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Total Lipid Extraction of Homogenized and Intact Lean Fish Muscles Using Pressurized Fluid Extraction and Batch Extraction Techniques

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The reliability and efficiency of pressurized fluid extraction (PFE) technique for the extraction of total lipid content from cod and the effect of sample treatment on the extraction efficiency have been evaluated. The results were compared with two liquid−liquid extraction methods, traditional and modified methods according to Jensen. Optimum conditions were found to be with 2-propanol/*n*-hexane (65:35, v/v) as a first and *n*-hexane/diethyl ether (90:10, v/v) as a second solvent, 115 °C, and 10 min of static time. PFE extracts were cleaned up using the same procedure as in the methods according to Jensen. When total lipid yields obtained from homogenized cod muscle using PFE were compared yields obtained with original and modified Jensen methods, PFE gave significantly higher yields, ∼10% higher (*t* test, $P < 0.05$). Infrared and NMR spectroscopy suggested that the additional material that inflates the gravimetric results is rather homogeneous and is primarily consists of phospholipid with headgroups of inositidic and/or glycosidic nature. The comparative study demonstrated that PFE is an alternative suitable technique to extract total lipid content from homogenized cod (lean fish) and herring (fat fish) muscle showing a precision comparable to that obtained with the traditional and modified Jensen methods. Despite the necessary cleanup step, PFE showed important advantages in the solvent consumption was cut by ∼50% and automated extraction was possible.

KEYWORDS: Lean fish; phospholipids; pressurized fluid extraction; PFE; total lipid content

INTRODUCTION

Sample preparation is a laborious, but necessary, step for characterization of the total lipid content of food or determination of specific analytes, when using exhaustive and nonselective extraction techniques. Many methods for the extraction of total lipids were developed during the last century by scientists such as Folch et al. (*1*), Bligh and Dyer (B&D) (*2*), and Jensen et al. (*3*, *4*). Organic solvents alone or in combination are used for these extractions. At that time, the laboratory workload and the amount of solvent consumed were much more than those nowadays. Moreover, there was no consciousness of the health risk and environmental pollution due to the continuous discharge of the organic solvents such as aromatic hydrocarbons and chlorinated solvents (*5*). It is also well-known that the choice of method for the extraction of analytes from the same tissue influences the yield. Although nonpolar solvents such as light petroleum ether are excellent solvents for nonpolar organics, their ability to extract polar organics is poor. Thus, quantitative total lipid extractions are often not possible because of the poor ability of these solvents to displace polar analytes, such as phospholipids, from stable matrix−analyte complexes (*6*).

Lipids exist in different chemical complexities. The simple lipids such as triglycerides are often part of large aggregates in storage tissue, from which they are extracted relatively easily. In contrast, complex lipids such as phospholipids and glycosphingolipids are usually constituents of membranes, where they occur in a close association with compound classes such as proteins and polysaccharides, with which they interact, and they are not extracted so readily (*7*).

Along with the demand for higher recovery, faster analysis, and increased possibilities for automation and reduced solvent usage, newer extraction techniques have been developed during the past decades including microwave-assisted extraction (*8*), supercritical fluid extraction (*9*), and pressurized fluid extraction (PFE). Several studies have shown that these methods in many cases can be more efficient than the traditional extraction methods (*5*, *6*, *10*−*14*). PFE utilizes extraction media including organic solvents at temperatures above the normal boiling point. The solvent is kept in a liquid phase due to high pressure even at temperatures much above the atmospheric boiling point. The

