ELSEVIER

PII: S0308-8146(97)00053-8

Food Chemistry, Vol. 61, No. 3, pp. 355–365, 1998

0308-8146/98 $19.00 + 0.00

Comparison between methods using low-toxicity solvents for the extraction of lipids from herring (*Clupea harengus*)

Ingrid Undeland,[a]* Magnus Härröd[b] & Hans Lingnert[a]

[a]*SIK — The Swedish Institute for Food and Biotechnology, Box 54 01, S-402 29 Göteborg, Sweden*
[b]*Department of Food Science, Chalmers University of Technology, c/o SIK, Box 54 01, S-402 29 Göteborg, Sweden*

(Received 7 January 1997; accepted 14 February 1997)

Three alkane/alcohol/water-based lipid extraction systems were evaluated to determine which would be the best replacement for the frequently used chloroform/methanol/water system, nowadays known to be very toxic. All the methods were applied to samples of minced herring (*Clupea harengus*) differing in quality and composition. In addition to comparisons of total lipid yield, the extracted lipids were compared with respect to content of triglycerides, phospholipids, free fatty acids, α-tocopherol, lipid hydroperoxides and conjugated dienes. The content of phospholipids was found to differ most between the lipids extracted by the four methods. Here, the chloroform/methanol/water system was the most efficient, followed by heptane/ethanol/water/sodium dodecyl sulphate (SDS) and then iso-propanol/hexane. However, by decreasing the level of SDS, the efficiency of the heptane/ethanol/water/SDS system in extracting phospholipids was increased to the same level as that of the chloroform/methanol/water system. This decrease in SDS also resulted in a higher recovery of free fatty acids. The lack of correlation between yields of phospholipids and yields of lipid oxidation products throughout this study was surprising because of the often-described susceptibility of phospholipids to oxidation.

INTRODUCTION

Fish lipids have received much attention owing to their positive effects on cardiovascular diseases (Bonekamp, 1990), but also because of their susceptibility to oxidation (Hultin, 1994). In investigations of lipid oxidation in fish, the solvent extraction step, which separates the lipids from the fish tissue, is a critical part of the analytical procedure. Lipids occur in the fish tissue in a wide variety of physical forms. Neutral lipids, or triglycerides, are parts of large aggregates in storage tissues and are extracted with relative ease. In contrast, phospholipids are more difficult to extract since they are constituents of membranes, where they occur in close association with such compounds as proteins and polysaccharides (Christie, 1982). Thus, the choice of solvents throughout the extraction has a great effect on the yields of various lipid classes, both intact and oxidised (Gunnlaugsdottir & Ackman, 1993; Randall *et al.*, 1991; Sahasrabudhe & Smallbone, 1983; Petterssen & Olsen, 1989).

*To whom correspondence should be addressed.

Among fish scientists, different modifications of the chloroform/methanol/water-based procedure of Folch *et al.* (1957), such as those developed by Bligh and Dyer (1959) and Hanson and Olley (1963), have been used extensively for the extraction of lipids. However, since chloroform is suspected of being a carcinogen (Radin, 1981) and methanol is well-known for its damage to the visual system (Hara & Radin, 1978), lipid investigators have sought less toxic, but still efficient, lipid extraction methods. To meet this need, alternative methods have been developed throughout the years (Radin, 1981; Hara & Radin, 1978; Burton *et al.*, 1985), most of which are based on alkane/alcohol/water mixtures such as hexane/iso-propanol/water. Alkane/alcohol/water mixtures have been proposed as solvent systems that are less toxic, cheaper, higher in UV transparency and lower extractability of non-lipids (Hara & Radin, 1978; Radin, 1981) as compared with chloroform/methanol/water. To evaluate the two solvent systems from these and other aspects, comparative investigations have been performed on beef (Sahasrabudhe & Smallbone, 1983), bluefish, sandworm, mussel (Randall *et al.*, 1991),

anchovy meal (de Koning & Mol, 1989), menhaden meal (Gunnlaugsdottir & Ackman, 1993) and giltheads (*Sparus auratus*) (Cabrini *et al.*, 1992). However, none of these investigations have focused on the efficiency of various methods in extracting lipid oxidation products. As compared with the native lipid, oxidation products are more polar in nature (Petterssen & Olsen, 1989) and it is believed that they are often associated with membranes (Halpin, 1984; Hardy *et al.*, 1979; Tichivangana & Morrisey, 1982). Special requirements must thus be placed upon the solvent mixture used for extraction of these products: it must make membrane lipids amenable to extraction and it must be polar enough to dissolve them. To avoid additional oxidation of lipids during the actual extraction procedure, the mixture used must also be effective at low temperatures.

In the present study, three methods based on solvents of low toxicity were chosen for comparison with a modified version of the Bligh and Dyer method in terms of their efficiency in extracting intact and oxidised lipids from minced herring (*Clupea harengus*). The four methods are summarised in Table 1. The first step was a screening for differences between and similarities among the four methods, after which the most promising alternative method was chosen for further improvement. The methods were compared with respect to amounts of total fat extracted, as well as the amounts of triglycerides, phospholipids, free fatty acids, α-tocopherol, lipid hydroperoxides and conjugated dienes in the fat obtained. The convenience of handling and practical use were other important aspects taken into account.

MATERIALS AND METHODS

Chemicals

n-Hexane, *n*-heptane, iso-propanol, methanol and water (for lipid class analyses) were all HPLC grade and obtained from Merck (Darmstadt, Germany). Iso-hexane was HPLC grade and was purchased from Fisons (Loughborough, UK). Chloroform was of pro-analysi grade (Merck, Darmstadt, Germany), acetic acid was of analytical grade (Prolabo, Paris, France) and the ethanol used was 99.5% and obtained from Kemetyl AB (Haninge, Sweden). Sodium dodecyl sulphate (SDS) was Molecular Biology Certified (Kodac Inc. Norwalk, CT, USA), and sodium sulphate and potassium iodide were both of pro-analysi quality (Merck, Darmstadt, Germany). α-Tocopherol was >95% (Merck, Darmstadt, Germany) and 13(S)HpODE was purchased from Cayman Chemical Company (Ann Arbor, MI 48108).

