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Improved lipid saponification for chromatographic quantification of fatty acids in porcine erythrocytes – an important lipidomic biomarker of the effectiveness of dietary fat supplementation in pigs as a large animal model for human studies

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ABSTRACT. The objectives of our study were to enhance and evaluate an improved saponification method for the quantification of fatty acids (FA), with a specific focus on highly-unsaturated long-chain polyunsaturated FA (LPUFA) that are easily peroxidised in pig erythrocytes. These erythrocytes serve as a valuable large animal model for human studies. We implemented a modified saponification procedure, involving a shorter initial saponification at 95 °C for 2 min, followed by overnight saponification at 22–25 °C, and subsequent gentle base and acid-catalysed methylation of FA. These analytical procedures proved to be suitable for gas-chromatographic analysis of highly-unsaturated LPUFA, which are prone to peroxidation in processed piglet erythrocytes. The modified initial saponification at 95 °C, followed by overnight saponification at 22–25 °C, and subsequent methylation, were used to assess the suitability of erythrocytes for the determination of FA, tocopherol and malondialdehyde (MDA) concentrations in monogastric mammalian tissues. The content of docosahexaenoic acid (DHA) in erythrocytes can serve as a marker for DHA bioaccumulation in animal tissues and dietary contents of n-3LPUFA (especially DHA). The concentrations of δ-, γ-, α-tocopherols, total cholesterol and MDA increased in piglet erythrocytes during 16 days of exposure to dietary fat. The levels of FA and tocopherols can therefore be used in erythrocytes as indicators of the bioaccumulation yield of these compounds in animal tissues. Additionally, we propose that FA and tocopherol profiles in erythrocytes may be used as indicators of the concentrations of these components in diets and the effectiveness of their bioaccumulation in animal tissues.

Introduction

The fatty acid profile of the diet affects the concentration of fatty acids (FA) present in the erythrocyte membrane (Baur et al., 2000; Harris et al., 2000). Erythrocytes, easily collectible cells for further physiological analyses, provide valuable insights into the FA profiles in less accessible tissues of animals and humans (Baur et al., 2000; Harris et al., 2000; Muhizi and Kim, 2021;

Boccardo et al., 2022). Additionally, numerous previous studies have demonstrated significant associations between erythrocyte FA profiles and the metabolic syndrome (MS), independent of well-known risk factors such as physical activity, alcohol intake, and smoking (Di Marino et al., 2000; Kabagambe et al., 2008; Gabriel et al., 2013; Pastorelli et al., 2021). In fact, the FA profile of erythrocytes may be a useful biomarker for assessing medium-term dietary fat intake (2–3 months for humans), and for comparing FA profiles in relation to their impact on cardiovascular diseases, diabetes or intermediate phenotypes (Enriquez et al., 2004; Poppitt et al., 2005; Baylin and Campos, 2006; Sun et al., 2007; Mužáková et al., 2019). For instance, recent prospective studies have shown a positive correlation ($R = 0.44$; $P < 0.01$) between dietary *trans*-FA (*t*-FA) concentrations and *t*-FA content in erythrocytes (Sun et al., 2007). FA composition of erythrocytes reflects FA consumed in the diet and can provide insights into certain mental diseases, such as schizophrenia, depression or anxiety, or metabolic disorders like diabetes (Bystrická and Ďuračková, 2016). Higher concentrations of unsaturated FA (UFA), especially polyunsaturated FA (PUFA), have shown a significant ($P < 0.05$) negative association in erythrocytes with systolic blood pressure, triglycerides and waist circumference, but a positive monotonic association with high-density lipoprotein (HDL) cholesterol content and low-density lipoprotein (LDL) cholesterol particle size (Kabagambe et al., 2008; Detopoulou et al., 2018; Ding et al., 2020). In addition, total polyunsaturated FA (ΣPUFA) and n-6PUFA (Σn-6PUFA), as well as the ratio of ΣPUFA to Σsaturated FA (ΣPUFA/ΣSFA) were found to be negatively correlated with the metabolic syndrome (MS) or its components (Kabagambe et al., 2008). Fortunately, short-term changes in diet composition (such as FA profile) have a smaller impact on the chemical composition of erythrocytes compared to blood plasma (Poppitt et al., 2005). The composition of plasma FA tends to fluctuate daily, whereas their content in human erythrocytes reflects long-term dietary fat intake over a period of 2–3 months. Interestingly, certain intestinal FA, especially short- and medium-chain FA and conjugated dienes, can be directly absorbed into the bloodstream and subsequently distributed to other tissues (Czauderna et al., 2004; Rozbicka-Wieczorek et al., 2014; Callahan et al., 2020; Jo et al., 2021; Trivedi et al., 2021). Thus, FA levels in erythrocytes can serve as an indicator of the effectiveness of lipases in the gastrointestinal tract and the FA profile in diets for humans and monogastric animals, particularly for pigs. In fact, pigs are frequently used as a biomedical large animal model for human studies due to its physiological and anatomical similarities to humans, especially in terms of the immune, cardiovascular and gastrointestinal systems (Goncharova et al., 2014).