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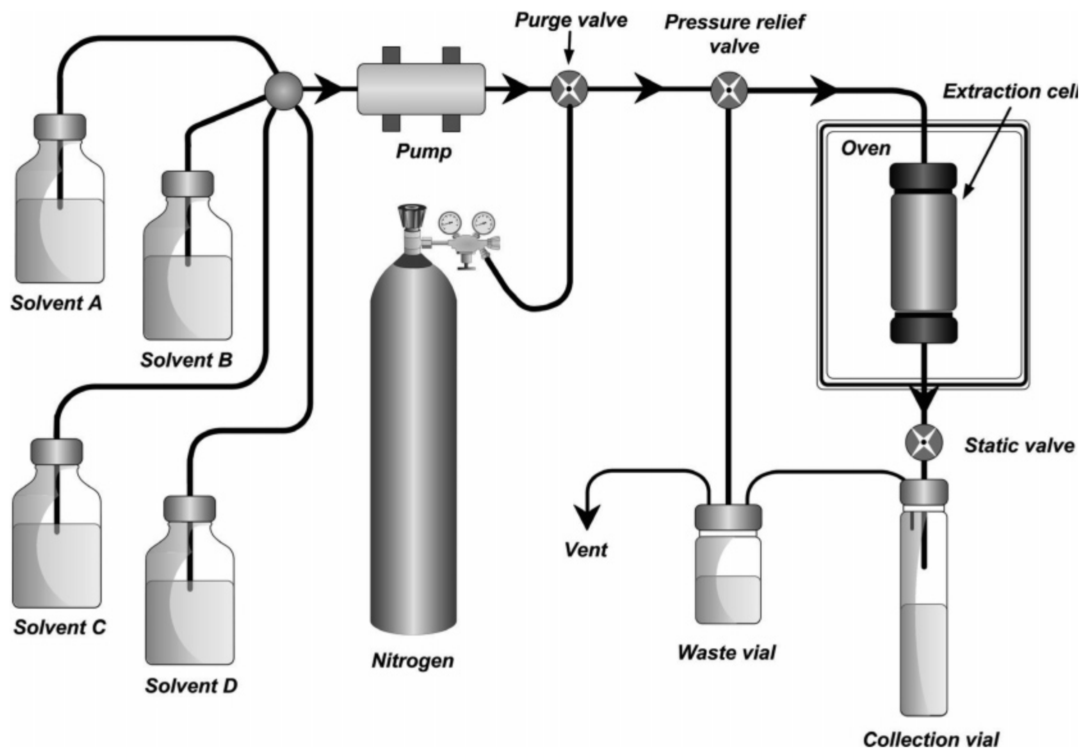

Figure 1. Schematic diagram of pressurized fluid extraction system, Dionex ASE 200.

elevated temperature, at which the extraction is conducted, increases the capacity of solvent to solubilize the analyte. Increased temperature is also known to weaken the bonds between the analyte and the matrix and to decrease the viscosity of the solvent with improved penetration into the matrix, resulting in an increased extraction yield (*15*).

It is well-known that the bioaccumulation and distribution of persistent organic pollutants (POPs) in marine organisms is influenced by several factors such as the lipid content of the organism and the physical−chemical properties of the compounds (water solubility and octanol/water partition coefficient). Some of these factors, mainly lipid content, must be taken into account when concentration data of organochlorine pollutants are interpreted (*16*). During the past 30 years, Sweden has employed an environmental monitoring system for the determination of chlorinated organic contaminants in biological matrices in the environment. The so-called Jensen (J) extraction, a traditional liquid−liquid extraction, has been used as a standard method (*4*) during the years for the extraction of total lipid content from wet fish muscle and for the analysis of POPs. However, there was uncertainty regarding the effectiveness of the total lipid extraction yield using the traditional J method, particularly in the case of lean fish muscle, where polar phospholipids dominate. Recently, the traditional J method has been modified to increase the total lipid recoveries from lean fish (*3*). From an ecological point of view, it is today accepted to be more relevant to express the residue level of lipophilic POPs on a fat weight basis than on a traditional fresh weight basis (*3*). It is of course imperative that the used extraction procedures lead to correct results for pollutants and for total lipid content (*17*).

In the present study, the reliability and efficiency of the PFE technique for extracting total lipid content from lean and fat matrices (cod and herring, respectively) and the effect of sample treatment on the extraction efficiency have been evaluated and the results compared to those obtained with the traditional and modified J methods. The influence of several experimental variables affecting the extraction efficiency of total lipids with PFE was also studied using the experimental design.

MATERIALS AND METHODS

Sample Treatment. Cod and herring samples were purchased from a local supplier (Ica, St. Per Gallery, Uppsala, Sweden). All samples were kept at −20 °C for 3 h to facilitate easy cutting into pieces. Three batches (batches I−III) of cod muscle and a sample of herring were used throughout the experiment. For batches I and II and the herring sample, the pieces were ground in a mixer with dry ice for ∼2 min. From this homogenate, portions of 3 and 10 g were weighed for PFE and J extractions, respectively. A subportion of the homogenate of batch II was freeze-dried. In batch III, the fresh whole cod muscle was divided into two equal sides. One side of the cod muscle was homogenized as above. From this homogenate, portions of 3 and 10 g were weighed for PFE and J extractions, respectively. The other side of the cod muscle was kept intact and untreated. Different spots from the front and back parts of the intact cod muscle were taken to avoid differences in lipid content. Portions of 3 and 10 g pieces were weighed for PFE and J extractions, respectively. All samples were stored at −20 °C until extraction. Homogenized and intact cod muscles had the same water contents.

