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Qualitative and Quantitative Liquid Chromatographic Analysis Methods for the Determination of the Effects of Feed Supplements on Hen Egg Yolk Phospholipids

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Quantitative and qualitative high-performance liquid chromatographic methods were utilized to separate phospholipid classes. After qualitative separation, the fatty acid moieties of each separated phospholipid class were determined using a gas chromatographic method. On the basis of these analyses, the effect of supplemented feeds on hen egg yolk lipids can be evaluated. The supplemented feeds contained 1−5% of vegetable-based or fish oils. The phospholipid content and composition were the same in all feeding groups, the proportions of phosphatidylcholines, phosphatidylethanolamines, and sphingomyelins being 70, 28, and 3%, respectively. In each feeding group, the fatty acid profiles of phosphatidylcholines and sphingomyelins were similar to each other and different from that of phosphatidylethanolamines. The supplemented feeds had a statistically significant ($p < 0.05$) effect on the fatty acid composition of phosphatidylcholines. The supplements decreased the proportion of saturated fatty acids in total fat, but this effect was not found in phospholipids.

KEYWORDS: Phospholipids; egg yolk; liquid chromatography; evaporative light scattering detector

INTRODUCTION

Typically, fats of hen eggs are constituted of approximately 30% of saturated, 50% of monoenoic, and 20% of polyenoic fatty acid residues (*1*, *2*). In the literature can be found several studies in which the fatty acids in hen eggs have been changed, for example, using supplements with *n*−3 fatty acids found in vegetable oils and fish oils (*3*−*8*) or oils with a high content of α-linolenic acid (ALA) (*9*−*12*). The intention of the supplemented feed is to change the fatty acid composition toward a more healthy choice for humans, and the fundamentals and practices of this idea have been reviewed recently (*13*). In these previous studies the fatty acid composition of egg yolk has been shown to change due to these changes in the fat composition of the feed. However, fewer data are available about the changes in the fatty acid moieties of phospholipids, which are relevant if the research is focused on the functional properties (*14*). The only publications showing phospholipid data so far are a paper by Jiang and co-workers (*10*), who used flax and other vegetable oils, and two papers on feeding studies showing the effect of 0−7% refined fish oil as a supplement (*15*, *16*).

In the present study we show the effects of oil-supplemented feeds on the phospholipid concentration and composition. Furthermore, the fatty acid composition of the phospholipids classes was determined in order to find out how much the changes in the fat source of the feed affect the fatty acid composition of phosphatidylcholines and phosphatidylethanolamines. Both of these phospholipid classes contribute to the functional properties of egg yolk, such as foaming and emulsion properties, which are discussed in more detail elsewhere (*14*). These molecules are also used as an essential fatty acid source in infant formulas, food additives, and ingredients of therapeutic and cosmetic products (*17*).

MATERIALS AND METHODS

Egg Yolk Samples and Reference Materials. Feeding experiments were performed at MTT Agrifood Research Finland, following the practices described elsewhere (*14*). A group of hens of the same age was equalized into five feeding groups, each containing ∼20 individuals. Group 1 remained with the basic feed. The supplemented feeds of groups 2−5 are described in **Table 1**. After 1 month, samples consisting of five eggs were taken from each feeding group, and the sampling was repeated after 1 week. The yolks of the five eggs of the same sample were pooled. Triplicate samples (10 g) were taken from the pooled yolks for phospholipid analyses. Total fat content and fatty acid determinations were performed as duplicates using the rest of the pooled yolks. Commercial reference compounds (purity > 99%) originating from egg yolk were purchased from Larodan Fine Chemicals (Malmö, Sweden) and Sigma-Aldrich (St. Louis, MO).