Taking into consideration the aforementioned information, the primary objective of the current trial was to examine the variations in the levels of physiologically important components (like FA, tocopherols or cholesterol) in erythrocytes (specifically in their membrane) during an experimental period of controlled fat feeding. The aim was to study the rate at which dietary supplements affect the concentrations of FA (particularly PUFA), tocopherols, cholesterol and malondialdehyde (MDA) (a marker of PUFA peroxidation) in erythrocytes. Additionally, the study sought to determine whether a qualitative biomarker for dietary supplements could be identified when the experimental diet was supplemented with n-3 long-chain, highly-unsaturated fatty acids (n-3LPUFA). Dietary enrichment with n-3 LPUFA has been demonstrated to exert positive effects on the health of humans, livestock and domestic animals. These highly unsaturated n-3LPUFA have also shown a significant positive impact on FA composition, oxidant-antioxidant balance, nitric oxide (NO) levels, anti-inflammatory cytokine synthesis, and the nerve network responsible for cardiac function (Jo et al., 2021; Trivedi et al., 2021). To this end, the concentrations of FA (especially n-3LPUFA), tocopherol, total cholesterol (T-Ch) and MDA were determined in erythrocytes of piglets fed a high fat diet (HFD) supplemented with fish oil (FO) rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The aim was to investigate the impact of this dietary supplementation on erythrocyte composition. Quantification of FA in erythrocytes, as well as in other biological samples, is usually carried out using capillary gas-chromatography with mass spectrometry (GC-MS). This analytical technique offers exceptional precision and accuracy, especially when handling small sample masses (Rozbicka-Wieczorek et al., 2014). Moreover, the extremely sensitive saponification and methylation method is essential for accurate and precise analyses of highly unsaturated long-chain PUFA in selectively isolated erythrocyte of piglets (Czauderna et al., 2007).

Therefore, the main aim of our study was to develop a novel pre-column method for GC analysis of FA (especially highly-unsaturated LPUFA) in erythrocytes of healthy piglets that would be based on improved initial saponification at 95 °C,

followed by overnight saponification and subsequent methylation (Czauderna et al., 2007). The key focus was on improving the method to prevent peroxidation of LPUFA, intra-isomerization and/or isomerization of PUFA, such as conjugated dienes (Czauderna et al., 2007), or the formation of biological artefacts in the assayed erythrocytes.

The second objective of the current study was to examine the effectiveness of improved pre-saponification at 95 °C, followed by saponification overnight and final methylations (Czauderna et al., 2007) to quantify FA, with particular emphasis on readily peroxidised LPUFA in erythrocytes of a healthy and growing piglet fed a high fat diet (HFD-20) supplemented with FO (rich in EPA and DHA) for a period of 16 days. To ensure consistency and eliminate individual variations, erythrocyte samples were collected from a single healthy piglet under similar pre-prandial conditions. Saponification (Czauderna et al., 2007) and methylation steps were pre-tested on red blood cells collected from eight piglets with pancreatic exocrine insufficiency (EPI) (Goncharova et al., 2014). These piglets were fed HFD-20 supplemented with pancreatic-like microbial enzymes and encapsulated FO (rich in EPA and DHA) for 4 weeks.

