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Three Extraction Methods for Determination of Lipids in Fish Meal: Evaluation of a Hexane/Isopropanol Method as an Alternative to Chloroform-Based Methods*

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Abstract: Three different extraction methods were compared for the determination of lipids in both oxidised and antioxidant-stabilised brown fish meal. The three methods under investigation were those of Smith Ambrose-Knobl using chloroform/methanol/water, Bligh and Dyer also using chloroform/methanol/water but monophasic, and Hara and Radin using hexane/isopropanol. The fat content, determined gravimetrically, was significantly lower ($P < 0{\cdot}05$) when hexane/isopropanol was used for lipid extraction from both oxidised and antioxidant-stabilised fish meal, than when either of the chloroform systems was used. Determination of lipid classes in the lipid extracts using silica gel thin-layer chromatography on Chromarods-SIII, with flame ionisation detection by Iatroscan, suggested that the lower lipid recovery by the hexane/isopropanol method may be due to a less effective extraction of the more polar lipid classes, including oxidation products, present in brown fish meal.

Key words: fish meal, lipid extraction, hexane/isopropanol, chloroform/methanol, TLC-FID, lipid classes.

INTRODUCTION

Fish meal is utilised chiefly as a source of protein and energy for domestic animals. The energy value of fish meal comes solely from its protein and lipid content (Barlow and Windsor 1983). The lipids in fish meal are especially important as nutritional sources of highly unsaturated *n*-3 fatty acids and also as an index of its energy value (Opstvedt *et al* 1970; Opstvedt 1973, 1974).

Numerous methods have been used for the determination of this lipid content in fish meal but there is still no general agreement on the best (Gunstone and Wijesundera 1978; de Koning *et al* 1985; de Koning and Mol 1989). Most methods are either unsatisfactory in terms of recovery or unsuitable for rapid and safe use. Methods using diethyl ether were commonly used in the past (Stansby and Clegg 1955; Wiechers *et al* 1964; Lee *et al* 1966). The International Association of Fish Meal Manufacturers (Hoval House, Potters Bar, Hertfordshire, UK), as recently as 1980 in their Technical Bulletin No. 13, recommended diethyl ether extraction for 6 h. These methods are now recognised as unsuitable for the determination of total lipids as not all lipids are considered to be equally well extracted by diethyl ether (Gunstone and Wijesundera 1978). Acetone is apt not to extract phospholipids (Christie 1982), even in the 16 h proposed for this method (AOAC 1984). Methods based on extraction with chloroform/methanol/water have been recommended for the analysis of total lipids in both oxidised and unoxidised fish meal (Ambrose and Knobl 1966; Ederzeel and Ritskes 1966; Olley *et al* 1966; AOAC 1984; de Koning *et al* 1985). Nevertheless, they are now undesirable as chloroform is suspected of being a carcinogen (Radin 1981; Nelson 1991). One reason for

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this multitude of methods is probably that oxidation occurring during drying has been found to cause a decrease in extractable lipid from fish meal (Williams 1959; de Koning and Mol 1989). This might be brought about by interaction of oxidation products with the protein (Pokorny *et al* 1983).

Lipid investigators have sought alternative methods for animal tissues. Low toxicity solvents such as hexane/isopropanol have been recommended (Hara and Radin 1978; Radin 1981). This study was carried out to compare three different extraction methods and to evaluate whether the method using hexane/isopropanol, not previously examined for lipid determination in fish meal, could be used for the extraction of this material. Both stabilised and unstabilised menhaden meals were tested after storage in various atmospheres over 60 days. The amounts of different classes of lipid components extracted by these three methods from a stabilised meal exposed to air have been compared by using silica gel thin-layer chromatography with flame ionisation detection (Iatroscan TLC-FID).

EXPERIMENTAL

Materials

Menhaden fish meal, from the Zapata Haynie Corporation, Reedville, VA, USA, was obtained during the final steps of a single production run and divided into two portions: one was immediately treated with 750 mg kg^{-1} ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) (WE): the other was not treated with antioxidant (WOE). Aliquots of both samples were packed in 300 × 500 mm Barrier Bags (Cryovac Division, W R Grace and Co, Mississauga, Ontario, Canada) under O_2, air or N_2 using a vacuum sealer. The samples were stored at room temperature for 60 days. The O_2 and air were replaced every 2 weeks. The control samples packed under N_2 were stored at −30°C. All fish meal samples were sieved through a 9 meshes to the inch (2 mm mesh opening) sieve immediately before extraction. The sieved product was then thoroughly remixed to ensure representative sampling for replicates of the three comparative analyses.