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Table 1. Brief Description of the Supplemented Feeds Given to the Hens ad Libitum

group 1	basic feed[a]
group 2	basic feed + linseed oil (5%) + vitamin E (150 mg/kg)
group 3	basic feed + rapeseed oil (5%) + vitamin E (150 mg/kg)
group 4	basic feed + fish oil (3%) + vitamin E (150 mg/kg)
group 5	basic feed + 15% (w/w) black currant press residue[b] + vitamin E (150 mg/kg)

[a] Basic feed consisted of barley and oats with limestone and PRE-MIX concentrate of vitamins, minerals, and amino acids. [b] Equal to ~1% of added black currant seed oil.

Isolation of Phospholipids. Phospholipids were extracted from each yolk sample (10 g) using glass-distilled grade acetone (Merck, Darmstadt, Germany) and ethanol (99.5%, Primalco, Finland) using the method adapted from Juneja (*2*). Acetone (1 × 20 mL, 2 × 30 mL) removed water from the samples and dissolved nonpolar lipids, such as triacylglycerols and carotenoids. More polar lipids were extracted from the residue using 2 × 25 mL of ethanol. Insoluble matter was filtered using glass microfiber filters (GF/C, Whatman International Ltd., Maidstone, U.K.). The ethanol solution was evaporated to dryness with a rotating evaporator, and the residue was weighed. Residue was dissolved in chloroform/methanol (2:1) (HPLC grade, Labscan, Dublin, Ireland) and stored in a freezer prior to the analyses.

Quantitative Determination of Phospholipids. Phospholipids were analyzed using a Shimadzu LC-9A HPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a low-pressure solvent mixing module and a 20 μL sample loop (Rheodyne, Cotati, CA). Separation was achieved using a silica column (Ultrasphere, 150 mm × 4.6 mm, d_p = 5 μm, Beckman Instruments Inc., San Ramon, CA) using a modification of a gradient elution system developed by Christie (*18*, *19*) for the separation of lipid classes. The ternary mobile phase gradient was composed using a low-pressure mixing unit (Shimadzu Corp.), and the flow rate was 1.5 mL/min. Eluent A was *n*-hexane, eluent B a mixture of 2-propanol and chloroform (4:1), and eluent C a mixture of 2-propanol and water (1:1) (HPLC grade, Rathburn Chemicals Ltd., Walkerburn, Scotland; and Labscan). The gradient elution program started with pure eluent A, resulting in the elution of neutral lipids. At 5 min the proportions of eluents A and B were 80 and 20%, respectively. During the subsequent 19 min, the proportion of eluent B was kept at 52%, and the proportion of eluent A was gradually decreased from 48 to 32%. Meanwhile, the proportion of eluent C was increased from 0 to 16%, resulting in the elution of phosholipids. After 24 min, the gradient was reversed, and the starting condition, pure eluent A, was reached at 30 min.

Aliquots of the sample and standard solutions (30−333 μg/mL) dissolved into chloroform/methanol (2:1) were injected in the column at ambient temperature. An evaporative light scattering detector (Sedex 55, SEDERE) was used for the quantitative determination. The instrument parameters used were air pressure = 0.26 MPa, temperature = 40 °C, and gain = 8. According to the operator's manual the intensity of the signal (I) is related to the mass of the analyte as follows: $I = km^b$, where m is the mass of the scattered particles and both k and b analyte and operational parameter-dependent constants. Hence, linear regression modeling was used to plot log peak area versus log quantity injected (ng) for each phospholipid class. The linear equations thus obtained were used for the quantitative measurements.

Qualitative Separation of Phospholipid Classes. Fractionation of phospholipid classes was achieved using an automated Shimadzu LC-10A system equipped with a Shimadzu diode array detector. Phospholipid classes were separated using a silica column (Superspher Si60, 240 mm × 4.0 mm, d_p = 5 μm, Merck) held in an oven at 30 °C. The isocratic elution solvent consisting of methanol, acetonitrile, and concentrated sulfuric acid in a ratio of 30:100:0.05 by volume at 1.5 mL/min was adapted from Kaduce and others (*20*). For the fractionation, the egg yolk sample solutions were evaporated to dryness, and the residues were dissolved in the elution solvent (5 mg/mL). For the fatty acid analyses 10−12 injections, 50 μL each, were needed to collect fractions of each phospholipid class.