Herring samples

In order to compare the extraction methods in more than one sample material, four different herring minces (A, B, C and D) were used in the present study. These minces varied in quality and composition according to the different catching grounds and storage conditions described in Table 2. The herring was supplied by local fishermen from the west and east coasts of Sweden. The fish were kept on ice and transported to SIK for mincing. Herring mince was prepared in a Baader meat-bone separator (Model 694 Allan Bratetröm o/c AB,

Table 1. The four extraction methods evaluated

Method	Bligh and Dyer (1959)	Hara and Radin (1978)	Burton *et al.* (1985)	Nilsson *et al.* (1994)
Solvents	Chloroform/methanol/water	*n*-Hexane/iso-propanol/water	*n*-Heptane/ethanol/water	*n*-Hexane/iso-propanol/water
Flow-chart	Fish, 20 g + chloroform, 50 ml + methanol, 100 ml + water, 40 ml ↓ homogenisation, 1 min ↓ + chloroform, 50 ml ↓ homogenisation, 30 s ↓ + water, 50 ml ↓ homogenisation, 30 s ↓ centrifugation (19 600 *g*), 15 min ↓ evaporation of chloroform phase	Fish, 20 g + *n*-hexane:iso-propanol (3:2), 180 ml ↓ homogenisation, 30 s ↓ + *n*-hexane:iso-propanol (3:2), 180 ml ↓ homogenisation, 30 s ↓ centrifugation (1 500 *g*), 5 min ↓ *n*-hexane phase shaken with aqueous Na_2SO_4 (0.067 mg/ml), 140 ml ↓ centrifugation (1 500 *g*), 5 min ↓ evaporation of *n*-hexane phase	Fish, 20 g + water, 80 ml ↓ homogenisation, 30 s ↓ + SDS-solution,[a] 80 ml ↓ homogenisation, 30 s ↓ + 99.5% ethanol, 160 ml ↓ homogenisation, 30 s ↓ + *n*-heptane, 160 ml ↓ homogenisation, 1 min ↓ centrifugation (19 600 *g*), 15 min ↓ evaporation of *n*-heptane phase	Fish, 20 g + iso-propanol, 32 ml ↓ homogenisation, 30 s ↓ + *n*-hexane, 64 ml ↓ homogenisation, 30 s ↓ centrifugation (19 600 *g*), 15 min ↓ evaporation of *n*-hexane phase

[a]0.1 M in the screening part; 0.2, 0.1, 0.05 and 0.025 M in the improvement part.

Table 2. Facts about the four herring minces used in the study

Herring mince	A	B	C	D
Geographical origin	Atlantic	Atlantic	Atlantic	Baltic sea
Frozen storage (months/°C)	6/−30	6/−18	Fresh	6/−18
Fat content (%)[a]	8.9	11.9	11.2	10.7
Screening-(S)/Improvement part (I)	S	S	I	I
Application	Total lipid	—	Total lipid	—
	PV/CD[b]	—	—	PV/CD
	α-Tocopherol	α-Tocopherol	α-Tocopherol	α-Tocopherol
	Lipid classes	Lipid classes	Lipid classes	Lipid classes

[a]As determined with the modified Bligh and Dyer method (1959) (mean value, $n=2$, $a=1$).
[b]PV, peroxide value; CD, conjugated dienes.

Stockholm, Sweden) using a hole diameter of 2 mm. The mince was then packed into 125 g-HDPE boxes (High Density Polyethylene) covered with polyamide laminated polyethylene film. Immediately after packaging, minces A, B and D were frozen at −40°C in a tunnel freezer. Following freezing, the samples were stored at −18 or −30°C for various periods of time, after which they were transferred to a −70°C freezer where they were kept until analysis. Mince C was used in a fresh state, and was stored for less than 2 h at 4°C prior to analysis.

Design of the experiments and evaluation of results

In order not to confuse the *number of lipid extracts* and the *number of analyses* made on each of the lipid extracts, n and a, respectively, were used for these parameters. For the measurements of total lipid yield, peroxide value and conjugated dienes, $n=2$ was used, whereas $n=1$ for the analyses of α-tocopherol, triglycerides, phospholipids and free fatty acids. However, in the cases in which $n=1$, the whole experiment, i.e. extraction and analysis, was repeated twice, using herring minces of two different backgrounds.

The amounts of total extracted lipids, individual lipid components and primary oxidation products were compared to evaluate the methods. Despite the lack of comparison with actual theoretical values, the word *yield* is used in the present paper when describing all of the results. The total lipid *yields* are expressed on a *herring mince basis* and the *yields* of different lipid components on a *lipid basis*.

Lipid extraction

The four extraction methods compared are described in Table 1. The Bligh and Dyer method (1959) was modified as described by Ekstrand *et al.* (1996, Personal Communication) for fatty fish, the Hara and Radin procedure (1978) was used as modified by Pickova (1995) for cod roe, the Burton *et al.* method (1985) was used as described by these authors for various rat tissues, and the Nilsson *et al.* procedure (1994) was used as described by these authors in their first mixing trial. In those methods that used water, this was deionised. Homogenisation was performed on ice, using a Sorvall Omnimixer (Ivan Sorvall Inc., Northwalk, CT, USA). 250 ml-polypropene centrifuge bottles and a refrigerated centrifuge (Sorvall Superspeed RC2-B, Instrument AB Lambda, Stockholm, Sweden) at 4°C were used to centrifuge the homogenates. The organic layers were evaporated to dryness in a rotary evaporator (Buchi 011, RE 111, Switzerland) equipped with a 28°C water bath.

Measurement of total lipid yield

Total lipid yield was determined gravimetrically after extraction of minces A (screening part) and C (improvement part) with the methods to be studied. On each occasion $n=2$, $a=1$. The lipid yields are expressed as $g\,kg^{-1}$ of herring mince.

Measurement of triglycerides, free fatty acids and phospholipids

For measurements of triglycerides, free fatty acids and phospholipids, total lipids were dissolved in iso-hexane to a concentration of $1\,mg\,\mu l^{-1}$. The samples were analysed by an HPLC (Shimadzu SCL6B, Tokyo) equipped with a diol column (Lichrosphere 100 diol, 5 m, 4×250 mm; Merck, Darmstadt, Germany) and a Light Scattering Mass Detector 750/14 (Applied Chromatography Systems Ltd, Macclesfield, UK). An integration system (Kontron Instruments, Milan, Italy) was used for data collection and integration. The samples were eluted by heptane, 2-propanol, water and acetic acid at a temperature of 55°C. The composition of the mobile phase was at the moment of injection 100/0/0/0 (v/v), but gradually changed to 100/0.2/0/0.2, 50/50/0/1.5 and 0/70/30/1.5 (see Table 3). The initial flow rate was $3\,ml\,min^{-1}$, but was changed during the run according to Table 3. Known amounts of standards were used for identification. Measurements were carried out using minces A and B (in the screening part) as well as C and D (in the improvement part). On each occasion $n=1$, $a=1$. The yields of triglycerides, phospholipids and free fatty acids are expressed as area units μg^{-1} of fat.