Lipid determination

Three different methods were used for lipid extraction: Smith–Ambrose–Knobl (SAK) using $CHCl_3$/methanol/water in a biphasic extraction (Smith *et al* 1964). Bligh and Dyer (BD) using $CHCl_3$/methanol/water in a monophasic extraction (Bligh and Dyer 1959). Hara and Radin (HR) using hexane/isopropanol (Hara and Radin 1978). The HR method was slightly modified for the purpose of extracting fish meal, ie water was added to the fish meal and the mixture sonicated for 15 min before extraction. In all three methods the extracted lipid material was freed of solvent at 30–40°C under vacuum using a rotary evaporator.

Each extraction was carried out in triplicate and the Tukey multiple comparison of means test was used to determine whether sample means were significantly different at the 5% level. All statistical analyses were performed using the Systat 4·0 computer program (Wilkinson 1988).

TLC-FID

The lipid extracts were examined for lipid classes by chromatography on silica gel (Chromarods-SIII) followed by quantitation on an Iatroscan TH-10 MKIII (Iatron Laboratories Inc, Tokyo, Japan; world distributor Newman-Howells Associates Ltd, Penybont Uchaf, Llanwrtyd Wells, Powys, UK) equipped with a flame ionisation detector. The Iatroscan was fitted with a push button switch to interrupt scanning anytime when required (Parrish and Ackman 1985); this was especially useful for partial scanning and redevelopment. The Iatroscan was operated with a hydrogen flow rate of 110 ml min^{-1}, air flow of 2000 ml min^{-1} and scan speed 2. The Iatroscan was connected to a Spectra-Physics SP 4270 computing integrator for recording of chromatograms and in order to provide digital integration of the peaks; the attenuation used was 16. The Chromarods were cleaned before use by soaking in concentrated nitric acid/water (35:65 v) overnight and then washed thoroughly with distilled water and acetone. The Chromarods were pre-scanned twice prior to use for cleaning and activation; after spotting and before each development the Chromarods were conditioned in a constant humidity chamber for 5–10 min over a saturated solution of NaCl and then immediately transferred to the developing tank.

Stock solutions were prepared for each lipid extract by accurately weighing approximately 150 mg of lipid into a 5 ml volumetric flask, adding chloroform, and storing at −20°C until analysed. Drummond disposable Microcap pipettes (Acadian Instruments Ltd, Etobicoke, Ontario, Canada) were used to apply the solutions to the Chromarods (normally 1 μl or cumulative applications of 1 μl).

Calibration of Chromarods

A stock solution of an eight-component composite standard, representing the major lipid classes found in fish meal (see below), was made up in chloroform and stored under nitrogen at −20°C. All standards used were obtained from the Serdary Research Laboratory (London, Ontario, Canada). Several different dilutions

of the stock solution were made and the standards used ranged in concentration from 0·1 to 20 $\mu g\,\mu l^{-1}$. Before making the composite standard, each component was run singly through the entire development procedure to determine purity. Calibration curves were compiled throughout the whole period of sample analyses. With each lot of 10 Chromarods, one rod, chosen randomly, was used for standards on each day of analyses. The total load applied to the Chromarods ranged from 0·1 to 20·0 μg.

Chromarod development

Three different solvent systems were used to obtain three chromatograms per Chromarod. The first development was carried out for 55 min in hexane/chloroform/isopropanol/formic acid (80:14:10·1 v). The Chromarods were then dried at 110°C for 3 min and partially scanned to a point just beyond the diglyceride (DG) peak to reveal neutral lipids. The Chromarods were then developed twice for 10 min in acetone (100%), dried at 110°C for 3 min and then scanned partially to the lowest point beyond the acetone-mobile polar lipid (AMPL) peak. Finally, the Chromarods were developed twice for 30 min in chloroform/methanol/formic acid/water (25:15:2:1 v), dried at 110°C for 5–7 min, and completely scanned to reveal phospholipids and less mobile materials.