Fatty Acid Analysis of Phospholipid Classes. The fractions obtained above were neutralized by adding 1 mL of sodium hydroxide (0.5 mol/L in methanol). Solvents were then evaporated at 65 °C using a stream of nitrogen. Fatty acid methyl esters (FAMEs) were prepared from dried phospholipid fractions using 1−2 mL of BF_3 (14% in methanol, Riedel-de-Haën, Seelze, Germany). Reaction was completed in 2 h at 65 °C. FAMEs were extracted from the cooled solutions into hexane. A Shimadzu GC-17A gas chromatograph equipped with flame ionization detector and splitless/split injector was used to analyze FAMEs. An HP Innowax (30 m × 0.32 mm, d_f = 0.25μm, Hewlett-Packard Co., Wilmington, DE) column was used with helium as a carrier gas. A multistep oven temperature program (120−240 °C) was chosen to achieve good separation of the FAMEs. The compounds were identified by comparing retention times with a GLC-68A standard solution (NuCheck-Prep, Elysian, MN), and relative proportions of identified FAMEs were calculated.

Total Fat Content and Total Fatty Acid Analyses. Lipids were extracted from egg yolk samples using chloroform/methanol (2:1) using a modified Folch method (*21*). The analysis procedure and results have been described in more detail elsewhere (*14*).

Statistical Analyses. The quantitative data of the phospholipid and phospholipid-derived fatty acid analyses of each supplemented feed group was tested against the basic feed group using Dunnett's t test (SAS System for Windows 6.11, SAS Institute Inc., Cary, NC). Significance is reported at $p < 0.05$.

RESULTS AND DISCUSSION

Chromatographic Analyses. At the beginning of this study an HPLC method for rapid and repeatable phospholipid separation and quantification was sought. The normal phase HPLC method (*20*) seemed to be appropriate for our purposes, because its solvent system was adequate for the subsequent fatty acid trans-methylation reaction. However, the peaks obtained using any fine adjustment of the method were too wide for reliable area calculation using UV detection at 214 nm. Another problem arises from the difference in the number of double bonds in fatty acid moieties of different feeding groups, which slightly changes the specific extinction coefficients of phospholipids. For that reason this isocratic normal phase LC method with polar mobile phase was used for qualitative separation and fractionation, and another HPLC method with light scattering detector was involved for quantitative determinations.

The gradient elution−normal phase LC method was found to be useful in the quantitative determinations of the egg yolk phospholipids as illustrated in **Figure 1**. Neutral lipids appeared in the front of the chromatogram, during 1−7 min. The total phospholipid content (grams per 100 g of fresh weight) of the egg yolk samples was determined by summing up the masses of the individual phospholipid classes. The phospholipid contents and the proportions of the phospholipid classes in each feeding group are listed in **Table 2**.

In our samples, the phosphatidylcholines constituted ~70% of the total phospholipids. The proportion of phosphatidylethanolamines was ~28% and that of sphingomyelins ~3%. In the literature the proportions of these compound groups are approximately 84, 12, and 2%, respectively; the residual ~2% proportion is constituted of lysophosphatidylcholine (*2*). Some differences in the proportions may arise from the isolation solvents used in each study. In this study a technological approach with solvents useful in production scale extraction were used. No lysophospholipids were observed in our study, even though concentrated samples were analyzed in order to quantify sphingomyelins. Christie observed that minor phospholipids owing proportions below 0.5% of total phospholipids were easily missed in routine analyses due to the nonlinear response of the light scattering detector (*18*). Actually, due to