Chemicals. Acetone (Ac), 2-propanol (IPR), normal hexane (n-Hx), diethyl ether (DEE), ethanol, methanol, phosphoric acid, sodium hydroxide, sodium carbonate, and sodium chloride were purchased from Merck AG, Darmstadt, Germany. Distilled water was obtained from a purification system (Milli-Q; Millipore, Bedford, MA), and nitrogen (99.996 vol %) was from AGA, Stockholm, Sweden.

Pressurized Fluid Extraction and Instrumentation. PFE extractions were carried out using an automated Dionex ASE 200 accelerated solvent extractor (Dionex Corp, Sunnyvale, CA) equipped with 11 mL stainless steel extraction cells. The commercial PFE instrument used in this study is a fully automated sequential extraction system connected to a solvent controller that allows automated switching and mixing of up to four different solvents. A schematic diagram of a PFE system is shown in **Figure 1**. The PFE system can operate with up to 24 samples containing extraction cells and up to 26 collection vials plus an additional 4 vial positions for rinse. A cellulose filter (Whatman, grade D28, 1.983 cm diameter) was placed at the bottom of the extraction

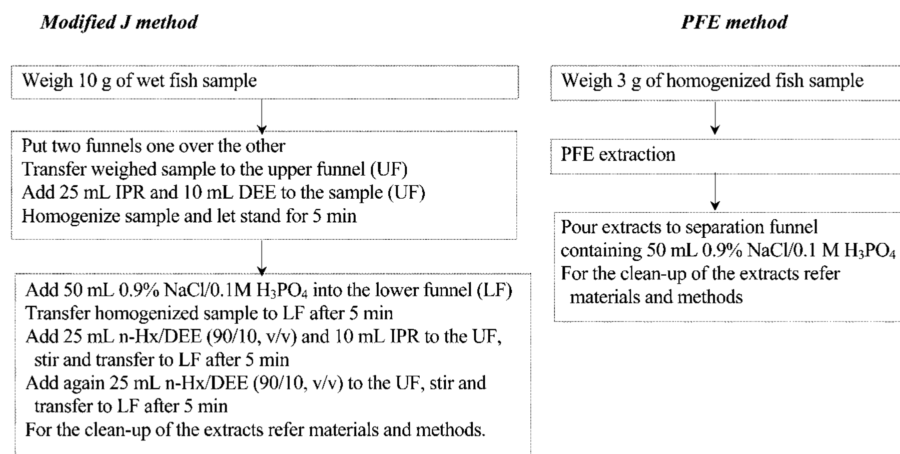

Figure 2. Comparison of the extraction scheme of the modified J and the developed PFE method for the gravimetric determination of total lipid content.

Table 1. Solvents Used for the Extraction of Total Lipid Content from Cod and Herring Samples in the Extraction Methods

	solvent mixture		
method	A	B	C
original J[a]	Ac, 25 mL n-Hx, 10 mL	n-Hx/DEE (90:10, v/v), 25 mL	n-Hx/DEE (90:10, v/v), 25 mL
modified J[a]	IPR, 25 mL DEE, 10 mL	n-Hx/DEE (90:10, v/v), 25 mL IPR, 10 mL	n-Hx/DEE (90:10, v/v), 25 mL
PFE	IPR/n-Hx (65:35, v/v), 20 mL	n-Hx/DEE (90:10, v/v), 20 mL	
PFE[a]	IPR/DEE (25:10, v/v), 20 mL	n-Hx/DEE (90:10, v/v), 20 mL	

[a] Solvent mixtures according to ref *3*.

cell before the sample was added to prevent clogging of the metal frit. The extraction cell was loaded with 3 g of homogenized wet or 1 g of freeze-dried sample. The extraction procedure started with a 5 min thermal equilibration time during which the cell was heated and continuously filled with solvent mixture A (**Table 1**). The extraction was continued under static conditions. The extraction temperature, static time, and extraction solvent were varied and optimized while the pressure and static cycle were kept constant for all experiments at 10 MPa and 2 cycles, respectively. The use of a static cycle introduces fresh solvent during the extraction process. When more than one cycle is used, the flush volume is divided by that number. Following the static extraction, the sample was rinsed with fresh solvent, 60% (v/v). Finally, the cell was purged with gaseous nitrogen for 60 s. The whole extraction procedure was repeated with solvent mixture B. The extracts from the sequential extractions were collected into the same vial, giving a total extraction volume of ~40 mL. The extraction scheme is shown in **Figure 2**.