Lipid classes and standards

Calibration of the Chromarod–Iatroscan system was effected for authentic peaks, supplemented for unknowns by material of similar chromatographic mobility. The lipid classes and standards were as follows: triglyceride (TG), tripalmitin; free fatty acid (FFA), stearic acid; free sterol (CHO), cholesterol; diglyceride (DG), 1,2-dipalmitin; acetone-mobile polar lipids (AMPL), monopalmitin; unidentified material (UIM), L-3-phosphatidylethanolamine (dipalmityl); phosphatidylcholine (PC), L-3-phosphatidylcholine (dipalmityl); oxidised polar lipid (OXPL), lysophosphatidylcholine (palmityl); and polar material (PM), lysophosphatidylcholine (palmityl). AMPL is a term introduced by Parrish (1987) as the designation for a particular peak, often well defined, first observed in ocean plankton lipid samples.

RESULTS AND DISCUSSION

The amounts of lipid extracted and determined gravimetrically from both oxidised and unoxidised menhaden meal by the three different extraction procedures under investigation are recorded in Table 1. When the lipid recoveries of the three different extraction procedures were compared, the recovered lipid was found to be significantly lower ($P < 0.05$) for all samples when the HR method was used for lipid extraction than when the SAK and BD methods were used. The HR method uses hexane/isopropanol as the extraction solvent system. Methods using only hexane as the extraction solvent have previously been shown to give lower lipid recoveries than the chloroform-based BD method (de Koning *et al* 1985; de Koning and Mol 1989). The SAK method gave the highest lipid recovery for all samples under investigation (Table 1). The differences between the gravimetrically determined lipid content by the SAK and BD methods in this study were small, and significant ($P < 0.05$) only in two instances. The main difference between the two methods is that the BD method is carried out using $CHCl_3$/methanol/water which, in the proportions 1:2:0·8 v, results in a monophasic extraction. On the other hand the SAK method uses $CHCl_3$/methanol/water in the proportions 1·5:1:0·4 v which results in biphasic extraction. An additional difference is that the extracted material is reblended in $CHCl_3$ in the SAK method. In the BD procedure, as used, the Waring Blendor was rinsed with $CHCl_3$ (50 ml) and this solvent was passed through the filter cake, which was firmly pressed dry. Generally, the SAK method requires a greater volume of $CHCl_3$ for each unit of fish meal extracted compared with the BD method. In fact, the total organic solvent requirement per unit was twice as great as either the HR or BD methods. It is considered that the BD procedure will recover virtually the same amount of lipid as the SAK procedure (Table 1) and uses appreciably less chloroform. Acccordingly, the authors

TABLE 1

Amount of lipid extracted ($g\,kg^{-1}$ meal) by three different procedures, Smith–Ambrose–Knobl (SAK), Bligh and Dyer (BD) and Hara and Radin (HR), from ethoxyquin-treated and untreated menhaden meal stored under N_2, air and O_2 for 60 days

Treatment and storage atmosphere	*Extraction method*[a]		
	SAK	*BD*	*HR*
With ethoxyquin			
Under N_2	132.6 ± 1.5^{a}	129.8 ± 1.3^{a}	113.1 ± 1.3^{c}
Under air	127.7 ± 2.2^{a}	124.6 ± 1.0^{a}	109.4 ± 1.7^{c}
Under O_2	129.9 ± 0.7^{a}	125.3 ± 0.7^{b}	110.5 ± 0.4^{c}
Untreated			
Under N_2	131.4 ± 0.4^{a}	129.0 ± 1.8^{a}	113.2 ± 0.8^{c}
Under air	125.9 ± 2.0^{a}	119.5 ± 1.4^{b}	110.7 ± 2.2^{c}
Under O_2	126.8 ± 1.3^{a}	122.5 ± 2.1^{a}	103.8 ± 1.3^{c}

[a] Means within a row having different superscripts are significantly different ($P < 0.05$). For each method, data are the mean ± SD of three determinations.