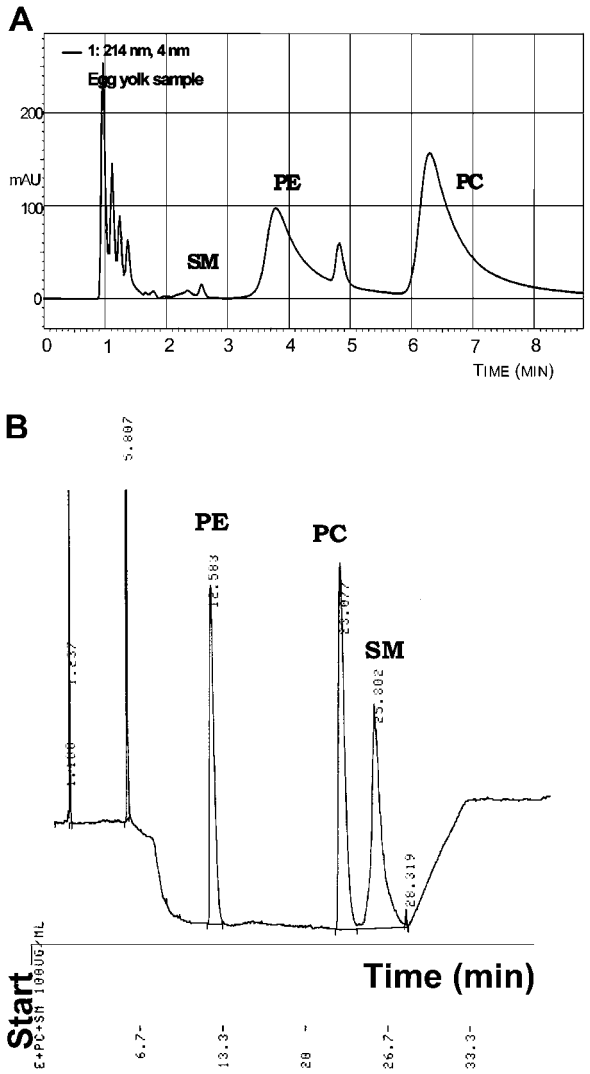

Figure 1. Typical HPLC chromatograms obtained in this study: (**A**) isocratic (qualitative) separation of egg yolk lipids with UV detection (214 nm); (**B**) gradient elution of a standard solution of phospholipids (2 μg each) with ELS detection. PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin. For detailed description of analysis conditions, see Materials and Methods.

Table 2. Phosholipid Content (PL) of an Egg Yolk Sample and Proportion of Each Phospholipid Class (PC %, PE %, and SM % of Total Phospholipids) in Different Feeding Groups

supplement	PL (g/100 g of fresh wt)	s[a]	PC %	s	PE %	s	SM %	s
no supplement	6.0	0.9	69.3	1.7	28.0	2.3	2.7	1.2
linseed oil	6.1	1.3	70.1	1.0	27.8	1.4	2.1	0.6
rapeseed oil	5.6	0.7	69.3	1.6	27.9	1.6	2.8	0.4
fish oil	6.3	0.9	69.8	1.4	27.5	1.4	2.7	0.3
black currant	5.6	0.3	69.6	1.4	27.6	1.5	2.8	0.3

[a] s, standard deviation of six replicates.

the low concentration of sphingomyelins in relation to PCs, each sample was to be analyzed using two dilutions. This was unexpected because it was believed that the log/log calibration curves would have been linear throughout the scale used (0.66−6.66 μg of each PL injected) and even more wide ranging. Due to logarithmic conversion even very small changes in the slope of the standard curves will result in relatively large variations in the resulting quantitative amount of the phospholipid. The poor repeatability may be due to relatively low amounts of injected phospholipid. In previous studies (*18*, *19*, *22*, *23*) much higher phospholipid concentrations were injected into system, because the standard response was found to be nonlinear at low concentrations. In our study it was seen that the major peaks, phosphatidylcholines and phosphatidylethanolamines, were quantitatively determined with acceptable variability (CV% about 2 and 5−8%, respectively), but the deviation observed in the sphingomyelins was relatively high (CV% = 10−40%). In our latter study, ≥10 μg of each phospholipid was injected into the same HPLC system, and similar variability was obtained (*24*).