J Methods. The J methods are described in more detail in refs *3* and *4*. Lipid extraction was performed on cod and herring muscles using the extraction method described in **Table 1** and **Figure 2**. About 10 g of the frozen tissue was homogenized for 1 min using an Ultra-Turrax (IKA-Werk) with solvent mixture A in a separatory funnel with a sintered glass filter at the bottom. The mixture was allowed to stand for 5 min. The lower funnel without a glass filter containing 50 mL of 0.1 M phosphoric acid in an aqueous 0.9% sodium chloride solution was mounted under the first one. The extract was transferred to the lower funnel through the filter by means of nitrogen pressure. The sample remaining in the top funnel was then stirred using a glass rod for 1 min with solvent mixture B and transferred to the lower funnel as above. Finally, the sample was stirred with solvent mixture C and transferred to the lower funnel.

Cleanup Procedure for the Extracts. Both the J and PFE extracts were cleaned up using the same procedure as in the traditional J method (*4*). Briefly, the extract containing 50 mL of 0.1 M phosphoric acid in an aqueous 0.9% sodium chloride solution was gently turned upside down ~20 times to avoid the formation of emulsion. After phase separation, the lower aqueous phase was released and kept. The remaining organic phase was transferred to a preweighed beaker. The aqueous phase was transferred back into the separatory funnel and gently turned upside down ~10 times with another 10 mL of n-Hx. After phase separation, the aqueous phase was discarded. The organic phase was combined with the first extract, and 10 mL of ethanol was added. The combined organic extract in the beaker was allowed to evaporate in a ventilation hood overnight at room temperature until dryness. The weight of the extracted lipids was then determined. Results from the freeze-dried extraction were calculated back to wet weight.

Analysis of Residue Using Infrared (IR) and Nuclear Magnetic Resonance (NMR) Spectroscopy. The residue was mixed with 20-fold of potassium bromide in a small mortar and subsequently compressed under vacuum in a hydraulic press for a study of the IR spectrum. IR spectra were obtained with a Perkin-Elmer 1760 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded for CD_3OD solutions at 499.94 and 125.72 MHz, respectively. Chemical shifts are reported in parts per million referenced to TMS via the solvent signal (^{1}H, CHD_2OD at 3.30; ^{13}C, CD_3OD at 49.0). NMR signals were assigned from COSY, HSQC, HMBC, NOESY, ROESY, and TOCSY (*18*) spectra. For NOESY and ROESY experiments, mixing times between 0.1 and 0.4 s were used.

Statistical Analysis. All results were expressed as mean value of total lipid content and compared by *t*-test analysis with an acceptance level of significant difference at $P < 0.05$ throughout this paper unless otherwise stated.

RESULTS AND DISCUSSION

Many papers have been published on the extraction of total lipid content using PFE from high-fat samples (*5*, *6*, *13*, *19*). In fat fish, the phospholipid fraction is a lower proportion of the total lipids compared to lean fish. Thus, recovery of phospholipids has less influence on the total lipid content from fat fish compared to lean fish. When the lipid contents of a lean and fat fish were examined using the B&D and J methods, it was found that the original J method gave the same recoveries as the B&D method in the case of lipids from fat fish, but from lean fish unacceptably low levels of lipids were obtained (*3*).