Material and methods

Reagents and chemicals

Encapsulated FO (dietary supplement) was purchased from Nutravita Ltd. The composition of FO composition in terms of its main fatty acids (mg/g FO) was as follows: palmitic acid (C16:0) 10.1, stearic acid (C18:0) 116.9, oleic acid (c9C18:1) 36.8, linoleic acid (c9c12C18:2) 27.8, αlinolenic acid (c9c12c15C18:3) 10.0, arachidonic acid (c5c8c11c14C20:4) 34.1, eicosapentaenoic acid (c5c8c11c14c17C20:5) 220.0; cis13docosaenoic acid (c13C22:1) 2.2, docosapentaenoic acid (c7c10c13c16c19C22:5) 62.5 and docosahexaenoic acid (c4c7c10c13c16c19C22:6) 330. HPLC acetonitrile (≥ 99.9%), methanol (≥ 99.9%) and GC nhexane (≥ 99.0%) were purchased from Lab-Scan (Dublin, Ireland). Nonadecanoic acid (C19:0; internal standard), cholesterol, a mixture of conjugated linoleic acid isomers, an standard FA mixture [37 FA methyl esters (FAME) mix], α-tocopherol (α-T), δ-tocopherol (δ-T), γ-tocopherol (γ-T), 2,6-di-tert-butyl-p-cresol, 25% aqueous 1,5-pentanedialdehyde solution, 1,1,3,3-tetramethoxy-propane (99%), cholesterol, 2,4-dinitrophenylhydrazine (containing ~30% water), trichloroacetic acid and 25% BF_3 in methanol were purchased from Sigma Aldrich (St Louis, MO, USA). Chloroform, dichloromethane (DCM), Na_2SO_4, NaOH and KOH were purchased from Avantor Performance Materials (Gliwice, Poland). All other reagents were of analytical grade. Water used to prepare mobile phases and chemical reagents was obtained using an Elix™ Essential Water Purification System (Merck KGaA, Darmstadt, Germany).

Pig management, experimental design and feeding

All experiments involving piglets were carried out at The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences (Jabłonna, Poland). The welfare guidelines and handling procedures for the piglets were strictly followed according to the recommendations of the Second Warsaw Local Ethics Committee for Animal Experimentation (Ciszewskiego 8 street, 02-785 Warsaw, Poland; permission number: WAW2/15/2017, February 2017) throughout the preliminary and experimental periods. The first experiment used one healthy piglet, and the second experiment utilised eight piglets with EPI. The piglets used in both experiments were crossbred (Polish Landrace × Yorkshire × Hampshire) male piglets (*Sus scrofa domesticus*) at 10 weeks of age weighing ~11.1 kg at the beginning of both experiments. All piglets were maintained on a 12-hour day-night cycle, with lights on from 06:00 to 18:00. They were housed in individual cages with dimensions of 130 cm (width) × 170 cm (length) × 150 cm (height). Each cage was equipped with a feeding trough, drinking nipple, and a heating lamp (150 W). All piglets were allowed to move freely within their cages and had visual contact with other piglets. All piglets had free access to fresh tap drinking water throughout the initial and experimental periods. Analyses of the standard cereal-based diet and cereal-based pelleted high-fat diet (HFD-20) were performed at The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences (Jabłonna, Poland). Neutral detergent fibre (2002.04; Mertens, 2002), as well as dry matter (934.01), crude protein (984.13), crude fibre (978.10), crude fat (920.39), ash (942.05), acid detergent fibre (973.13) and acid detergent lignin (973.13) in pigs' feed were analysed according to the AOAC (2005).

The composition of the standard cereal-based diet (Morawski Plant, Kcynia, Poland), used as the

adaptation diet in the first experiment on a healthy piglet (without EPI), is presented in Table 1. The diet contained the following components: 16% crude protein, 34% starch, 2% crude fat, 42% crude fibre, 4.8% mineral-vitamin-micronutrients and 1.2% amino acids. HFD-20 (Morawski Plant, Kcynia, Poland; Table 2) contained: 17.5% crude protein, 3.9% crude fibre, 20% crude fat, and 5.2% ash, as well as 5000 IE vitamin A/kg, 500 IE vitamin D/kg and 85 mg vitamin E/kg.

