydroxide pellets while cooling under running tap water. The alkaline solution was extracted with three 50 cm^3 portions of hexane; the combined hexane extracts were washed once with water and dried over anhydrous sodium sulfate. After filtration, the hexane was evaporated using a water jet pump on a rotary evaporator (temperature below 60 °C) and the residue taken up in a small volume (about 0.5 cm^3) of isooctane. This solution was injected into the gas chromatograph. The temperature was programmed from 100 to 150 °C rising at 4 °C min^{-1} while the injection port remained at 200 °C. Quantification was carried out with a Spectra-Physics 4270 integrator. A minimum of three injections were routinely carried out and the mean was calculated.

The procedure was checked on a matured untreated meal spiked with EQ in the range 150–850 mg kg^{-1}.

Chromatographic Properties of the EQ-dimer (2), the Quinolone (3) and EQ on Alumina

The following experiments were carried out to determine whether the EQ-dimer and the quinolone were sufficiently separated on alumina in the routine determination of EQ.

Separation of the EQ-dimer (**2**) *and EQ*

(*i*) A mixture of 1.06 mg of the dimer and 1.94 mg of EQ was placed on a column of active alumina (Grade I, 10 × 2 cm) dissolved in 2 cm^3 of hexane. Elution with hexane and hexane–diethyl ether (50 + 1 v/v) failed to elute any fluorescent material. Elution with 100 cm^3 of hexane–diethyl ether (4 + 1 v/v) eluted the dimer, as was evident from its absorption maximum at 375 nm. The absorbance at 375 nm of the 100 cm^3 solution was 0.190, corresponding to 0.99 mg (93%) of the EQ-dimer.

Elution with 100 cm^3 of diethyl ether yielded EQ. The absorbance of this solution at 362 nm was 0.252, corresponding to 1.68 mg (87%) of EQ.

(*ii*) A mixture of 0.95 mg of the dimer and 2.37 mg of EQ was separated on alumina (Grade IV, 10 × 2 cm) with hexane–diethyl ether (9 + 1 v/v) as in the normal routine determination of EQ. Separation, although more difficult to achieve than with Grade I alumina, was possible. The dimer solution (100 cm^3) had an absorbance at 375 nm of 0.158, which corresponds to 0.83 mg (87%) of the dimer. The EQ solution (100 cm^3) had an absorbance of 0.308 at 362 nm, corresponding to 2.06 mg of EQ (87%).

Separation of the quinolone (**3**) *and EQ*

A mixture of 1.09 mg of the quinolone and 1.20 mg of EQ was placed on a column of deactivated alumina (Grade IV). The fluorescent material was eluted with a mixture of hexane–diethyl ether (9 + 1 v/v) and collected in a 100 cm^3 calibrated flask. The absorbance at 362 nm was 0.159, corresponding to 1.07 mg of EQ (89%). Elution with hexane–diethyl ether (3 + 7 v/v) eluted the yellow quinolone band which was collected in

a 50 cm³ calibrated flask. The absorbance at 345 nm was 0.550, corresponding to 0.96 mg of the quinolone (88%).

Determination of the EQ-dimer (2) by Gas Chromatography

The EQ-dimer content of a meal was determined by separating the dimer together with EQ from other hexane-soluble compounds using chromatography on alumina. The resulting solution was treated with the MQ-dimer (**5**) as internal standard and the mixture analysed by gas chromatography.

A 5.0 g amount of a fish meal was poured onto the top of a column of alumina (Grade IV, 10 × 2 cm) in hexane and all the fluorescent material eluted with hexane–diethyl ether (9 + 1 v/v). To this solution was added an accurately weighed amount of the MQ-dimer (1–2 mg weighed on a five decimal place balance). The solvent was evaporated on a rotary evaporator (temperature below 60 °C) and the residue taken up in about 0.5 cm³ of isooctane. This solution was injected into the gas chromatograph with the temperature being programmed from 100 to 250 °C rising at 16 °C min^{-1} while the injection port remained at 200 °C. The method was verified on an untreated meal spiked with the EQ-dimer in the range 50–400 mg kg^{-1}.

Determination of the Quinolone (3) by Gas Chromatography

The quinolone was determined in the sample of meal that was analysed for EQ. This means, therefore, that MQ was used as internal standard in the determination of the quinolone. The recovery of the quinolone was determined in meals spiked in the range 10–300 mg kg^{-1}.

Results and Discussion

Synthesis of the EQ-dimer (2), the MQ-dimer (5) and the Quinolone (3)

The preparation of the EQ-dimer and the quinolone from EQ proceeded as described by Thorisson.[3]

The structure of the MQ-dimer (**5**) follows unambiguously from its preparation and its 200 MHz ^{1}H NMR spectrum which, like its EQ counterpart, had a broad NH signal integrating for one proton at 4.03 ppm and a coupling constant of approximately 2.8 Hz for aromatic protons H$^{b'}$ and H$^{c'}$.