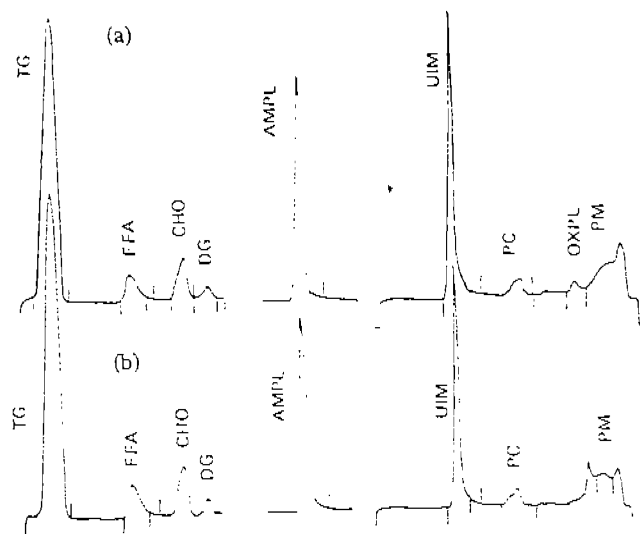

Fig 1. Separation of lipid classes in unstabilised (WOE) menhaden meal stored under O_2 for 60 days. Each rod was scanned twice partially and once completely, resulting in three chromatograms per sample application. Development is from right to left, scanning from left to right. Attenuation is the same for all chromatographs. Lipid class abbreviations are explained in the text. (a) Lipid extracted using the Smith–Ambrose–Knobl method, and (b) lipid extracted using the Hara and Radin method.

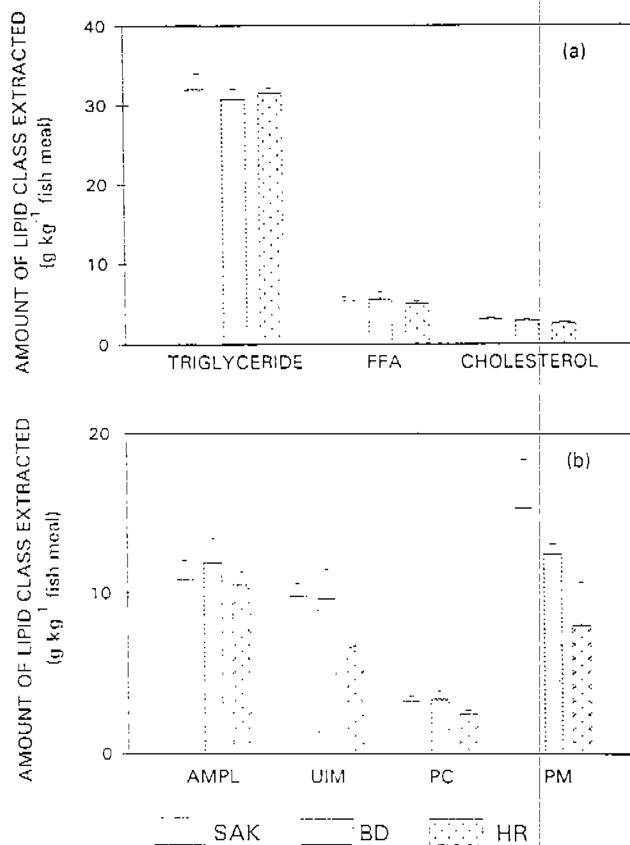

Fig. 2. Amounts of lipid classes recovered, using three different extraction procedures, from antioxidant-stabilised (WE) menhaden meal stored under air. Data points are the mean ± SD of three lipid extracts. (a) Non-polar lipid classes (FFA—free fatty acids) and (b) polar lipid classes (AMPL—acetone-mobile polar lipid, UIM—unidentified material, PC—phosphatidylcholine, PM—polar material).

concur with de Koning *et al* (1985) and de Koning and Mol (1989) that the extraction of lipid from fish meal is best carried out by the Bligh and Dyer (1959) procedure. This is also the conclusion of researchers using foods (Daugherty and Lento 1983) and other materials when procedures are strictly standardised (Randall *et al* 1991).

In order to attempt to explain the lower lipid recovery of the hexane/isopropanol system (HR method), TLC-FID was used to compare the types and amounts of lipid components extracted by the different extraction methods. To clarify qualitative differences between lipid extracts obtained using the chloroform-based methods and the hexane/isopropanol method, chromatograms of lipids obtained from a WOE menhaden meal sample extracted by the SAK and the HR methods are compared in Fig. 1. The chromatograms obtained for the first two partial scans, where the non-polar lipids are separated in the first scan and the redeveloped AMPLs in the second scan, show that the same major lipid components are present in both of the lipid extracts. The third scan shows the separation of polar lipid classes; in both lipid extracts peaks identified as UIM, PC and PM were observed. However, a peak designated as OXPL was seen only in the lipid extract obtained using the SAK and BD methods; it was not seen in the HR lipid extract. This might suggest that OXPL was not extracted from the oxidised fish meal sample or that it was present in such a small amount that it was not detected as a separate peak and therefore overlaps the PM peak.