Measurement of α-tocopherol

α-Tocopherol was determined by normal-phase HPLC according to the method of Piironen *et al.* (1984) with minor modifications as described by Undeland and Lingnert (1996, submitted). The determinations of α-tocopherol were carried out using herring samples A and B (in the screening part) and C and D (in the improvement part). On each occasion $n=1$, $a=1$. The repeatability of the method for α-tocopherol analysis was measured to be RSD% 3.0 (Relative Standard Deviation) ($n=1$, $a=6$). The levels of α-tocopherol are expressed as g kg^{-1} of fat.

Table 3. Design of mobile phase composition and flow gradients during lipid class analysis

	Composition in the bottles (v/v)			
	A	B	C	D
Heptane	100	100	50	—
HAc	—	0.2	1.5	1.5
2-propanol	—	0.2	50	70
Water	—	—	—	30

	Gradients				
Time (min)	Mobile phase (%)				Flow rate (ml min^{-1})
	A	B	C	D	
0	100	0	—	—	3
5	100	0	—	—	—
6	90	10	—	—	—
30	90	10	—	—	—
31	50	50	—	—	—
44	0	100	0	—	—
54	—	96	4	—	—
64	—	81	19	—	—
72	—	0	100	0	—
73	—	—	99	1	—
79	—	—	99	1	—
90	—	—	90	10	—
97.5	—	—	—	—	3
100	—	—	64	36	—
101.3	—	—	—	—	2.47
102	—	—	—	—	2.33
103.2	—	—	—	—	2.16
104	—	—	0	100	—
104.2	—	—	—	—	1.98
105.3	—	—	—	—	1.72
106.2	—	—	—	—	1.6
107	—	—	—	—	1.54
107.6	—	—	—	—	1.5
115	—	—	0	100	1.5
115.1	—	—	0	100	1.5
115.7		0	100	0	—
117.5	—	50	50	—	—
118.1	—	—	—	—	1.5
119.9	—	—	—	—	1.75
120.5	—	—	—	—	2.2
121	—	50	50	—	3
121.5	—	65	35	—	—
130	0	100	0	—	—
133	35	65	—	—	—
135	100	0	—	—	—
145	100	0	—	—	3

Measurement of peroxide value

The peroxide value (PV) was determined by the AOCS Cd-8-53 titrimetric method (1990) using minces A (in the screening part) and D (in the improvement part). On each occasion $n=2$, $a=2$. The method was slightly modified in that 0.5 g of fat was used instead of 1 g. Furthermore, 1 ml potassium iodide and 25 ml water were added rather than 0.5 ml and 30 ml, respectively. The repeatability of the method for analysing PV was RSD% 3.1 ($n=1$, $a=6$), and the results are expressed as Meq peroxide kg^{-1} of fat.

Measurement of conjugated dienes

Oxidised fatty acids containing conjugated double bonds absorb UV light strongly between 230 and 375 nm and, when containing dienes, at 234 nm (Chan & Lewett, 1977; Situnayake *et al.*, 1990). This property has been used as the basis for their detection in biological samples by simple spectrophotometry (Parr & Swoboda, 1976). In the present study, absorption at 234 nm was measured using FIA (Flow Injection Analysis) as described by Undeland and Lingnert (1996, submitted). Samples from mince A were used in the screening part of the present study, while samples from mince D were used in the improvement part. On each occasion $n=2$, $a=2$. The repeatability of the method for analysing conjugated dienes was RSD% 4.5 ($n=1$, $a=6$), and the results are expressed as area units μg^{-1} of fat.

RESULTS

Comparison between the four extraction methods

Total lipid yield, α-tocopherol and lipid classes

The yields of total lipids from herring mince A obtained with the four extraction methods are listed in Table 4. The Bligh and Dyer method gave the highest yield, being between 15 and 30% higher than the yields obtained with the other three methods. The levels of α-tocopherol and various lipid classes in the fat extracted with the four methods are shown in Fig. 1 (herring mince A) and Fig. 2 (herring mince B). With respect to α-tocopherol yields, both these figures show the same tendency, which is that fat extracted with the Hara and Radin method contained the highest levels of α-tocopherol. The effect

Table 4. Total lipid yield (g kg^{-1} of herring mince)[a] obtained from extractions of mince A

Extraction method	Total lipid yield
Bligh and Dyer	89 ± 6
Hara and Radin	67 ± 3
Burton *et al.*	74 ± 1
Nilsson *et al.*	78 ± 2

[a]Mean ± (maximum−minimum value)/2, ($n=2$, $a=1$).

of each extraction method on the distribution of triglycerides, phospholipids and free fatty acids also resulted in nearly the same patterns in fat from both herring minces. The levels of triglycerides and, except in one case, free fatty acids seemed to be unaffected by the extraction method applied. This was in contrast to the yields of phospholipids, which were greatly affected by the extraction procedure. The Bligh and Dyer method extracted by far the largest amounts of phospolipids from both herring minces followed by, in descending