In the literature, nonlogarithmic linear standard curves, or even only two-point calibrations, have been used in the calculations to obtain very good results. Indeed, the response of the detector can be linear if the concentration range is very narrow. This linearity can be used in the calculations if the concentrations of the phospholipids in the sample solutions have been carefully equalized with those of reference mixtures (*23*, *25*, *26*). However, such a procedure was not adopted during our study, because the intention was to use the same analytical method later for the analysis of samples, in which the phospholipid composition and concentration are more variable than in the present study (*24*). Wang and co-workers (*27*) analyzed soybean phospholipid molecular species by liquid chromatographic method using light scattering detector. They have discussed in more detail the alternative procedures for the determination of the constants of the power function used in quantification.

Comparison of Feeding Groups. The proportion of phospholipids is about one-third of the total lipids of the yolk. The total phospholipid content per fresh weight of egg yolk samples from different feeding groups is shown in **Table 2**. The differences between the feeding groups were not statistically significant. The major fatty acids of phospholipid classes are shown in **Figure 2**. The primary observation is that the fatty acid compositions of sphingomyelins and phosphatidylcholines are relatively similar and different from phosphatidylethanolamines. One of the major differences between phospholipid classes is that in the cholines fraction and in the sphingomyelin fraction the ratio of palmitic acid to stearic acid was >2, but in the ethanolamines fractions this ratio was ∼1. Similar results of phosphatidylcholines have been reported by Amate and others (*28*) and Cossignani and others (*16*), but in their studies the ratio of 16:0/18:0 in the ethanolamines fraction was <1. When a single phospholipid class is considered, the variation between feeding groups is somewhat limited because 85−94% of fatty acids is constituted of palmitic, oleic, stearic, and linoleic acids. However, in phosphatidylethanolamines the content of minor fatty acids (i.e., proportion of a fatty acid is <10%) is more pronounced; that is, the changes in fatty acid composition have more relevance when nutritional and functional properties are considered.

When every supplemented feed group was tested against the control feed group, there was no statistically significant difference in fatty acid composition of sphingomyelins, although some changes are visible in **Figure 2C**. The lack of significance is due to the large experimental error, which can be seen as a result of the low concentration of the sphingomyelins and the fact that each sphingomyelin molecule contains only one fatty acid moiety.

Several fatty acids showed statistically significant changes ($p < 0.05$, $n = 6$) in phosphatidylcholines and phosphatidylethanolamines, and these are marked with an asterisk in **Figure 2A**,**B**, respectively. The linseed oil supplement (group 2)