Optimization of the PFE Parameters. In a screening study, different solvent mixtures were investigated to determine the

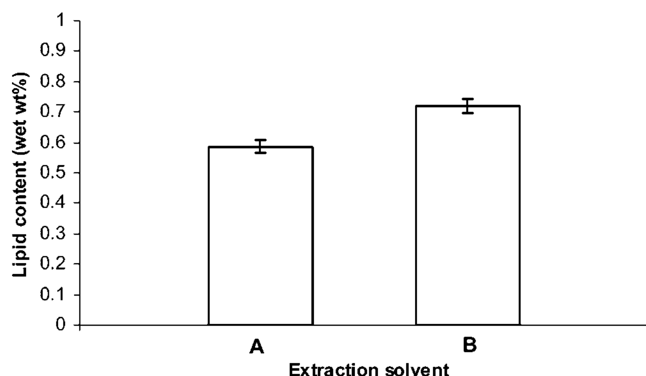

Figure 3. Amount of total lipid extracted using (**A**) Ac/n-Hx (70:30), n-Hx/DEE (90:10, v/v) (SD = 0.02, $n = 6$) and (**B**) IPR/n-Hx (70:30, v/v), n-Hx/DEE (90:10, v/v) (SD = 0.02, $n = 8$). PFE conditions: 3 g of homogenized cod, 130 °C, 5 min, 2 cycles, 10 MPa, 60% flush volume, 1 min of N_2 purge. Error bars represent SD values.

extraction solvent mixture that gives highest total lipid yield. The amount of total lipid extracted using different solvent mixtures is shown in **Figure 3**. It was found that the combination of the extraction solvents IPR/n-Hx (70:30, v/v) as a first and n-Hx/DEE (90:10, v/v) as a second solvent mixture yielded maximum total lipids.

To fully understand the way in which PFE operating variables affect extraction, individual operating variables must be considered along with nonlinear effects and interaction terms (*20*). An optimization study was performed to investigate the optimum values of the different variables expected to have the largest effect on the extraction efficiency of total lipid content from cod muscle. The most important operating parameters, extraction solvent, temperature, and extraction time (*11*), were studied using a full factorial (3 × 3 × 2) design (Unscrumbler, CAMO, ASA, Oslo, Norway). To determine the effect of each parameter on the extraction efficiency, the amount of n-Hx in IPR was varied from 10 to 60%, temperature from 100 to 140 °C, and extraction time from 5 to 10 min. The parameters pressure and number of cycles were kept constant. Changing the pressure has very little influence on analyte yield, and it was not considered to be a critical experimental parameter (*11*, *21*). The main effect of pressure is to maintain the solvents in liquid state above their atmospheric boiling points.

Many solvents or solvent combinations can be used to extract lipids from tissues. The neutral lipids dissolve well in nonpolar organic solvents, but the polar lipids, especially the phospholipids, dissolve in relatively polar solvents. Solvents to extract total lipids must demonstrate a high solubility for all lipid compounds and must be sufficiently polar to remove the lipids from their association with cell membranes and lipoproteins (*22*, *23*). Yet they must not be so polar that they react chemically with the lipids or that moderately hydrophilic lipids such as triglycerides do not dissolve and are left adhering to the tissue (*7*). Thus, it is necessary to use solvents that not only dissolve the lipids readily but also overcome the interactions between the lipids and the tissue matrix, and it is essential to perturb both the hydrophobic and polar interactions at the same time (*7*). Increased temperature may disrupt the strong solute−matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attraction of the solute molecules and active site on the matrix (*24*) and must therefore be considered as an important parameter to investigate. Certain sample matrices can retain analytes within pores or other structures. Increasing the extraction time can allow complete diffusion of extraction solvent into the sample matrices. The effect of extraction time should always be considered in conjunction with number of cycles, to produce complete and efficient recovery.

Table 2. Result of the Experimental Design Used To Study the Influence of Extraction Parameters Percent of n-Hx in IPR, Temperature, and Time on the Extraction Yield of Total Lipid Content from Cod Muscle (Batch I) Using PFE

expt	% of *n*-hexane in IPR	temp (°C)	time (min)	lipid content (wet wt %, $n = 2$)	
1	30	120	10	0.84	0.83
2	10	140	5		0.74
3	60	140	5	0.68	0.74
4	60	100	5	0.71	0.78
5	60	100	10	0.79	0.79
6	30	120	5	0.84	0.84
7	10	120	5	0.74	0.75
8	10	100	5	0.75	0.78
9	30	100	10	0.80	0.83
10	10	120	10	0.81	0.70
11	10	140	10	0.76	0.78
12	60	140	10	0.72	0.81
13	30	140	5	0.75	0.71
14	30	100	5	0.79	0.85
15	30	140	10	0.79	0.79
16	60	120	10	0.75	0.85
17	60	120	5	0.84	0.75
18	10	100	10	0.81	0.72

Table 3. Multilinear Regression Results from the Full Factorial Design for Total Lipid Extraction from Cod Muscle Using PFE

term	coefficient	*p* value
constant	0.827605	0.000
H	0.006592	0.439
T	−0.017563	0.046
t	0.011917	0.091
$H \times H$	−0.047302	0.004
$T \times T$	−0.029092	0.051
$H \times T$	−0.002077	0.841
$H \times t$	0.004510	0.591
$T \times t$	0.009925	0.247

[a] *H*, percent of *n*-hexane in IPR; *T*, temperature; *t*, time.