The supplemented FO was administered to the piglets in the form of capsules. Each capsule, containing 1 g of FO, was cut into several pieces prior to the administration of the first half of the piglets' ration.

The first experiment was carried on a single healthy and growing piglet to ensure the stability and uniformity of the erythrocyte samples for analysis. The piglet had an initial weight of 11.2 kg at the start of the experiment and was fed a standard cereal-based diet (Table 1). The diet was provided at a daily amount to 4% of the piglet's body weight, with the first half of the ration given in the morning (at 09:00) and the second half in the afternoon (at 17:00). After a three-week adaptation period, the piglet was fed HFD-20 (Table 2) twice daily (2.0% body weight per meal), at 09:00 and 17:00 for 16 days. The first half of the morning ration was supplemented with 5 g of encapsulated FO (5 g FO/piglet/day) containing 1.10 g of eicosapentaenoic acid (EPA; c5c8c11c14c17C20:5) and 1.65 g of docosahexaenoic acid (DHA; c4c7c10c13c16c19C22:6). Three blood samples (~2 ml) from the healthy piglet (without EPI) were collected every day (day 1 to 16) before morning meal in Experiment 1.

Table 1. Composition of the standard cereal-based diet (Morawski Plant, Kcynia, Poland)[1]

Ingredients	g/kg
Wheat	402.75
Barley	400.00
Soybean meal	120.00
Fish flavour	30.00
Monocalcium phosphorum	20.00
Fodder chalk	10.00
Fodder salt	2.70
L-Lysine 78%	5.00
DL-Methionine 99%	2.70
L-Treonine 98.5%	2.00
L-Tryptophan 98%	2.00
Organic acids	1.30
Zinc oxide	0.42
Vitamin premix	0.13
Micronutrients	1.00

[1] Basal diet for pigs or piglets is a standard diet balanced according to the nutrient requirements of pigs (NRC, 2012)

Table 2. Ingredients, chemical composition, and nutritional value of cereal-based pelleted high-fat diet (HFD-20) (Morawski Plant, Kcynia, Poland)

Ingredients	g/kg
Barley	261.00
Wheat	274.80
Soybean meal	220.00
Rapeseed oil	200.00
Premix 0.5% grower[1]	5.00
Fodder salt	2.00
Zink oxide	0.17
Lysine	5.00
Methionine	2.00
Threonine	2.00
Acidifier	1.00
Chemical composition[2]	
dry matter	905
ash	24.3
organic matter	881
crude protein	166
ether extract (fat)	216
crude fibre	33.5
starch	311
lysine	13.20
methionine	4.65
threonine	7.80
P	6.97
Ca	2.0
Na	1.14
Energy, MJ/kg	16.8 (4012.7 kcal)
Lysine / energy, g/MJ	0.79

[1] contained per kg of diet: µg: vit. A 2250, vit. D_3 37.5; mg: Fe 152, Zn 231, Cu 24.9, Mn 50.7, I 0.69, Se 0.23, Co 0.08, Mg 1.24, Na 1.14, vit. E 63.2, vit. K_3 1.1, vit. B_1 4.27, vit. B_2 4.46, vit. B_6 5.34, vit. B_{12} 2.71, biotin 0.21, folic acid 1.87, nicotinic acid 51, calcium-D pantothenate 15.3, choline chloride 1300; g: Ca 2.0, P 6.97, K 6.12, S 1.56, NaCl 1.80; [2] concentration of main fatty acids: g/kg high fat diet (HFD-20): caprylic acid (C8:0) – 0.402, capric acid (C10:0) – 0.043, lauric acid (C12:0) – 0.213; miristic acid (C14:0) – 0.236, C16:0 – 11.26, cis-9-C16:1 (c9C16:1) – 0.265, stearic acid (C18:0) – 2.796, oleic acid (c9C18:1) – 77.32, linoleic acid (c9c12C18:2; LA) – 102.1, gamma-linolenic acid (c6c9c12C18:3) – 0.621, α-linolenic acid (c9c12c15C18:3; αLNA) – 20.63, arachidic acid (C20:0) – 0.286, behenic acid (C22:0) – 0.215, arachidonic acid (c5c8c11c14C20:4; AA) – 0.06, erucic acid (c13C22:1) – 0.236 and eicosapentaenoic acid (c5c8c11c14c17C20:5, EPA) – 0.031

