The behavior of tristearin obtained from crystallization of solvents in the absence and presence of various emulsifiers at different crystallization conditions has been tested. The results indicate clearly that solvent crystallization under any circumstances will yield only the β-form and that the presence of any of the emulsifiers in any concentration does not affect the crystal structure. The lack of effect in the presence of emulsifiers is quite disappointing, because in our previous study on the effect of modifiers on fatty acids, we detected significant influence of the emulsifier on the fatty acids crystallized from solvents.

In a separate set of experiments, we tried to melt and cool for resolidification the tristearin obtained previously from solvent crystallization in the presence of an emulsifier. Upon resolidification, the α-form was obtained as usual. However, it was found, surprisingly, that when testing subsequent melting by DSC, only part of the α-form converted into the β-form indicating the existence of the emulsifier as an impurity in the tristearin that was previously crystallized from solvent by the analogy in thermal behavior previously described. The fact that the emulsifier was absorbed in the tristearin during the crystallization in sufficient amounts to affect the solidification process in the later stage may serve as a method for evaluation of the amounts of emulsifier capable of incorporation in the tristearin.

This study on the effect of various food emulsifiers on the crystal-structure modifications of tristearin shows that several emulsifiers, such as sorbitan monostearate and other monoglyceride derivatives of citric acid, can serve as α-form crystal preservatives, preventing the transformation of the α-form into the most thermodynamically stable β-form. The enthalpy of α-form melting (ΔH_α) and β-form melting (ΔH_β) and the exothermic transition (ΔH_t) which have been measured and calculated helped to evaluate the amount of β-form obtained upon heating. In our previous study on the crystallization of stearic acid* (13), it was shown that one can predict the activity of a given emulsifier to serve as a modifier if both bulkiness of the hydrophilic groups and the right length of the hydrophobic groups exist in the tested emulsifier. The effective emulsifiers, as found in the present study, have the same characteristics.

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Ethoxyquin in Fish Meal

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ABSTRACT

Four reliable methods are described for the determination of ethoxyquin antioxidant in fresh fish meal. These include an accurate chromatographic laboratory method, a colorimetric method, and 2 rapid factory methods, one a quantitative titration technique and the other suitable for spot checks for ethoxyquin in "go–no go" situations. With the laboratory method, 6 or 8 chromatographic columns can be handled simultaneously. The 2 rapid methods are based on 1,1-diphenyl-2-picrylhydrazyl and may be used routinely in the factory to determine the antioxidant content of several hundred samples.

Fish meal manufactured from the pelagic anchovy, pilchard and mackerel of the Southern Hemisphere contains up to 10% of a highly unsaturated oil which can cause spontaneous heating. Partly for this reason, such meals are treated with 6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline (ethoxyquin, EQ), a highly effective antioxidant which renders the meal safe for storage and transport, and which maintains a higher calorific and nutritional value than cured meals.

This investigation was motivated by the need for a rapid, reliable method for the determination of EQ in fish meal. Existing methods proved too time-consuming (1) as the number of analyses increased, and all methods suffer from disappearance of the antioxidant in reactive fish meals (2-5). Because the original dosage cannot be determined after a few days, and because the recoverable EQ is changing at a rate which differs for each sample, pin-point accuracy is deemed less important than maximal recovery and simplicity of method. An accuracy of plus or minus 10 mg/kg at the 400 mg/kg level is quite acceptable in meal production.

The official method for EQ of the Association of Official Analytical Chemists (6) involves extraction with petroleum ether (7) chromatography and subsequent measurement by fluorimetry. The method requires extreme care and skill, and is intended for determination at low levels, e.g., on apples. Alternative methods not using chromatography (8) are unsuitable as blank values are large in older meals and not possible to determine, because the "blank" requires an untreated meal, of obviously different history.

This communication describes the methods that are routinely used for determining EQ in fresh fish meal, at both laboratory and factory levels. For laboratory use, the chromatographic method is rapid, relatively simple and reliable. The 2 rapid methods for factory tests, i.e., quantitative titration and spot check for EQ, use 1,1-diphenyl-2-picrylhydrazyl (DPPH). With certain provisos, both can be used by unskilled personnel.