Table 2 shows the results of the optimization study for the determination of total lipid content from cod muscle (batch I) using the PFE technique. Statistical treatment of the data is shown in **Table 3**. It showed that at a 95% confidence level ($p < 0.05$) the nonlinear interaction of the amount of n-Hx in IPR and temperature is significant. Extraction time has no significant effect on total lipid recovery and was kept at 10 min. A close investigation of the response graph showed that total lipid extracted reached an upper plateau at a temperature of 115 °C with extraction solvent IPR/n-Hx (65:35, v/v) (**Figure 4**). The final operating parameters for validation of the new PFE method were set as follow: 3 g of homogenized wet or 1 g of freeze-dried fish sample was extracted for 10 min with IPR/n-Hx (65:35, v/v) at a temperature of 115 °C and a pressure of 10 MPa. The flush volume was set at 60% (v/v) in two cycles, and a purge of 60% was used. The whole extraction process was repeated using n-Hx/DEE (90:10, v/v) as a second extraction solvent. The second sequential extraction improved the yield of total lipids by 5%. A repeatability study was performed at the optimum conditions using cod muscle (batch I). The average total lipid content was found to be 0.78% with an RSD value of 4.2% ($n = 5$).

Comparison between the J Methods and the Optimized PFE Method. In a second experiment, cod muscle was extracted for total lipid content, and the results were compared to original and modified J methods. In addition, comparison with a fat fish,

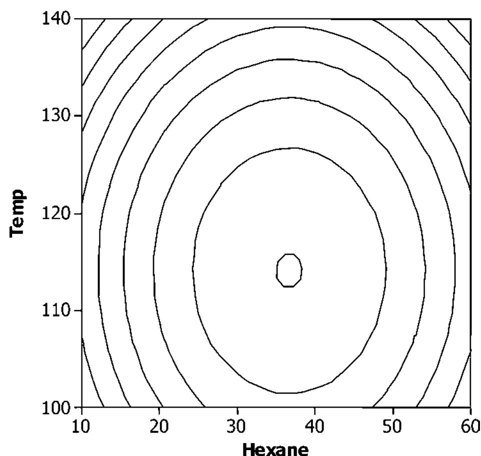

Figure 4. Response surface of the percent of total lipid content extracted from cod muscle as a function of extraction solvent and temperature.

Table 4. Comparison of Total Lipid Extracted Using the Original J, Modified J, and PFE Methods from Cod Muscle (Batch II), Low-Fat Fish Tissue[a]

extraction method	lipid content (wet wt %)	RSD ($n = 5$) (%)
original J[a]	0.38	6.3
modified J[a]	0.55	2.8
PFE[a,b]	0.62	3.1
PFE[b,c]	0.41	2.5

[a] Homogenized wet cod muscle. [b] PFE conditions: IPR/n-Hx (65:35, v/v) and n-Hx/DEE (90:10, v/v), 10 min, 115 °C, 10 MPa, 60% flush volume, 2 cycles, and 1 min of N_2 purge. [c] Freeze-dried cod muscle.

herring muscle, was performed. **Table 4** shows the amount of total lipid extracted from cod muscle (batch II) using the original J, modified J, and PFE methods. The original and modified J methods were compared to PFE using homogenized wet cod muscle. Significantly higher total lipid yields were achieved with PFE (0.62%) than with the original (0.38%) and modified J (0.55%) method when using homogenized wet cod muscle. The difference between the original and modified J methods earlier reported by Jensen et al. (*3*) was confirmed. When the Ac was replaced with IPR and n-Hx with DEE (modified J method), the yield of total lipids increased significantly from 0.38 to 0.55% compared to the original J method. The lower yield obtained with the original J method used on tissues high in phospholipids could be due to poor solubility in Ac (**Table 4**). Acetone dissolves simple lipids and glycolipids, but it will not dissolve phospholipids readily and indeed is often used to precipitate them from solution in other solvents (*7*).