Antioxidant Properties

Fish oil treated with either EQ, the EQ-dimer (**2**) or the quinolone (**3**) at the 0.01% level gave induction times at 50 °C of 23.4 h (EQ), 8.2 h (EQ-dimer), 17.4 h (quinolone) and 4.5 h (control). These results are noteworthy. The dimer, for instance, is expected to have an induction time of about half that of EQ (11.7 h), as it has only one intact EQ residue with an NH group and is twice the relative molecular mass of EQ. The value of 8.2 h is reasonably close to the calculated value of 11.7 h, from which it can be deduced that the EQ-dimer has approximately one-third of the antioxidant activity of EQ. It is, however, surprising that Thorisson[3] claims that the EQ-dimer has no antioxidant activity. The quinolone has, unexpectedly, a very marked activity which is approximately three-quarters of that of EQ.

Interference of the EQ-dimer (2) and the Quinolone (3) in the Determination of EQ

The UV/VIS spectra of EQ, the EQ-dimer and the quinolone are shown in Fig. 1. These spectra clearly show a large degree of overlap and both the dimer and the quinolone, unless they are separated from EQ, will interfere with the determination of EQ.

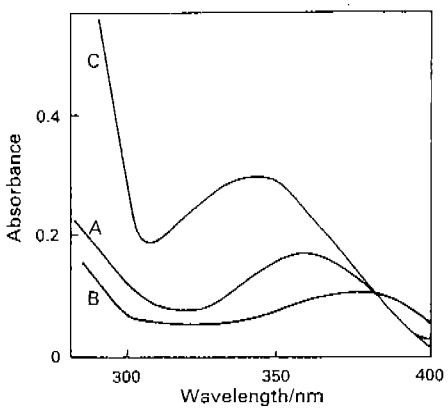


Fig. 1 Absorption spectra of A, EQ; B, the dimer (**2**); and C, the quinolone (**3**)

Table 1 Recovery of EQ added to fish meal using MQ as internal standard

EQ added/ mg kg^{-1}	EQ recovered/ mg kg^{-1}	Recovery (%)
158	148	95
212	214	101
250	262	105
400	394	99
412	440	107
633	660	104
836	870	104

Separation of EQ and the EQ-dimer (**2**)

The chromatographic behaviour of the EQ-dimer and EQ on alumina shows that the dimer is eluted first from the alumina. By using active alumina (Grade I), a mixture of hexane–diethyl ether (4 + 1 v/v) readily separated the dimer from EQ. Use of deactivated alumina (Grade IV) and hexane–diethyl ether (9 + 1 v/v), as in the routine determination of EQ, also gave sufficient separation of the dimer and EQ, although the two bands were close together and a clear-cut separation was more difficult to achieve. The results clearly indicate that the presence of the EQ-dimer can be a possible source of interference. It should be mentioned here that Spark[1] advises the inclusion of the dimer with EQ. It appears from our results that this should not be recommended if the intention is to obtain the genuine EQ content. If, however, it is the intention to obtain the antioxidant content expressed as EQ there is a case for the inclusion of the dimer as it has a reasonable antioxidant activity.

Separation of EQ and the quinolone (**3**)

This separation presented no difficulties. Calculation clearly showed that 89% of the EQ was recovered from the alumina uncontaminated by the quinolone by the normal routine procedure of eluting with hexane–diethyl ether (9 + 1 v/v). The quinolone required the much more polar solvent mixture of hexane–diethyl ether (3 + 7 v/v) to be eluted from the alumina. This result clearly demonstrates that the quinolone does not interfere with the determination of EQ in fish meal by chromatography on alumina.

Gas Chromatographic Procedure for the Determination of EQ With MQ as Internal Standard

The separation of MQ and EQ on the 15 m capillary column was excellent, MQ emerging after 3.23 min and EQ after 4.32 min. It must be mentioned here that the temperature of the injection port and the column should not exceed 200 and 150 °C, respectively, as MQ and EQ decompose at higher temperatures, which becomes evident in the appearance of

Table 2 Recovery of the EQ-dimer (2) added to fish meal using the MQ-dimer (5) as internal standard

EQ-dimer added/ mg kg^{-1}	EQ-dimer recovered/ mg kg^{-1}	Recovery (%)
53	50	94
166	153	92
200	220	110
294	272	93
376	384	102