EXPERIMENTAL

Materials and Methods

Chromatography columns, 1.5 × 30 cm, with sintered glass frit (porosity 1) and stopcock (preferably Teflon) were

used. The ultraviolet lamp was a hand-held, low power type, shortwave (250 nm). The spectrophotometer, UV/visible (220-700 nm), was used with appropriate cells. Volumetric flasks were 100 mL each.

Alumina, 80-200 mesh, Grade III (Brockmann [9]) was prepared as follows: acid-washed alumina, 80-200 mesh, was heated to 600 C for 6 hr, and then cooled in a desiccator. Distilled water was added at the rate of 9 mL to 91 g, and the mixture was stored in sealed containers for at least 24 hr before use. "Alumina for chromatography," 80-200 mesh, was received as Grade II (Brockmann and Schodder [9]). Half of the bottle contents was removed, wetted with methanol, and dried in air to produce Grade IV. This was returned to the half-bottle remaining, the bottle sealed, shaken and stored for at least 24 hr before use.

Hexane was redistilled (bp 68 C). Diethyl ether (to be used with the usual precautions associated with highly inflammable solvents) was peroxide-free, redistilled and stored over ferrous sulfate. Sodium sulfate (anhydrous) was dried at 105 C for 2 hr. Ethoxyquin was redistilled (bp 108 C/0.5 mm Hg).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemical Co.) was used as received (90% pure) and as recrystallized from ether/hexane. DPPH was stored at 0 C under a nitrogen atmosphere. Solutions of DPPH in ethanol were made by predissolving the DPPH in a minimal quantity of diethyl ether and adding ethanol to volume.

Treatment of Fish Meal

Fish meal, treated with 400 mg/kg ethoxyquin or untreated, was obtained fresh from a local plant and transported in full, sealed containers. Laboratory addition of EQ was usually within .5 hr of production.

The method used for adding and distributing EQ in fish meal was: the appropriate amount of EQ for the amount of meal to be treated (viz., 100, 200 or 400 mg/kg) was dissolved in a small amount of diethyl ether and the solution was evaporated into a thin film on the wall of a suitable-sized glass beaker (1 L for 500 g fish meal). The fish meal was then poured into the beaker and stirred with a glass rod until no fluorescence due to EQ could be detected by UV light on the walls of the beaker. Five min was ample time.

Chromatographic Determination of EQ

The chromatography column (Fig. 1) was filled with hexane and excess pressure below the frit was released by running out hexane until the level was about 15 cm, with occasional closing of the tap. Alumina Grade III (Brockmann and Schodder [9]) was added slowly with tapping to avoid trapped air voids. The alumina column was 8-12 cm high. About 2 cm of anhyd sodium sulfate was added to the top of the alumina.

Fish meal (5 ± 0.05 g) was poured into the hexane layer and elution was started. Successive small amounts of hexane were added to the top of the fish meal until all the (dark) extract had passed through the sodium sulfate. The fish meal could be stirred with a glass rod if fines blocked the surface, as disturbance of the sodium sulfate layer was not important. Hexane was then run through until neutral fats were no longer in the eluant—as shown by no residue when a drop of eluant was evaporated on a clean, ground-glass stopper. EQ, if present above 5 mg/kg in the meal, could be seen as a blue fluorescent band under short-wave ultraviolet (UV) light.

The EQ was then eluted with 10% diethyl ether in hexane, following the movement, by minimal use of the UV lamp. Occasionally, a second (minor) fluorescent band was observed. This was thought to be a reaction product of EQ

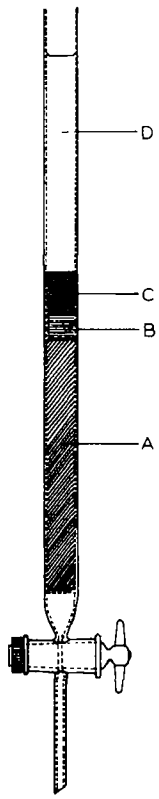

FIG. 1. Chromatographic column for determination of EQ. A – alumina; B – sodium sulfate anhyd; C – fish meal; D – eluant (hexane/petroleum ether [60-80 C] or hexane/petroleum ether [60-80 C]/ether [10:1]).