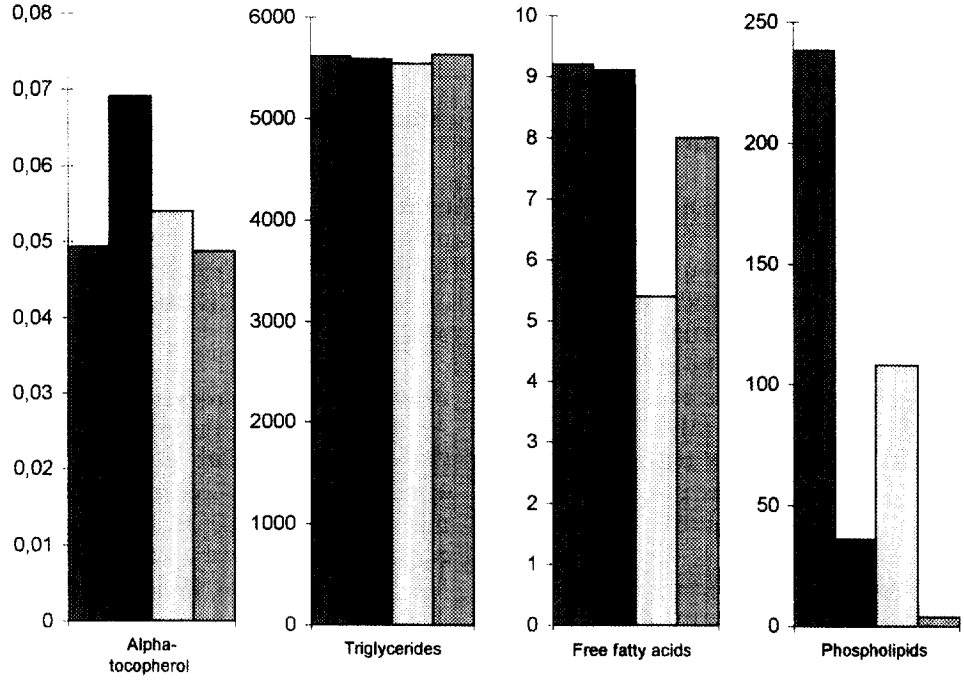

Fig. 1. Influence of the extraction method on the amounts of α-tocopherol (g kg^{-1} fat), triglycerides (area units μg^{-1} fat), free fatty acids (area units μg^{-1} fat) and phospholipids (area units μg^{-1} fat) in fat from herring mince A. (■) Bligh and Dyer; (■) Hara and Radin; (□) Burton *et al.*; and (▨) Nilsson *et al.*

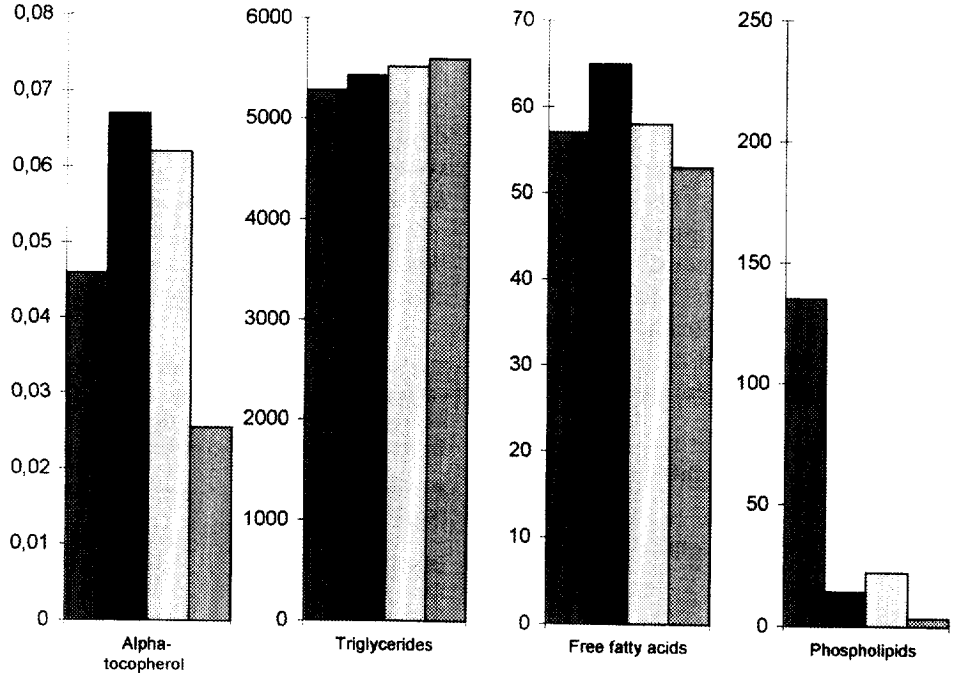

Fig. 2. Influence of the extraction method on the amounts of α-tocopherol (g kg^{-1} fat), triglycerides (area units μg^{-1} fat), free fatty acids (area units μg^{-1} fat) and phospholipids (area units μg^{-1} fat) in fat from herring mince B. (■) Bligh and Dyer; (■) Hara and Radin; (□) Burton *et al.*; and (▨) Nilsson *et al.*

order, the Burton *et al.* method, the Hara and Radin method and the Nilsson *et al.* method.

Primary oxidation products

The efficiency of the four extraction methods for extracting lipid hydroperoxides and conjugated dienes from herring mince A is illustrated in Table 5. The peroxide values in the lipids extracted with the four methods were very similar and the same was true for the levels of conjugated dienes.

Table 5. Peroxide value (Meq peroxide kg^{-1} fat)[a] and level of conjugated dienes (area units μg^{-} fat)[a] in fat extracted from mince A

Extraction method	Peroxide value	Conjugated dienes
Bligh and Dyer	4.6 ± 0.3	8.3 ± 0.1
Hara and Radin	5.3 ± 0.6	9.0 ± 1.4
Burton *et al.*	5.9 ± 0.9	7.9 ± 0.5
Nilsson *et al.*	5.0 ± 1.4	9.0 ± 0.2

[a]Mean ± (maximum − minimum value)/2, ($n = 2$, $a = 2$). A mean value of the two analyses was used to establish this sample variation.

Improvement of the Burton *et al.* procedure

On the basis of the results of the phospholipid measurements in the first part of this study, the Burton *et al.* procedure was selected for further improvement. As described by the authors of this method, SDS in high concentrations may cause the formation of soluble mixed lipid–SDS micelles to which the phospholipids are attracted. This property was studied by changing the concentration of SDS from the original 0.1 M, to 0.2, 0.05 and 0.025 M. The impact of this change in SDS concentration on the yields of various intact and oxidised lipid components was then evaluated.

Total lipid yield, α-tocopherol and lipid classes

Table 6 shows total lipid yields from extractions of herring mince C with the four Burton *et al.* procedures and the Bligh and Dyer method. In accordance with the results from the screening part of this study (Table 4), the Bligh and Dyer method gave higher yields of lipids (20–40% higher) than did the various Burton *et al.* procedures. Table 6 also shows that the change in SDS concentration did not seem to affect the efficiency of the Burton *et al.* method in extracting total lipids. Figures 3 and 4 show the lipid class distributions in fat extracted from minces C and D, respectively. From both minces, all five methods extracted fat containing nearly the same concentrations of α-tocopherol and triglycerides. A few exceptions were the slightly higher yields of α-tocopherol in fat obtained from the two minces extracted with 0.2 M SDS and from mince C extracted with 0.05 M SDS. In contrast to these results, the yields of more polar lipid components, such as phospholipids and free fatty acids, were largely dependent on the solvents used for extraction. A decreasing concentration of SDS correlated positively with an increasing yield of both phospholipids and free fatty acids. Using 0.025 M SDS, the Burton *et al.* method actually gave the same yields of both these lipid classes as did the Bligh and Dyer method.