Experiment 2 involved eight piglets with EPI (Goncharova et al., 2014) weighing 11.1 ± 0.9 kg at the start of the experiment. The piglets with EPI at the beginning of the experiment were fed daily a ration of HFD-20 (Table 2) corresponding to 4% of their body weight; the first half of the ration was given in the morning (at 09:00), and the second half of the ration in the afternoon (at 17:00). After a four-week adaptation period, EPI piglets were fed HFD-20 supplemented with pancreatic-like microbial enzymes, including bacterial (*Chromobacterium viscosum*) L1 with

a specific activity of ≥ 2500 units/mg (Sigma), and fungal (*Rhizopus oryzae*) L2, with a specific activity of 4019.3 units/mg (Amano). The enzymes were added at a concentration of 0.8 g per kg of HFD-20. Additionally, the piglets were supplemented with 5 g of encapsulated FO per day, which contained 1.10 g of EPA and 1.65 g of DHA.

The health status of all piglets was monitored on a daily basis by professional veterinary staff at The Kielanowski Institute of Animal Physiology and Nutrition, PAS (Jabłonna).

Preparation of erythrocyte samples

Blood samples from each piglet (without or with EPI) were collected via a jugular vein catheter into heparinized tubes, just before morning feeding (at 08:30) on each day of the experimental period. The collected blood samples were immediately placed on ice and then centrifuged at 3000 × g for 15 min at 4 °C. After centrifugation, the plasma layer (supernatant) and buffy coat (white blood cells and platelets) were carefully separated from red blood cells (residue). To obtain the residue containing erythrocytes, 1 ml of saline solution was added, mixed and centrifuged at 4000 rpm (rotor radius – 4.5 cm) for 10 min at 4 °C. The washing procedure was repeated three times. Selectively isolated erythrocytes (RBC) were stored in sealed vials and frozen at −32 °C until further chemical analyses. All piglet erythrocyte samples were analysed individually.

The concentrations of FA, total cholesterol (T-Ch), tocopherols and MDA in erythrocytes were expressed on a fresh weight basis (i.e. per 1 g of fresh erythrocytes).

Preparation of fatty acid methyl esters (FAME) in processed erythrocyte cells

The improved saponification and extraction of free fatty acids from erythrocyte cell hydrolysates were conducted as follows: red blood cells (40–60 mg) were placed in a vial and treated with 1 ml of 2 M KOH in water and 1 ml of 1 M KOH in methanol. Next, 25 µl of the internal standard solution (17 mg × ml^{-1} C19:0 in chloroform) was added, and the resulting mixture was protected from light. Subsequently, the mixture was carefully flushed with argon (Ar) for 4–5 min, vigorously vortexed for 1–2 min in a sealed vial, and then heated under Ar at 95 °C for 2 min. The resulting solution was immediately cooled on ice to room temperature and sonicated for 10 min. Finally, the resulting hydrolysate was protected from light and stored in a tightly sealed vial under Ar at 22–25 °C overnight. The next day, water (1.5 ml) was added to this hydrolysate, and the solution was again vigorously vortexed. This solution was then acidified with 4 M HCl to an approximate pH of 2.0, and free fatty acids were extracted four times with 1.5 ml of DCM. To avoid loss of free FA, the extraction was repeated four times using 1.5 ml n-hexane. The upper layer of n-hexane was combined with a layer of DCM, and the resultant organic phase was dried using ~100 mg of Na_2SO_4. Organic solvents were removed under a stream of Ar at room temperature. The obtained residue (I) was stored at −32 °C until mild methylation catalysed by base or acid.