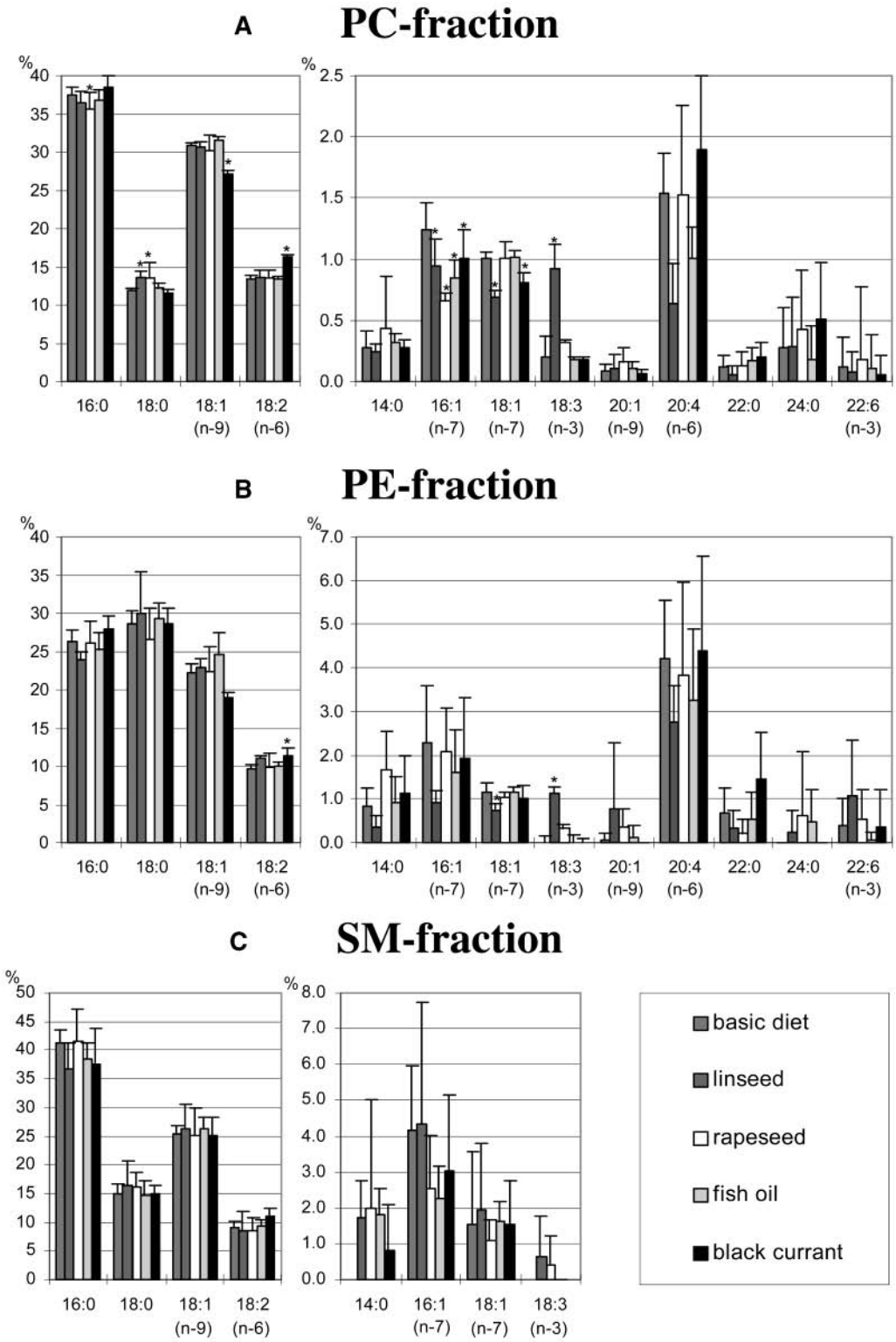

Figure 2. Fatty acid composition of phospholipid classes. Standard deviation ($n = 6$) is shown as error bars. Statistically significant differences between a control and the supplemented feed groups are marked with and asterisk (∗).

increased markedly the concentration of ALA and decreased the $n-7$ fatty acids. Similar results have been reported in other studies, which were mentioned in the Introduction. One further detail not shown in **Figure 2** is that in phosphatidylethanolamines of the linseed group there was ∼0.4% of eicosapentaenoic acid (EPA, 20:5$n-3$). EPA was not found exceeding the limit of quantification (0.02%) in the other feeding groups or other phospholipid classes.