(10) and was normally included in the collected EQ band. The excessive use of UV can affect the recovery of EQ.

Upon elution, the EQ was collected in a 100-mL volumetric flask. When elution was complete (no fluorescence in eluate), the tip of the column was rinsed into the flask (a "ring" of EQ was visible in UV) and the solution made-up to volume.

EQ absorbance was determined at 362 nm against hexane as a reference.

Colorimetric Determination of EQ

Fish meal (5 ± 0.01 g) was mixed with anhyd sodium sulfate (2-3 g) and poured into a chromatography column (Fig. 1) filled to ca. 10 cm with ethanol with 2-3 cm of anhyd sodium sulfate at the bottom. Elution was continued with successive small additions of ethanol and exactly 100 mL was collected.

DPPH solution (0.05-0.07 mg/mL) in ethanol (absorbance 0.2-0.3) (5 mL) was mixed with the EQ extract (1 mL) and the absorbance at 517 nm determined. A blank of 5 mL DPPH solution and 1 mL of ethanol was also measured at 517 nm. The difference in absorption was recorded.

Titration of EQ

Fish meal (1 g) was weighed into a test tube. In the field, this can be conveniently done using a jeweler's hand-held carat balance (1 g = 5 carats). Alcohol (2 mL) was added to wet the meal. DPPH solution (0.292 g/L) was titrated rapidly into the meal until the purple remained. This was done with continuous shaking. In practice, it is found that a "dead stop" method gives greater accuracy than dropwise addition as the end-point is somewhat subjective. At this concentration of DPPH, 5 mL is equivalent to 400 mg/kg. A concen-

tration of 0.234 g/L would give an equivalence of 1 mL to 100 mg/kg, but the stronger solution was used here as it is the same strength as that used for the rapid check method, and also the greater concentration renders the end-point more sensitive.

Rapid Colorimetric Determination of EQ

A solution of 0.292 g/L of DPPH in alcohol was made. Fish meal (1 ± 0.05 g) was added to 5 mL of a solution of 0.292 g/L DPPH and shaken for 2 sec. The meal was allowed to settle and the supernatant was examined for traces of the characteristic purple color. Any purple color remaining indicates an EQ content of less than 400 mg/kg in the meal.

Calibration and Recovery of EQ

All methods were calibrated with pure EQ solutions in either hexane or ethanol, as appropriate. Recovery of EQ solutions with known concentrations after chromatography was determined. Recovery of EQ from fish meal involved measurement after addition of EQ to meals either containing no EQ or meals previously treated with EQ at the factory or in the laboratory.

RESULTS AND DISCUSSION

Chromatographic Determination of EQ

The absorbance of EQ in hexane at 362 nm is twice as much as the absorbance of the same amount of EQ in acid solution at 296 nm. Also, the 362-nm wavelength is in a cleaner part of the spectrum than 296 nm, the 296 nm being close to the general absorption caused by organic materials. In contrast to the direct extraction and measurement in acid at 296 nm, no blank value is required, as essentially pure EQ is being measured.

The calibration graph (Fig. 2) shows that the absorbance of EQ at 362 nm is linear with EQ concentration in hexane. The factor is EQ (mg/mL) = 0.074 × absorbance at 362 nm in hexane. The presence of 10% diethyl ether in the hexane had no effect either on the absorbance maximum or the calibration curve.

The use of UV irradiation to visualize EQ on the chromatographic column must be kept to a minimum. Figure 3 indicates the decay with time of the EQ absorbance at 362 nm as determined in the spectrometer by irradiation at 260 nm and measurement at 362 nm. While it is clear that the dose in such a circumstance is greater than that received during chromatography, a related effect is obvious and irradiation should be kept to a minimum, including subdued lighting, and preferably with any fluorescent lights switched off.