Figure 2 shows a comparison between the modified J and PFE methods for the gravimetric determination of total lipid content. Although the cleanup step is a bottleneck for J and the current method, solvent consumption was reduced by 50% per extraction in PFE. PFE has the additional advantage in terms of automation and reduced solvent exposure of the operator. With the solvent controller up to four solvents can be mixed and delivered to the instrument. It decreases the amount of time spent on laborious tasks such as measuring and mixing solvents and reduces errors.

To investigate if the developed PFE method could also be applied on high-fat tissue, the total lipid content of homogenized herring sample was examined, and yields were compared to the original J method. As can be seen in **Table 5**, total lipid extracted using PFE gave a significantly higher yield (7.8%) compared to original J method (6.9%). A comparison of total lipid extraction efficiency of the PFE and conventional J was

Table 5. Comparison of Total Lipid Extracted Using PFE and Original J from Homogenized Herring Tissue

extraction method	lipid content (wet wt %)	RSD ($n = 5$) (%)
PFE[a]	7.8	8.5
original J	6.9	2.2

[a] PFE conditions: IPR/n-Hx (65:35, v/v) and n-Hx/DEE (90:10, v/v), 10 min, 115 °C, 10 MPa, 60% flush volume, 2 cycles, and 1 min of N_2 purge.

Table 6. Comparison of Total Lipid Content Extracted Using Modified J and PFE from Cod Muscle (Batch III)

extraction method	lipid content (wet wt %)	RSD ($n = 5$) (%)
modified J[a]	0.62	1.4
PFE[a−c]	0.68	3.7
PFE[b−d]	0.36	2.9
PFE[a,b,e]	0.68	8.5
PFE[b,d,e]	0.51	18.0

[a] Homogenized wet cod muscle. [b] PFE conditions: 10 min, 115 °C, 10 MPa, 60% flush volume, 2 cycles, and 1 min of N_2 purge. [c] Solvent: IPR/n-Hx (65:35, v/v) and n-Hx/DEE (90:10, v/v). [d] Intact wet cod muscle. [e] Solvent: IPR/DEE (25:10, v/v) and n-Hx/DEE (90:10, v/v).

conducted by Saito et al. (*25*). They obtained consistent lipid content by both methods from tissue samples of swine heart, kidney, and liver and cattle adipose tissue using dichloromethane/acetone (1:1) as solvent mixture for PFE.

A subportion of the homogenate cod muscle was freeze-dried and extracted using the developed PFE method. These samples gave clear extracts, but the recovery was low (**Table 4**). This effect is probably because the extraction solvent compositions in the freeze-dried and wet samples are different as the wet sample contains water as an indigenous solvent.

Yield Comparison between Homogenized and Intact Tissues. To investigate the effect of sample treatment on the extraction efficiency and avoiding the homogenization step, as is used in both the J and proposed PFE methods, another whole cod was employed (batch III). One side of the cod muscle was homogenized while the other side was used without any pretreatment. The PFE results were compared with those obtained with the modified J method. **Table 6** shows the amount of total lipid extracted using homogenized and intact cod muscle.

Significantly higher total lipid yields were achieved with PFE (0.68%) than with the modified J method (0.62%) when using homogenized wet cod muscle. The solvent mixture IPR/n-Hx (65:35, v/v) may able to solubilize only a relatively small amount of the indigenous water, and there is a possibility of two-phase formation giving a lower extraction yield. IPR/DEE (25:10, v/v) used in ref *3* dissolves the indigenous water and remains in one phase during the extraction. Interestingly, this solvent mixture together with n-Hx/DEE (90:10, v/v) gave a significantly higher yield than IPR/n-Hx for intact cod muscle, 0.51% compared to 0.36%, respectively. However, when using homogenized tissue the statistical *t* test showed no significant difference in yields between the two extraction solvents (**Table 6**). The lower yield obtained when intact wet cod muscle was extracted might be due to the proteins in the sample coagulating to a very hard pellet during extraction, making the diffusion of the solvent into the matrix not efficient enough to extract all of the lipids. Moreover, phospholipids such as phophoinositides are most likely bound to other cellular biopolymers by ionic bonds, and these are not easily disrupted by simple solvation with organic solvents. Purely mechanical factors such as the formation of inclusion complexes can also limit the extractability of lipids by limiting their accessibility to solvents (*7*). Another

reason for the low yields by PFE when using intact wet cod muscle might be caused by the large difference in particle size, leading to a more inhomogeneous diffusion path distribution through the formation of channeling (*26*). The increased efficiency when the cod muscle was homogenized (**Table 6**) could be mainly because finely divided particles are more easily dissolved and extracted due to their large surface area-to-volume ratio. Moreover, no channeling problem was observed when using homogenized compared to intact wet sample.