Table 6. Total lipid yield (g 'kg^{-1} herring mince)[a] obtained from extractions of mince C with the modified Bligh and Dyer method and the four Burton *et al.* procedures comprising various SDS concentrations (M)

Extraction method (SDS concentration)	Total lipid yield
Bligh and Dyer	111 ± 5
Burton *et al.* (0.2)	78 ± 1
Burton *et al.* (0.1)	90 ± 5
Burton *et al.* (0.05)	86 ± 4
Burton *et al.* (0.025)	85 ± 7

[a]Mean ± (maximum − minimum value)/2, ($n = 2$, $a = 1$).

Primary oxidation products

As was found for the first part of the present study (Table 5), the nature of the solvents used for lipid extraction had no effect on the yields of primary oxidation products (Table 7). Both the PV and the level of conjugated dienes were very similar among the various fats extracted from mince D with each of the five methods.

DISCUSSION

From the number of lipid extraction comparisons carried out in recent years (Hara & Radin, 1978; de Koning & Mol, 1989; Randall *et al.*, 1991; Cabrini *et al.*, 1992; Sahasrabuhe & Smallbone, 1983; Gunnlaugsdottir & Ackman, 1993), it is obvious that there is a desire to find solvent systems with low toxicity to replace the hazardous but commonly used chloroform/methanol/water system (Folch *et al.*, 1957; Bligh & Dyer, 1959; Hanson & Olley, 1963). However, because of the high efficiency of this system for extracting both polar and unpolar lipids, no alternative system has this far been fully successful. In the present comparison, unlike previous ones,

Table 7. Peroxide value (Meq peroxide kg^{-1} fat)[a] and level of conjugated dienes (area units μg^{-1} fat)[a] in fat extracted from mince D with the modified Bligh and Dyer method, and the four Burton *et al.* procedures comprising various SDS concentrations M

Extraction method (SDS concentration)	Peroxide value	Conjugated dienes
Bligh and Dyer	10.5 ± 1	20 ± 0.1
Burton *et al.* (0.2)	9.5 ± 0.5	22 ± 0.3
Burton *et al.* (0.1)	11.5 ± 0.3	20 ± 1.5
Burton *et al.* (0.05)	10.5 ± 1.7	23 ± 0.2
Burton *et al.* (0.025)	9.0 ± 1.3	21 ± 0.2

[a]Mean ± (maximum − minimum value)/2, ($n = 2$, $a = 2$). A mean value of the two analyses was used to establish this sample variation.

consideration was taken to the efficiency of various methods in extracting lipid oxidation products. Special attention was therefore paid to the concentrations of the more polar components in the lipids. This approach led to some notable results, in most cases seen contemporaneously in lipids extracted from herring minces with completely different backgrounds, a fact which greatly strengthens some of the conclusions drawn.

Comparison between the four extraction methods

In accordance with the results of Gunnlaugsdottir & Ackman (1993), who compared two chloroform/methanol/water based systems (Bligh & Dyer, 1959; Smith *et al.*, 1964) with the method of Hara & Radin (1978), the present study showed that chloroform/methanol/water gave the highest yields of total lipids as

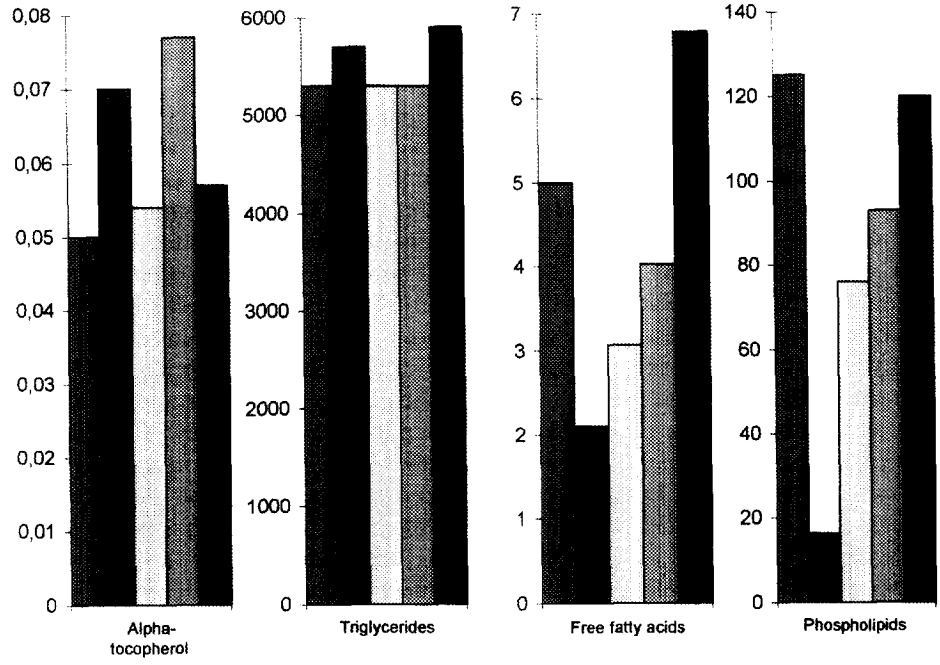

Fig. 3. Influence of the extraction method on the amounts of α-tocopherol (g kg^{-1} fat), triglycerides (area units μg^{-1} fat), free fatty acids (area units μg^{-1} fat) and phospholipids (area units μg^{-1} fat) in fat from herring mince C. (▩) Bligh and Dyer; (■) Burton *et al.*; (0.2 M SDS); (□) Burton *et al.* (0.1 M SDS); (▨) Burton *et al.* (0.05 M SDS); and (■) Burton *et al.* (0.025 M SDS)