When the oil content of the fish meal is high (12% or more) the oil can partially elute the EQ and repeat chromatography may be necessary. The amount of fish meal can be increased or decreased for very low or very high EQ levels.

Recovery after chromatography of known amounts of EQ standards was very good. Only at very low levels did losses go above 5% (Table I). Reproducibility of six determinations of the same amount of EQ after chromatography was good (Table II).

Recovery of EQ from fish meal was good (Tables III and IV), considering the methods of mixing and possible sampling errors. To indicate the variation expected, Table V shows the results of 6 determinations (2 sets of 3 in parallel) carried out as nearly simultaneously as possible on one factory-treated sample of meal. The results were considerably better than expected, despite factory conditions and variations in mixing of EQ into the commercial fish meal.

Colorimetric Determination of EQ

Using the same solutions as in Table I, a linear relationship was demonstrated for an absorbance loss of 0.1-0.3 units. Below a loss of 0.1, the pure DPPH line was linear, but that

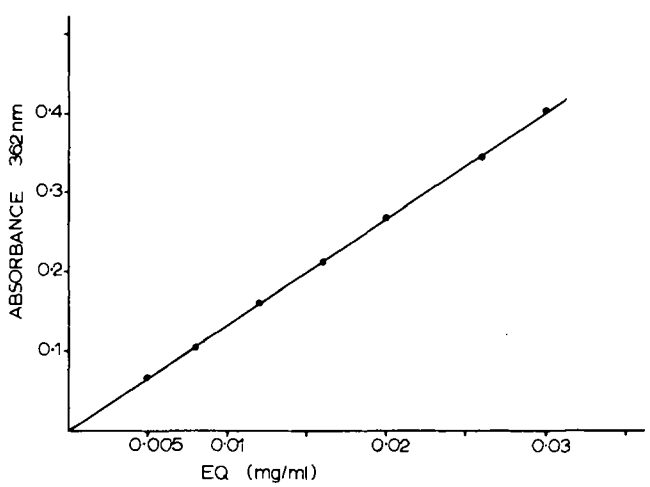

FIG. 2. Absorbance (Log I/I_0) at 362 nm of EQ in hexane (petroleum ether 60-80 C).

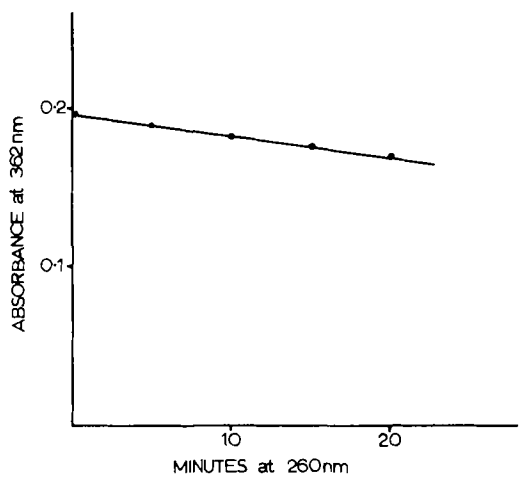

FIG. 3. Change in absorbance (Log I/I_0) of EQ in hexane (petroleum ether 60-80 C) with time of irradiation at 260 nm.

TABLE I

Recovery of EQ after Chromatography of Standard Solutions

EQ added to column (in mL), mg	0.12	0.225	0.300	0.600	1.20
EQ recovered	0.10	0.216	0.290	0.592	1.196
% Loss	17	4	3	1	0.5

TABLE II

Reproducibility of Recovery of EQ from Chromatography of 0.225 mg EQ Samples in 50 mL Solvent

EQ recovered (mg)	0.217	0.216	0.220
	0.228	0.219	0.220
Average	0.220	–	–
St. dev.	0.0042	–	–
Relative st. dev.	1.9%	–	–

of the 90% material was not (Fig. 4). Because DPPH is unstable, it is recommended that users construct their own calibration curve or recrystallize the DPPH each time. Solutions of DPPH should not be kept for more than a few days, even at reduced temperature.