The main limitation of the developed PFE method is the low total lipid recovery from intact cod muscle. Different sample pretreatments were investigated to increase the recovery. These include on-line preheating for 5 min at 100 °C, ultrasonication with water and without water in the cell prior to extraction, decreasing the extraction temperature to 40 and 60 °C, and increasing the extraction time to 20 min at 60 °C. All of these attempts were performed to increase the penetration of solvent to the intact wet sample. However, none of the above treatments improved the total lipid yield.

Additional Extractable Materials from PFE. Because there is a significantly higher yield obtained when homogenized cod muscle is extracted by the PFE technique compared to the liquid−liquid extraction used in modified J, we suspected that there might be coextracted other unknown species, raising the yield. This could be possible because PFE applies higher temperature and pressure. To investigate possible coextracting compounds that inflate gravimetric results, the total lipid extracts were redissolved in 20 mL of IPR/DEE (25:10, v/v) at room temperature and centrifuged. The supernatant was transferred to a preweighed beaker. The precipitate was further redissolved in 20 mL of n-Hx/DEE (90:10, v/v) and centrifuged. The supernatant was combined to the above preweighed beaker and evaporated overnight to determine the lipid content. The remaining residual from both solvent mixtures readily dissolves in water-forming foams, 2 M sodium hydroxide 2 M sodium, and partially in methanol. This residual represents ∼10% of the original PFE extract. The amount of the residual was ∼10% regardless of whether the material was homogenized or not for both cod and herring samples.

The residual was further investigated by IR and ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. All techniques indicated that the material contained a reasonably homogeneous material. In IR, there were observed strong CH_2 stretching signals (2925 and 2854 cm^{-1}), suggesting long-chain aliphatic chains, strong C=O vibrations at 1736 cm^{-1} (ester groups), and intense broad OH-stretching at 3400 cm^{-1}, indicating partial hydrogen bonding.

The observed NMR shifts were as follows: ^{1}H NMR δ 5.28, 4.42, 4.23, 4.03, 2.36, 1.59, 1.28, 0.87 (H, CH_3); ^{13}C NMR δ 71.1 (CH or CH_3), 66.3 (CH_2), 65.9 (CH_2), 63.9 (CH_2), 59.6 (CH_2), 54.1 (CH_2), 33.9 (CH_2), 31.5 (CH_2), 29.8 CH_2, 22.6 (CH_2), 13.7 (CH_3); ^{31}P NMR δ-1 (broad).

It was not the objective of the present study to elucidate in more detail the chemical composition of the additional extract obtained using PFE as compared to traditional liquid−liquid extraction. However, it appears to be likely, taking all spectroscopic techniques into consideration, that the "extra" material is of phospholipid nature and possesses ester-bound long-chain aliphatic residues. The character of the headgroup is more uncertain. However, the presence of several hydroxyl groups suggests that the lipids are of inositidic or glycolipid nature, tallying with the observed emulsifying properties. It is possible that these highly polar lipids require higher temperatures/pressures to become released from the matrix.

Conclusion. Optimized PFE affords yields of total lipids from fish muscles exceeding those of conventional batch extractions (modified J methods) by a factor of ∼10% due to the higher temperature, regardless of the fish fat contents. Preliminary IR and NMR studies suggest the presence of inositidic or glycositidic phospholipids in this residual fraction.

One obvious advantage of PFE compared to the batch extraction methods is the lower requirement of solvents (reduced by 50%) and reduced solvent exposure of the operator. Another principal advantage of PFE is its inherent suitability to allow for automation. However, the present study shows that the time-consuming and laborious homogenization step in the PFE procedure is not easily omitted. The current cleanup step could be further investigated, and more studies with respect to automation fitness are required.

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