Preparation of fatty acid methyl esters (FAME)

Two millilitres of 2 M NaOH in methanol was added to residue I, and the resulting solution was carefully de-aerated by a stream of Ar for ~5 min, followed by the reaction for 1 h at 40 °C. After cooling, 1 ml of 25% BF_3 in methanol was added to the reaction mixture, and carefully flushed with a stream of Ar for ~5 min and reheated for 1 h at 40 °C. Subsequently, water (2.5 ml) was added to the cooled reaction mixture and FAME were extracted with 1 ml of n-hexane. The clear supernatant was transferred to a GC vial.

Gas chromatography

The analyses of all FAME in processed erythrocytes were performed using a GC-MS-QP2010 Plus EI (SHIMADZU; Tokyo, Japan), equipped with a BPX70 fused silica capillary column (120 m × 0.25 mm i.d. × 0.25 µm film thickness; SHIM-POL), a quadrupole mass selective (MS) detector (Model 5973N; SHIMADZU; Tokyo, Japan) and an injection port. Helium as the carrier gas was operated at a constant pressure of 223.4 kPa and an initial flow rate of 1 ml/min. The temperature and split ratio of the injector were 200 °C and 1:1, respectively. Injection volumes were 1–2 µl. The transfer line was maintained at 240 °C. The MS was operated in the EI mode and full scan monitoring (m/z 30–350). The ion-source temperature was set to 240 °C, and the electron energy was equal to 70 eV. Total FAME profiles in processed samples were determined using the column temperature gradient programme. The column was initially operated at 70 °C for 4 min, and then the temperature of the column was increased according to the following temperature gradient program: 12 °C/min to 150 °C, held for 6 min, increase at a rate of 8 °C/min to 168 °C, held for 27 min, increase at a rate of 0.75 °C/min to 190 °C, held for 10 min, increase at a rate of 1.8 °C/min to 210 °C, held for 15 min, increase at a rate of 6 °C/min to 234 °C, held

for 4 min, increase at a rate of 6 °C/min to 236 °C, held for 20 min.

The identification of FAME in erythrocyte samples was verified based on electron impact ionization spectra of FAME and compared with FAME standards and the NIST 2007 reference mass spectra library (SHIMADZU; Tokyo, Japan).

MDA analysis in processed erythrocytes using C18-UFLC-DAD

MDA concentration in erythrocyte samples was determined after saponification, fol-lowed by derivatisation according to the procedure described by Czauderna et al. (2011). Separation of derivatised MDA from endogenous species in processed erythrocytes was conducted using a C18-liquid chromatographic system (Czauderna et al., 2011). The concentration of MDA in erythrocytes was analysed using a liquid chromatographic apparatus (SHIMADZU; Tokyo, Japan) with two LC-20ADXP pumps (UFL-CXR), SIL-20 ACXR autosampler (LFL-CXR), CBM-20A communications bus module, CTO-20A column oven, C18-column (Synergi, Hydro-RP; particle size: 2.5 μm; 100 Å; 100 mm × 2 mm i.d.; Phenomenex; Torrance, CA, USA), DGU-20A5 degasser and SPD DAD.

Analysis of tocopherols and T-Ch in processed erythrocytes using liquid chromatography

Tocopherols (α-T, δ-T and γ-T) and T-Ch in erythrocyte samples (40–60 mg) were analysed according to the method described by Czauderna et al. (2009). T-Ch, α-T, δ-T and γ-T were fractionated from endogenous components in processed erythrocyte samples using a reversed-phase (C18) liquid chromatography system (SHIMADZU; Tokyo, Japan). The liquid chromatographic instrument used consisted of an ultra-fast liquid chromatography system (C18-UFLC-DAD), containing two LC-20ADXP pumps (UFL-CXR), SIL-20 ACXR autosampler (LFL-CXR), CBM-20A communications bus module, CTO-20A column oven, Kinetex C18-column (the particle size: 2.6 μm; Hydro-RP, 150 mm × 2.1 mm i.d.; Phenomenex; Torrance, CA, USA), DGU-20A5 degasser and SPD photodiode array detector (DAD).