The reaction of EQ with DPPH is essentially instantaneous and is unlike BHA or BHT, which can take up to 1 hr for complete reaction. Thus, this method might be used to determine EQ in the presence of other antioxidants (1).

The same fish meals used in the chromatographic calibration (Tables IV and V) were examined for EQ by the DPPH absorbance loss method. Again the repeated determinations gave good results on the factory-treated meals (Table V). The increase on average was not considered significant. When older meal was included (Table VI), an unusually high recovery was apparent. This same meal gave a lowered recovery by chromatography (Table IV) and after addition of 400 mg/kg EQ, the recovery of the extra EQ was satisfactory. This finding has significance for the use of the method for meal treated with EQ more than 24 hr before analysis and will be discussed in a later paper. The colorimetric method or any method involving DPPH is not applicable to "older" meal.

TABLE III

EQ Recovered from Laboratory-Treated Fish Meal (Chromatographic Method)

EQ added (mg/kg)	100	200	400
EQ recovered (mg/kg)	96	195	388

TABLE IV

EQ Recovered from Factory-Treated Meal, before and after Addition of EQ in Laboratory (Chromatographic Method)

EQ recovered from meal (mg/kg)	365	380	248[a]
EQ added in laboratory (mg/kg)	100	200	400
EQ recovered after addition (mg/kg)	461	575	620[a]

[a]Sample about 3 days old.

TABLE V

Reproducibility of EQ Recovery from Factory Meal[a] (Nominal 400 mg/kg)

Method	No. analyses[a]	Range	Average	Relative st. dev. (%)
Chromatographic	6	376-431	401	5.3
Colorimetric	6	390-428	410	4.6
Titration (single operator)	8	390-420	406	3.5

[a]All analyses were done on subsamples of one meal.

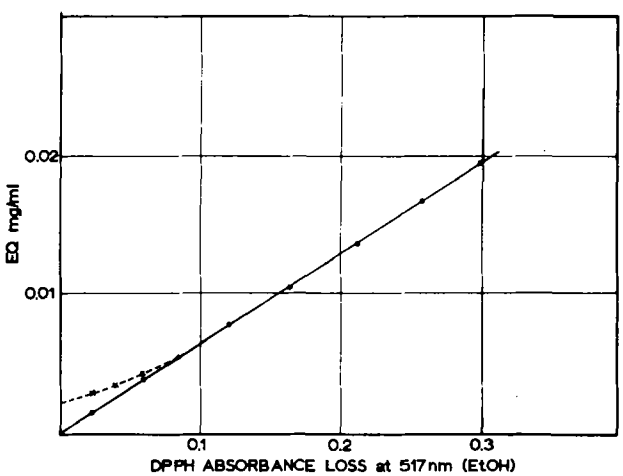

FIG. 4. DPPH absorbance ($\log I/I_0$) loss at 517 nm in ethanol after reaction with EQ. Dotted line: 90% DPPH. Solid line: recrystallized DPPH.

TABLE VI

Recovery of EQ from Factory-Treated Meal with EQ Added in Laboratory (DPPH Colorimetric Method)

EQ recovered from factory meal (mg/kg)	390	410	648[a]
EQ added in laboratory (mg/kg)	100	200	400
EQ recovered after addition (mg/kg)	481	595	1010[a]

[a]Meal was about 3 days old.

RAPID METHODS

Titration

This method was developed for field application and gives adequate results for control purposes, but requires a subjective assessment of the end-point. In skilled hands, the accuracy of ± 15 mg/kg at the 400 mg/kg level is attainable. The dark brown dipicrylphenylhydrazine masks the end-point above about 500 mg/kg. For rapid, multiple analyses, the method has been found to be very useful. Reproducibility for one operator is shown in Table V.

Spot Check

The rapid check for adequate EQ addition is used routinely in several plants in South Africa and as a routine laboratory check. Because the lifetime of DPPH solution is limited, a system of preweighed capsules is used, backed by standards, to check the DPPH solution.

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