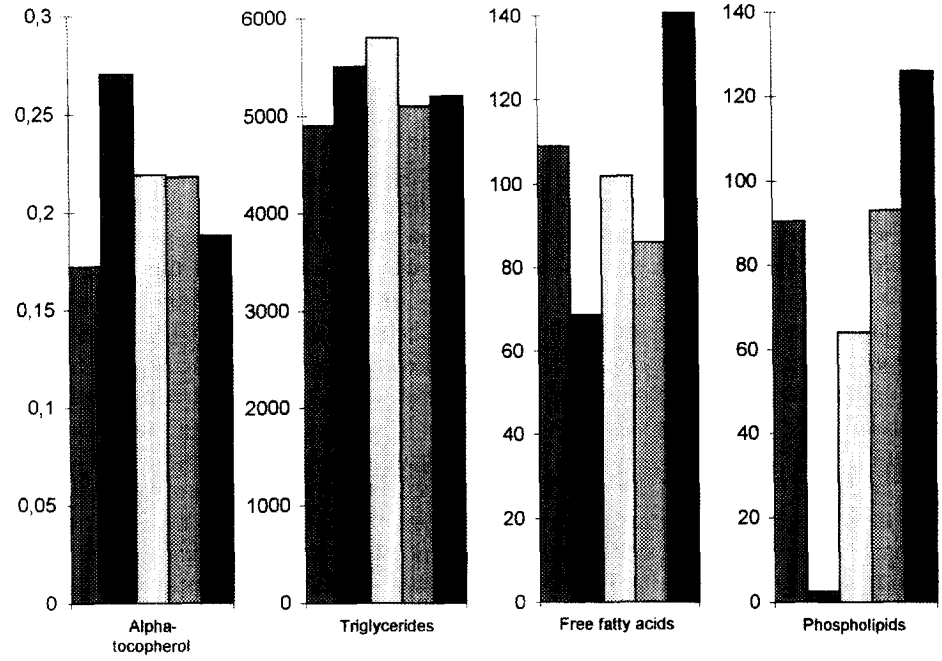

Fig. 4. Influence of the extraction method on the amounts of α-tocopherol (g kg^{-1} fat), triglycerides (area units μg^{-1} fat), free fatty acids (area units μg^{-1} fat) and phospholipids (area units μg^{-1} fat) in fat from herring mince D. (▩) Bligh and Dyer; (■) Burton *et al.* (0.2 M SDS); (□) Burton *et al.* (0.1 M SDS); (▨) Burton *et al.* (0.05 M SDS); and (■) Burton *et al.* (0.025 M SDS)

compared with the various alkane/alcohol/water mixtures. However, these results do not agree with those obtained in comparisons between the Bligh and Dyer method and methods based on heptane/ethanol/water/SDS (Burton *et al.*, 1985; Cabrini *et al.*, 1992), *n*-hexane/ethanol/water (Cabrini *et al.*, 1992) and *n*-hexane/iso-propanol/water (Sahasrabudhe & Smallbone, 1983). In these evaluations, total lipid yields obtained with the various methods could not be distinguished from one another. It has been proposed that these inconsistencies in the results can be explained by differences in the levels of lipid oxidation products (de Koning & Mol, 1989; Pokorny *et al.*, 1983) and polar lipids (Christie, 1982) among the samples. As the chloroform/ methanol/water system extracts polar compounds more efficiently than the alcohol/alkane/water system does, high levels of these compounds will affect the results significantly.

In the present study, the efficiency of the chloroform/methanol/water system in extracting polar lipids was obvious. Actually, only the yields of these lipids were considerably affected by the nature of the solvent system (Figs 1 and 2), but here the differences were huge. As an example, the Nilsson *et al.* method gave phospholipid yields that were only 2.5% (mince A) and 1.7% (mince B) of those obtained with the Bligh and Dyer method. Similar results, although not as pronounced, have been obtained by many scientists (Cabrini *et al.*, 1992; Sahasrabudhe & Smallbone, 1983; Gunnlaugsdottir & Ackman, 1993), and one explanation has been the bilayer arrangement of the membranes in which the phospholipids are situated (Randall *et al.*, 1991). Because of this arrangement, which only exposes the polar lipid ends, it is understandable why polar solvents are needed for extraction and, furthermore, why physical forces must also be overcome (Randall *et al.*, 1991). According to Bligh & Dyer (1959) and Christie (1989), the monophasic system obtained using chloroform:methanol:water in the proportions 1:2:0.8 has all these required properties. It thus overcomes the interactions between lipids and the tissue matrix and also readily dissolves the lipids (Christie, 1989). The lower efficiency of the alkane/alcohol/water mixtures for the extraction of phospholipids can be explained by two reasons: the polar part of these mixtures (in this study ethanol and iso-propanol) is less polar than the methanol, which constitutes the polar part of the Bligh and Dyer system (Zief & Kiser, 1990) further, solubility of polar lipids is poorer in hydrocarbon solvents, such as hexane, than it is in chloroform (Christie, 1982).

With both herring minces (Figs 1 and 2), the four methods were ranked in the same order as regards their efficiency in extracting α-tocopherol, the order being: Hara and Radin > Burton *et al.* > Bligh and Dyer > Nilsson *et al.* As in the present results, Cabrini *et al.* (1992) also found that alkane/alcohol/water extracted α-tocopherol more efficiently than chloroform/methanol/ water. These authors evaluated the Bligh and Dyer, Burton *et al.* and Katayama *et al.* (1980) (*n*-hexane/ethanol/water) methods for yields of lipophilic antioxidants from gilthead liver and rat liver. The following order of efficiency was found for the α-tocopherol yield: Katayama *et al.* > Burton *et al.* > Bligh and Dyer. Burton *et al.* (1985) also compared their method with that of Bligh and Dyer for extractability of α-tocopherol. Here, the Burton *et al.* procedure was found to be the most efficient one. All the above results might be explained by the nonpolar nature of α-tocopherol and, thus, its need for more nonpolar solvents in order to dissolve, a need which is fulfilled by the various alkane/alcohol/water mixtures.

The results of the analyses of peroxide value and conjugated dienes in the various lipid extracts were somewhat surprising. Despite the fact that one important lipid class, the phospholipids, was more or less lost in some of the extractions (Figs 1 and 2), the level of primary oxidation products was nearly the same in all of the four extracts obtained (Table 5). As has been described by many authors (Hardy *et al.*, 1979; Tichivangana & Morrisey, 1982; Halpin, 1984), the phospholipids are particularly susceptible to lipid oxidation, which is why a significant amount of the primary oxidation products formed would be expected to originate from the phospholipid fraction. Lower extraction yields of phospholipids would then be expected to result in lower levels of primary oxidation products. However, this correlation was not seen in the present study. One explanation for this could be that the phospholipid fraction is a very small part of the total lipids in herring (Eliasson & Nilsson, 1994). Thus, even if the phospholipids are relatively more oxidised than the neutral lipids, the methods used for measurement of PV and conjugated dienes may not be sensitive enough to monitor total changes caused by differences in the contributions of primary phospholipid oxidation products. This should imply that, among all the lipid hydroperoxides formed, the largest part originates from the neutral lipids. However, the phospholipids could still be the most important lipid class regarding initiation of lipid oxidation. Furthermore, we cannot exclude the possibility that the primary phospholipid oxidation products are so polar that they are not fully recovered by any of the extraction methods tested. To elucidate this, more detailed studies of oxidation in various lipid classes and of the extractability of primary oxidation products remain to be conducted.