The oil supplement of feeding group 5 was dissimilar to the other groups because it was not pure oil but a press residue obtained from the black currant juice manufacturing process, giving only ∼1% of supplemented oil to the feed. Still, it seemed to have a strong influence on the fatty acid composition. Although black currant seed oil contains γ-linolenic acid (GLA, 18:3$n-6$), it was not found in the egg yolk samples, but the proportion of arachidonic acid (20:4$n-6$) was somewhat larger

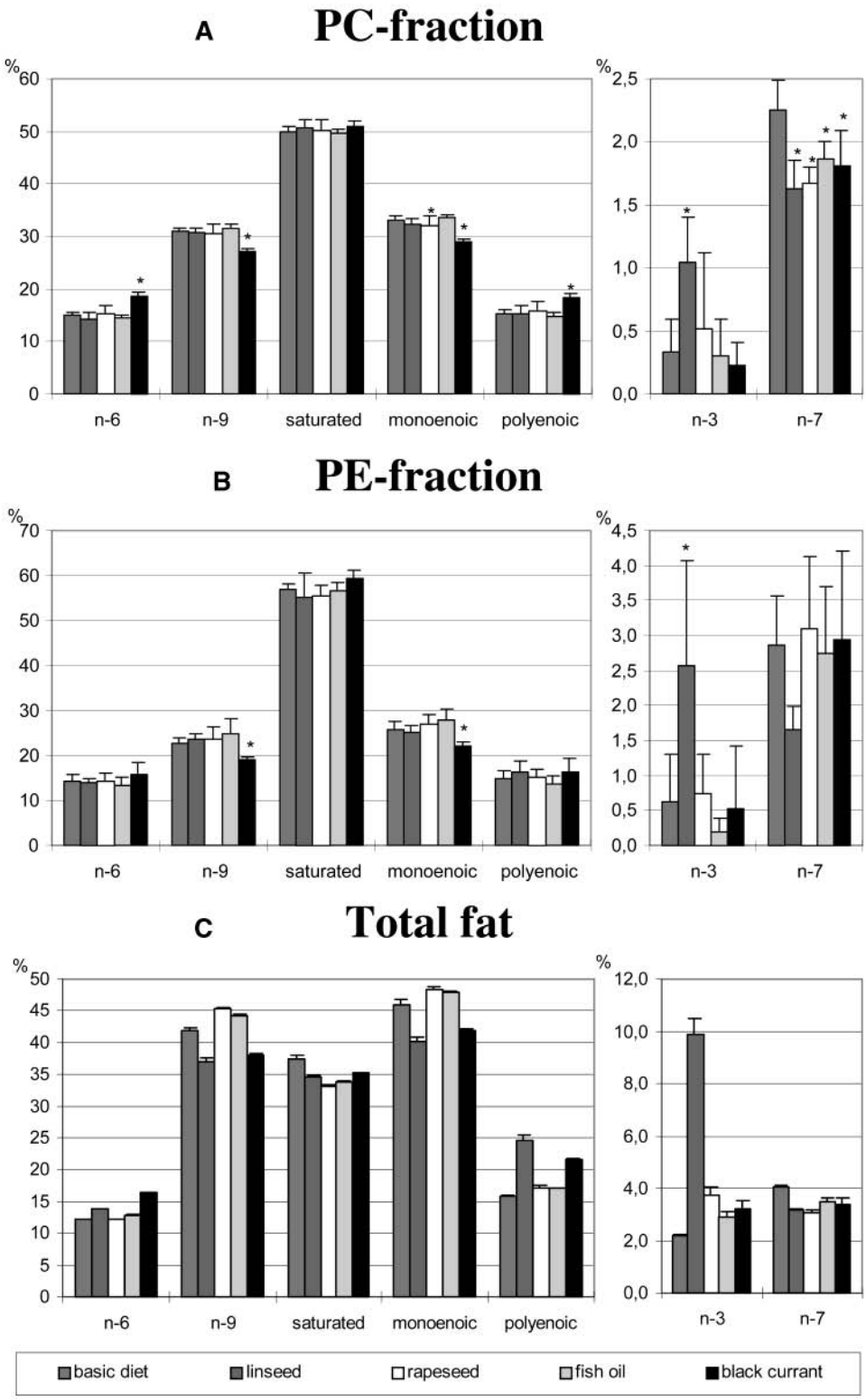

Figure 3. Fatty acids of phosphatidylcholines (PC fraction) and ethanolamines (PE fraction) and total fat. Total fat data are adapted from ref *14*. Standard deviation (for phospholipids, $n = 6$, and for total fat, $n = 2$) is shown as error bars. Statistically significant differences between a control and supplemented feed groups are marked with an asterisk (∗).