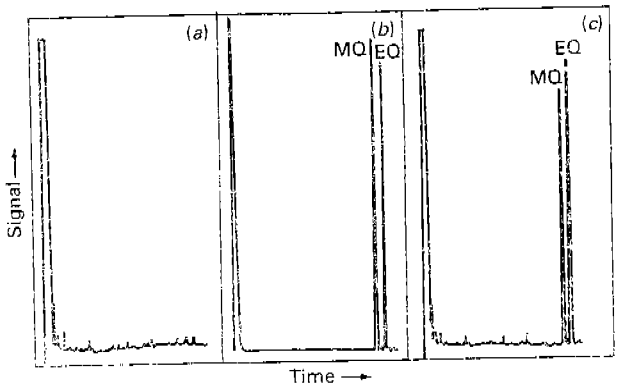


Fig. 2 Chromatograms of (*a*) meal only; (*b*) the MQ-dimer (5) and the EQ-dimer (2) only; and (*c*) meal treated with the MQ-dimer (5) and the EQ-dimer (2)

Table 3 Recovery of the quinolone (3) added to fish meal using MQ as internal standard

Quinolone added/ mg kg^{-1}	Quinolone recovered/ mg kg^{-1}	Recovery (%)
10	5	50
106	63	58
146	86	59
200	96	48
254	165	65
338	185	52

satellite peaks for both compounds. The decomposition of MQ and EQ probably yields a molecule of methane and 6-(m)ethoxy-2,4-dimethylquinoline.[9]

The recovery of EQ in spiked meals is presented in Table 1. Recovery ranged from 95 to 107% with a mean of 102% and a standard deviation of 3.8%, which is clearly sufficiently accurate for this work. In view of the good recovery and the specificity of the procedure the results obtained with this method were always taken as the correct results when comparing the two methods.

Determination of the EQ-dimer (2) by Gas Chromatography

By using the MQ-dimer (5) as internal standard, the EQ-dimer (2) could be readily determined. Under the conditions of the procedure, outlined under Experimental, the dimers of MQ and EQ had retention times of 7.42 and 7.80 min, respectively. The recovery of the EQ-dimer from the spiked meals is presented in Table 2. The recoveries ranged from 92 to 110% with a mean of 98% and a standard deviation of 6.9%. Examples of the separation of the two dimers in a meal together with the absence of any interfering substances in the meal are shown in Fig. 2.

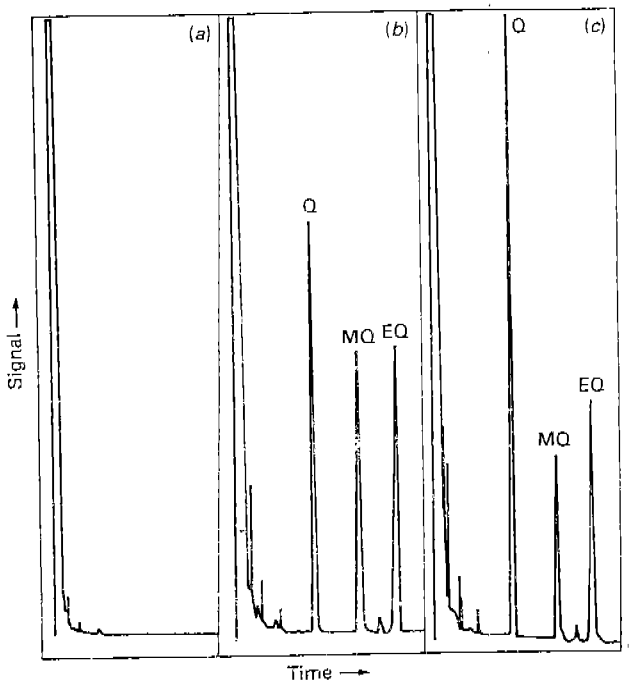


Fig. 3 Chromatograms of (*a*) meal only; (*b*) the quinolone (Q) (3), MQ and EQ only; and (*c*) meal plus Q, MQ and EQ

Determination of the Quinolone (3) by Gas Chromatography

In the determination of EQ in fish meals using MQ as the internal standard, the quinolone (3) appeared as a peak with a retention time of 1.96 min. The recovery of the quinolone from a spiked meal was, however, not quantitative; values of between 48 and 65% with a mean of 55% and a standard deviation of 5.9% were obtained (see Table 3). This is due to unequal distribution of the quinolone and MQ in hexane and the aqueous acidic or alkaline solutions. The values obtained in the determination of the quinolone were, therefore, always corrected for by multiplication by a factor of 1.8. As the amounts of the quinolone in meals were low (see below) this seems acceptable for our purposes. Examples of the chromatograms obtained when taking (*i*) a fish meal, (*ii*) the quinolone, MQ and EQ and (*iii*) a meal treated with the quinolone, MQ and EQ through the procedure are shown in Fig. 3.