In terms of handling and practical use, each method showed advantages and disadvantages. Regarding collection of the organic layers, all three alkane/alcohol/water mixtures were more convenient than the Bligh and Dyer method. This was because the alkane layers were obtained as upper phases, in contrast to the chloroform of the latter method. Concerning evaporation of the organic solvents, less than 4 min was required for chloroform, whereas more than ten min was needed for hexane and heptane. This period of time, elapsing during the evaporation step, may be an

important factor to take into consideration as a means of avoiding unwanted oxidation of the lipids. One possible improvement that would decrease this risk is a replacement of hexane and heptane with petroleum ether, since this solvent has a lower boiling point.

According to this discussion, it is difficult to rank the four methods with respect to the convenience of their performance. However, in general, the simplest method to use was that of Nilsson *et al.*, since it required small amounts of solvents and, further, because of its speed.

Improvement of the Burton *et al.* procedure

In the first part of this study, the comparison between four extraction methods, the only yield that was considerably affected by the nature of the extraction solvents was the phospholipid yield (Figs 1 and 2). Concerning this lipid class, the Bligh and Dyer method proved to be superior to the three alternative methods. However, since the aim of the present study was to replace chloroform/methanol, the second most efficient method for extractions of phospholipids, the Burton *et al.* procedure, was selected for further improvement.

As described by Burton *et al.* (1985), their method combines the property of the detergent SDS to dissociate and solubilise membrane proteins with the extraction capability of the aqueous alcohol/alkane mixture ethanol/heptane. The efficiency of this method was confirmed for human plasma, red blood cells, homogenates of various tissues from rat and various multilamellar vesicles. They proved the SDS method to be superior in terms of recovery of α-tocopherol and total lipids as compared with the Bligh and Dyer procedure. However, according to the authors, it is important to bear in mind that mixed lipid–SDS micelles may form when there is sufficient SDS present. As a result, it is believed that the phospholipids are retained within these micelles upon addition of ethanol and *n*-heptane. With this background, the second part of this study was aimed at evaluating the effect of various SDS concentrations on the yield of different lipid components, in particular the phospholipids.

As was seen in Table 6, all four SDS concentrations tested in relation to Burton and colleagues' method (0.2, 0.1, 0.05 and 0.025 M) resulted in lower total lipid yields as compared with the Bligh and Dyer method. According to Figs 3 and 4, the most probable explanation for this is not the failure of the SDS method to extract polar lipids. Rather, the explanation is incomplete removal of the organic phase due to precipitate formation (probably consisting of SDS and proteins) at the interface between the aqueous and the organic layers.

It seems likely from Figs 3 and 4 that the suggestions of Burton *et al.* regarding the formation of mixed SDS–lipid micelles and their attraction to phospholipids, were correct. There was a clear inverse correlation between SDS concentration and yield of phospholipids. As a result, the same high phospholipid yield obtained with the Bligh and Dyer method could be obtained by decreasing the level of SDS from the original 0.1 to 0.025 M. With only one exception (Fig. 4), there was also an inverse correlation between SDS level and yield of free fatty acids. This is probably also a result of the fairly high polarity of the free fatty acids, meaning that they were retained in the lipid–SDS micelles together with the phospholipids. At the SDS level of 0.025 M, the efficiency of the Burton *et al.* procedure for extracting free fatty acids actually seemed slightly higher than that of the Bligh and Dyer method. The present results are not in complete accordance with those obtained by Burton *et al.* (1985), who found that recoveries of total and individual fatty acids from rat liver were insensitive to SDS concentrations between 0.05 and 0.2 M. However, a marked change in the total fatty acid composition occurred at SDS concentrations higher than 0.2 M. The authors associated this change with a decline in phospholipid recovery. Similar results were obtained by Cabrini *et al.* (1992). Using a wide range of SDS levels, they found nearly the same yields of various lipid components in fat from giltheads (*Sparus auratus*) and rat livers, as well as from giltheads white muscle. According to Burton *et al.* (1985), proteins may act as a "buffer" or "sponge" to SDS, and consequently, differences in SDS sensitivity appear to be related to the amount of proteins present in the extracted material. As described by these authors, the concentration of free monomeric SDS does not reach the 'critical micelle concentration' until the proteins are completely saturated. However, from the present results, this does not seem to apply for herring mince, since this is a material which is rich in proteins but still very sensitive to small variations in SDS. The nature of the proteins and phospholipids present may be another explanation of the obvious variations in SDS sensitivity among different materials. As regards the phospholipids, Burton *et al.* (1985) reported that both the type of head group and fatty acid tail, to a large extent determine its retention in the micelles.

In agreement with the study of Burton *et al.* (1985), various SDS concentrations did not seem to affect the yields of more unpolar lipid components, in this case α-tocopherol and triglycerides. There were no common trends among the lipids extracted from minces C and D that correlated with the SDS concentration used. The same was true when the various Burton *et al.* methods were compared with the Bligh and Dyer method. Thus, as was previously seen in the first part of this study, these methods extracted unpolar lipid components with almost equal efficiency.

In terms of the extractability of primary lipid oxidation products, the more or less surprising results obtained in the screening part of this study were repeated in the improvement step (Table 7). Thus, neither the peroxide value nor the level of conjugated dienes was affected by the nature of the extraction solvents. Despite the very low yield of phospholipids and free fatty acids using 0.2 M SDS as compared with 0.025 M, the two lipid

extracts had the same levels of primary oxidation products. Possible explanations for this have previously been discussed.

CONCLUSIONS

The use of alkane/alcohol/water mixtures for extractions of intact and oxidised lipids from minced herring has the advantage that the solvents are relatively low in toxicity and, in some respects, are very convenient to use. However, as compared with chloroform/methanol/water, these mixtures were found to extract a somewhat smaller amount of total lipids, and a much smaller amount of phospholipids. The Burton *et al.* procedure (heptane/ethanol/water/SDS) was the only alkane/alcohol/water method that extracted reasonable amounts of phospholipids. This method was therefore selected for further improvement in order to give the same yield of phospholipids as did the chloroform/methanol/water system. This objective was achieved by decreasing the SDS concentration from 0.1 to 0.025 M, which also positively affected the extractability of free fatty acids. The fairly low yield of total lipids was unaffected by this modification, and was explained instead by the handling process. As a consequence, the Burton *et al.* procedure is not useful for analyses of total fat, but, in this modified way, could be highly recommended for measurements of α-tocopherol, various lipid classes and primary lipid oxidation products. As regards the primary lipid oxidation products, a surprising observation was that their yields were unaffected by the large variations in recoveries of phospholipids obtained.