The amounts of lipid classes extracted from an oxidized menhaden meal are illustrated in Fig. 2. All three procedures extracted about the same amount of the major non-polar lipid classes, ie TG, FFA and CHO, almost certainly (Walton *et al* 1989) > 95% cholesterol (Fig 2(a)). However, the hexane/isopropanol method tended to extract less of the polar lipid classes AMPL, UIM, PC and PM than the chloroform-based methods. This trend is particularly evident for the more polar lipid classes including UIM, PC and PM (Fig 2(b)). Similar results were obtained for unoxidised fish meal (Gunnlaugsdottir 1992). The TLC-FID determination of the lipid extracts obtained using the three different extraction procedures suggests that the lower lipid recovery of the hexane/isopropanol method may be due to a less efficient extraction of the polar lipid classes present in both oxidised and unoxidised fish meals.

Thus the lower yield of the hexane-based extraction method might be explained by the different solubilities of lipid components in polar and non-polar solvents, as polar lipids are thought to be only slightly soluble in hydrocarbon solvents such as hexane (Christie 1982;

Nelson 1991). Extraction of lipids from any fish or other muscle tissue requires both polar and non-polar solvents (Christie 1982). The polar solvent used in the HR procedure is isopropanol, whereas methanol is the polar solvent used in both the BD and SAK procedures. Of these two polar solvents, isopropanol is less polar than methanol (Zief and Kiser 1990), although it may have virtues in other circumstances (Peuchant *et al* 1989). Both of these factors, that is the poor solubility of polar lipid components in hydrocarbon solvents, and the lower polarity of isopropanol compared with methanol, might, in part, explain the less efficient extraction of polar lipid classes and consequentially the lower lipid recovery of the hexane/isopropanol method. In the past, evaluation of the types of lipid classes extracted from fish meal has been incomplete (de Koning *et al* 1985, 1986). Sahasrabudhe and Smallbone (1983) compared the amount of neutral and polar lipids extracted from lean, medium- and high-fat ground beef using seven different extraction methods. They used a silica acid column to separate the lipid classes in a purified lipid sample. Two of the seven extraction methods investigated were the BD and the HR extraction methods. The HR method was found to extract a smaller amount of the polar lipids in medium and high fat ground beef than the BD method. The results for fish meal in this study are therefore in agreement with the results of Sahasrabudhe and Smallbone (1983) for meat. Minor components may of course not all respond in the same way as acylated glycerol materials (Randall *et al* 1991; Cabrini *et al* 1992).

The occasional slightly lower recovery of lipid by the BD solvent system (Table 1) may also be partly due to a lesser recovery of polar material (Fig 2), since recoveries of the other six lipid classes seemed to be similar to those from the SAK extractions. Since the respective lipid recoveries from each solvent system, applied to the WE and WOE meals stored in different atmospheres, showed no systematic differences beyond the expected role of air (and O_2) and benefit of ethoxyquin, it appears that most of the lipid oxidation products and their interactions with amino acids take place in the drying stage of fish meal production or shortly thereafter. This is also suggested by the recent investigations of fish meal by Saito and Udagawa (1992) employing nuclear magnetic resonance. It may be significant that the lower temperature meal (ie meal dried at the lower temperature of 90°C instead of the conventional 100°C) gives better growth in salmon aquaculture (Pike *et al* 1990). Recovered FFAs were minor components (Fig 1). This lipid class may be especially prone to oxidation and interaction with amino acids (de Koning *et al* 1986) and there are indications that there is a temperature effect especially affecting FFAs (Koizumi *et al* 1986).

The samples of commercial fish meal were received approximately 1 week after manufacture and antioxidant had been added to the appropriate (WE) sample immediately after the post-drying cooling step of manufacture. The storage and distribution of fish meal is often a matter of months and the authors feel that their respective analyses were carried out on fish meal realistically mimicking commercial samples. Oxidation processes are modified as to time of initiation by antioxidants, but eventually even protected meals may be as susceptible to the same total oxidation as unprotected meals, accounting for the similarities in lipid recovery of Table 1 for the SAK and BD methods applied to the air- and O_2-stored samples compared with the N_2-stored samples.

CONCLUSIONS

Hexane/isopropanol extracts a significantly ($P \leqslant 0.05$) smaller amount of lipid from both oxidised and unoxidised menhaden meal than chloroform-based methods. TLC-FID determination of the lipid extracts suggests that the lower lipid recovery with hexane/isopropanol may be due to less efficient extraction of polar lipid classes. Thus, hexane/isopropanol cannot be recommended as a viable alternative to chloroform-based methods for lipid determination in brown fish meal.

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