(nonsignificant) than in the control group, but seemingly higher than in the other supplement feed groups (not tested statistically). In addition, the other $n-6$ fatty acid, linoleic acid, was significantly increased in both the phosphatidylcholines and phosphatidylethanolamines of feeding group 5.

The fatty acid composition of phospholipid classes was further compared with that of total fat. To simplify the comparisons, the total fat-derived fatty acids and fatty acids of phospholipids were combined into three groups, that is, saturated, monoenoic, and polyenoic fatty acids; and the unsaturated fatty acids were divided further to subgroups ($n-3$), ($n-6$), ($n-7$), and ($n-9$). These groups are shown in **Figure 3**. The more detailed description and discussion related to the data shown in **Figure 3C** are published elsewhere (*14*). The total fat-derived fatty acids

were analyzed as duplicates; therefore, no statistical analyses were performed. The fatty acid groups (phospholipids) showing statistically significant differences are marked with an asterisk ($p < 0.05$, $n = 6$). Sphingomyelins were excluded from this comparison, but their pattern is comparable with that of phosphatidylcholines, as discussed above.

The main difference in the fatty acid composition of total fat and phospholipids is in the proportion of saturated and monoenoic fatty acids. About 50% or more of phospholipid-derived fatty acids and about 33−37% of total lipid fatty acids are saturated. On average, this means each lipid molecule contains at least one saturated fatty acid moiety. All of the supplements decreased the proportion of saturated fatty acids in total fat, but no statistically significant differences were found in phospholipid classes. The proportion of saturated fatty acids even increased in phospholipids; for example, **Figure 2** shows significant increases of stearic acid in phosphatidylcholines in feeding groups 2 and 3. This result can be related to the current opinion of the positional distribution of fatty acids, reviewed by Juneja (*2*) and Gober and others (*29*). The change in fatty acid composition affected by the feed supplements is perhaps more visible in triacylglycerols than in phospholipids, because in the hen egg yolk both unsaturated and saturated fatty acids are found in the sn-3 position of triacylglycerol molecules. The degree of unsaturation of the fatty acid in the sn-1 and sn-2 positions is probably less likely changed by the feed supplements, because the sn-1 position of triacylglycerol and phosphatidylcholine molecules is more specifically occupied by saturated fatty acids and the sn-2 position by unsaturated fatty acids.

Grouping of the fatty acid information may hide some of the statistically significant differences. However, it can be seen in **Figure 3** that the changes due to black currant press residue (15%), yielding an oil additive of 1% in the feed, had at least as strong an effect on fatty acid moieties as the purified oils added at a 3−5% level. Whether the other constituents of the press residue have some effects on the fatty acid profiles or the lower oil content is sufficient for changing the fatty acid composition, this finding needs to be checked by further feeding studies using purified black currant oil.

Using relatively straightforward and simple qualitative liquid chromatographic fractionation of phospholipid classes and subsequent gas chromatographic separation of phospholipid-derived fatty acid methyl esters, we could quantitatively determine statistically significant ($p < 0.05$, $n = 6$) differences in fatty acid compositions of egg yolk lipids obtained through oil-supplemented feed. This study was performed using relatively low amounts of lipids injected into chromatographic systems; thus, its methods may be applicable to the analysis of other samples of limited quantity. Quantitative determination of phospholipid classes did not yield significant differences between the basic and supplemented feed groups. However, statistically significant changes in the proportions of fatty acid moieties derived from fractionated phospholipid classes were found using even as low as 1% (w/w) of supplement oil.

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