Comparison of the Gas Chromatographic Procedure and Chromatography on Alumina for the Determination of EQ in Fish Meal

The results of the determination of EQ by the two methods on five fish meals of different origin and stored at 25 °C are presented in Table 4. The results showed that for EQ contents above approximately 30 mg kg^{-1}, chromatography on alumina tended to yield lower values than the gas chromatographic (GC) method, whereas for EQ contents below 30 mg kg^{-1} the reverse applied. Statistical analysis of the results from the meals with EQ contents above 30 mg kg^{-1} gives the following expression:

$$\text{EQ (by GC)} = 1.1 \times \text{EQ (by alumina)} - 2.1 \; (r = 0.99; n = 23)$$

This means that the value obtained for EQ by chromatography on alumina should be corrected for by multiplying by a factor of 1.1. The values obtained by chromatography on alumina for meals with EQ contents below approximately 30 mg kg^{-1} are much too high and the results are to be regarded as unreliable. For these meals the gas chromatographic method should be used.

Table 4 Ethoxyquin, the quinolone (**3**) and the EQ-dimer (**2**) in fish meal during storage at 25 °C

Meal history	Storage time/d	EQ/mg kg^{-1} Chromatography on alumina	EQ/mg kg^{-1} Gas chromatography	Quinolone/ mg kg^{-1}	EQ-dimer/ mg kg^{-1}	Total EQ equivalents mg kg^{-1}*
(1) Anchovy/pilchard factory meal; approximately 400 mg kg^{-1} of EQ added by factory personnel	1	198	230	2	+†	—
	212	79	83	9	86	118
	279	75	81	9	114	126
	357	80	76	9	101	116
	479	78	68	5	120	112
	573	76	61	4	120	104
(2) Maasbanker factory meal; approximately 1000 mg kg^{-1} of EQ added by factory personnel	1	938	1022	0	0	—
	184	288	297	29	92	349
	260	214	260	23	84	305
	359	208	244	7	96	281
	453	177	150	5	102	188
(3) Anchovy factory meal; 400 mg kg^{-1} of EQ added in the laboratory	20	403	394	5	0	—
	244	285	323	14	74	358
	100‡	155	238	26	98	290
	145‡	182	205	0	57	224
	216‡	146	145	7	72	174
	283‡	135	89	5	116	131
(4) Anchovy factory meal; 400 mg kg^{-1} of EQ added in the laboratory	59	164	185	21	111	238
	133	105	150	13	64	181
	186	134	134	3	98	169
	259	117	100	5	78	130
	347	79	87	22	96	136
(5) Anchovy factory meal; 400 mg kg^{-1} of EQ added in the laboratory	68	68	87	17	104	134
	181	62	30	9	124	78
	248	44	25	7	93	61
	321	62	20	4	70	46
	365	51	20	2	76	47

*See text for explanation.
† Dimer present but not determined quantitatively.
‡ Storage temperature raised to 70 °C.

Included in Table 4 are the amounts of the quinolone (**3**) and the EQ-dimer (**2**) that were generated during storage at 25 °C. There was a small and variable amount (4–30 mg kg^{-1}) of the quinolone formed in the stored meals, indicating that the quinolone is an intermediate product in the oxidation of EQ. It is not known which products are formed on further oxidation of the quinolone, but it seems unlikely that these products will interfere with the determination of EQ by chromatography on alumina, as the quinolone itself is not eluted from alumina under the conditions of the analysis.

The dimer, on the other hand, is formed in larger amounts (up to about 100 mg kg^{-1}) in the meals. All meals, except for the second meal, eventually contain amounts of the EQ-dimer which match and even exceed those of EQ. The presence of these relatively large amounts of the dimer in aged meals is probably responsible for the high values obtained by chromatography on alumina.

By using the data on the relative antioxidant efficacies of the quinolone (**3**) and the dimer (**2**) (see above), *viz.*, the quinolone has approximately three-quarters and the dimer approximately one-third of the efficacy of EQ, it is possible to calculate the antioxidant content expressed as EQ. These calculated total EQ equivalents are included in Table 4. It is evident from the results that the EQ content of a meal is normally an underestimate of its antioxidant content. It also seems unlikely that any of the three meals will be entirely deprived of antioxidant in the foreseeable future. Even if the EQ content were to drop to negligible levels there will probably still be sufficient dimer to protect the meal lipids against oxidation.

It can also be concluded that in the absence of EQ the presence of the EQ-dimer may serve as evidence to establish whether an aged meal has originally been treated with the antioxidant EQ. This question has on a few occasions been answered successfully in this Institute by analysing the meal for the EQ-dimer.

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