Using the Bligh and Dyer method as a reference, this screening study revealed the Burton *et al.* procedure to be a very promising method for lipid extractions from fatty fish. However, to make this a standard method, a deeper evaluation of critical steps and optimal conditions is recommended.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Ingela Gangby and Annika Rutgersson for their skilful assistance in the laboratory. Thanks are also due to Abba seafood AB, and the Swedish Council for Forestry and Agricultural Research for their financial support of this study.

REFERENCES

Anonymous (1990). Peroxide value acetic acid–chloroform method. AOCS Official Method Cd-8-53.

Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. of Biochem. Physiol.*, **37**, 911–917.

Bonekamp, G. (1990). Sill och strömming. Wezäta AB, Göteborg, Sweden.

Burton, G. W., Webb, A. & Ingold, K. U. (1985). A mild rapid and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids*, **20**, 29–39.

Cabrini, L., Landi, L., Stefanelli, C., Barzanti, V. & Sechi, A. M. (1992). Extraction of lipids and lipophilic antioxidants from fish tissues: a comparison among different methods. *Comp. Biochem. Physiol.*, **101 B**, 383–386.

Chan, W. H-S. & Lewett, G. (1977). Autoxidation of methyl linoleate. Separation and analysis of isomeric mixtures of methyl linoleate hydroperoxides and hydroxy linoleates. *Lipids*, **12**, 99–104.

Christie, W. W. (1982). *Lipid Analysis*, 2nd ed. Pergamon Press, New York, NY, USA.

Christie, W. W. (1989). Fatty acids and lipids: structures, extraction and fractionation into classes. In *Gas Chromatography and Lipids*. The Oily Press, Ayr, Scotland, pp. 11–42.

de Koning, A. J. & Mol, T. H. (1989). Lipid determination in fish meal: an investigation of three standard methods applied to stabilised and non-stabilised anchovy meals at increasing stages of maturity. *J. of Sci. Food Agric.*, **46**, 259–266.

Eliasson, M. & Nilsson, K. (1994). Determination of the peroxide value of solid phase separated polar and non-polar fat from herring. Degree project, Swedish Institute of Food and Biotechnology, Göteborg, Sweden.

Folch, J., Lees, M. & Sloaney Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. of Biol. Chem.*, **226**, 497–509.

Gunnlaugsdottir, H. & Ackman, R. G. (1993). Three extraction methods for determination of lipids in fish meal: evaluation of a hexane/isopropanol method as an alternative to chloroform-based methods. *J. of Sci. Food Agric.*, **61**, 235–240.

Halpin, B. E. (1984). On the possible role of the sarcoplasmatic reticulum in lipid oxidation in fish. M. S. Thesis, University of Massachusetts, Amherst, USA.

Hanson, S. W. F. & Olley, J. (1963). Application of the Bligh & Dyer method of lipid extraction to tissue homogenates. *Biochem. J.*, **89**, 101–102.

Hara, A. C. & Radin, N. S. (1978). Lipid extractions of tissues with a low-toxicity solvent. *Anal. Biochem.*, **90**, 420–426.

Hardy, R., McGill, A. S. & Gunstone, F. D. (1979). Lipid and autoxidative changes in cold stored cod (*Gadhus morhua*). *J. of Sci. Food Agric.*, **30**, 999–1006.

Hultin, H. O. (1994). Oxidation of lipids in seafoods. In *Seafoods Chemistry, Processing Technology and Quality*, ed. H. H. Huss, M. Jakobsen & J. Liston. Elsevier, Amsterdam, pp. 125–138.

Katayama, K., Takada, M., Yuzurya, T., Abe, K. & Ikenoya, S. (1980). Simultaneous determination of ubiquinone-10 and ubiquinol-10 in tissue and mitochondria by high performance liquid chromatography. *Biochem. Biophys.*, **95**, 971–977.

Nilsson, M., Hansson, G. & Johansson, L. (1994). Utveckling av gaskromatografisk analysmetod för fettsyror i biologiska prover. Degree project, University of Göteborg, Sweden.

Parr, L. J. & Swoboda, P. A. T. (1976). The assay of conjugable oxidation products applied to lipid deterioration in stored foods. *J. of Food Technol.*, **11**, 1–12.

Petterssen, J. & Olsen, S. (1989). Rancidity and its measurements in foods. Proceedings from 15th Scandinavian Symposium on lipids, Rebild Bakker, Denmark, 11–15 June, pp. 528–539.

Pickova, J. (1995). Fatty acid composition in eggs from cod (*Gadhus morhua*) originating from four different groups. Publ. no 35, Dep. of Food Science, Swedish Institute of Agricultural Sciences.

Piironen, V., Naro, P., Syväoja, E-L., Salminen, K. & Koivistionen, P. (1983). High-performance liquid chromatographic determination of tocopherols and tocotrienols and its application to diets and plasma of Finnish men. *Int. J. of Vit. Nutr. Res.*, **53**, 35–40.

Pokorny, J., Novotna-Moravkova, E., Alexova, H. & Davidek, J. (1983). Oxidation of polyunsaturated lipids in presence of nonlipidic components. *Dev. Food Sci.*, **11**, 603–618 (CA 106: 1747722j).

Radin, N. S. (1981). Extraction of tissue lipids with a solvent of low toxicity. In *Methods in Enzymology*, Vol. 72, ed. J. Lownstein. Academic Press, New York, pp. 5–7.

Randall, R. C., Lee II, H. & Ozretich, R. J. (1991). Evaluation of selected lipid methods for normalizing pollutant bioaccumulation. *Environmental Technology and Chemistry*, **10**, 1431–1436.

Sahasrabudhe, M. R. & Smallbone, B. W. (1983). Comparative evaluation of solvent extraction methods. *JAOCS*, **60**, 801–805.

Situnayake, R. D., Crump, B. J., Zezulka, A. V., Davis, M., McConcey, B. & Thurnham, D. I. (1990). Measurment of conjugated diene lipids by derivative spectroscopy in heptane extracts of plasma. *Ann. Clin. Biochem.*, **27**, 258–266.

Smith, P., Ambrose, M. E. & Knobl, G. N. (1964). Improved rapid method for determining total lipids in fish meal. *Comm. Fish Rev.*, **26**, 1–5.

Tichivangana, J. Z. & Morrisey, P. A. (1982). Lipid oxidation in cooked fish muscle. *Ir. J. of Food Sci. Technol.*, **6**, 157–163.

Undeland & Lingnert (1996). *J. Sci. of Food Agric.*, submitted.

Zief, M. & Kiser, R. (1990). An overview of solid phase extraction for sample preparation. *Am. Lab.*, **22**, 70–83.