Statistical analysis

All statistical analyses of the effects of dietary additives were carried out using the Statistica 12.5 PL software package (StatSoft Inc., Tulsa, OK, USA). The significance of differences in the content of selected lipid compounds/biomarkers during the first experiment (between-day) with a healthy piglet was determined using the Friedman ANOVA test. A P-value ≤ 0.05 was considered significant.

The statistical analysis of the effects of initial saponification at 95 °C for 2 min and 10 min (Table 4) on the concentrations of selected FA in erythrocytes of eight piglets with EPI was carried out using the non-parametric Mann-Whitney U test. Differences were considered significant at $P \leq 0.05$.

Results

No adverse effects (like vomiting or diarrhoea) were observed in eight piglets with EPI and the healthy piglet (without EPI) fed diets with 5 g of encapsulated FO (5 g FO/piglet/day). Moreover, the analysis revealed that the levels of EPA, DPA and DHA contained in 5 g of FO capsules were sufficient to increase the concentrations of these fatty acids in erythrocytes above the limit of quantification (LOQ) (Tables 3, 4 and 5).

Effect of initial saponification time at 95 °C on FA concentrations in erythrocytes

Experiment 1 in healthy piglet. To investigate the susceptibility of unstable arachidonic acid (AA; c5c8c11c14C20:4), EPA, DPA, and DHA in erythrocytes to oxidation and decomposition. We first attempted to quantify the methylated FA formed after initial saponification at 95 °C for 2, 10 and 20 min, followed by saponification overnight, and subsequent methylation (Czauderna et al., 2007). Unfortunately, shorter time (< 2 min) of initial saponification resulted in the formation of a visible precipitate derived from the assayed erythrocytes in the KOH solution used for hydrolysis. This precipitate persisted even after mild base and acid methylations were performed.

The results summarized in Table 4 show that the shortest duration (2 min) of initial saponification at 95 °C resulted in higher concentrations of AA, EPA, DPA, DHA, tetracosapentaenoic acid (c9c12c15c18c21C24:5; C24:5n-3) and tetracosahexaenoic acid (c6c9c12c15c18c21C24:6; C24:6n-3) in erythrocytes compared to the concentrations observed with longer times (10 and 20 min) of erythrocyte processing. On the other hand, the concentrations of other FA, i.e. saturated FA (SFA) and UFA containing 18 or fewer carbon atoms in UFA chains in the processed erythrocytes were not dependent on the duration of pre-saponification (i.e. 2, 10 or 20 min). These findings indicated that initial saponification at 95 °C for 2 min reduced the degradation efficiency of LPUFA, which are susceptible to oxidative damage when compared to longer durations of initial saponification (10 and 20 min).

Table 3. Calibration equations, correlation coefficients, limits of detection (LOD) and limits of quantification (LOQ) of FA determined in erythrocytes and inter-assay coefficient of variations (C.V., %) from the measurements of fatty acid (FA) concentrations in three erythrocyte samples from piglets with pancreatic exocrine insufficiency (EPI) fed high fat diet (HFD-20) supplemented with pancreatic-like microbial enzymes and fish oil (FO)

FA	Calibration equation[1], y(ng) = a x Sn	Linearity, r	Detector response to ng FA[1]	FA range (µg) in processed standards	LOD[2], ng	LOQ[3], ng	2 min C.V., %	10 min C.V., %
C10:0	0.00009335 x Sn	0.9996	386089	0.5–50.0	0.10	0.32	3.11	2.96
C14:0	0.00008415 x Sn	0.9998	420393	2.5–25.0	0.09	0.29	4.08	3.86
C16:0	0.00010711 x Sn	0.9999	330446	10.0–300.0	0.11	0.36	3.92	3.91
C18:0	0.00008008 x Sn	0.9999	438989	10.0–400.0	0.12	0.39	4.89	4.69
C20:0	0.00010625 x Sn	0.9987	331007	0.5–5.0	0.18	0.58	2.88	3.04
c9C14:1	0.00007820 x Sn	0.9968	447261	5.0–50.0	0.10	0.33	2.93	3.63
c9C16:1	0.00007874 x Sn	0.9978	444329	1.0–10.0	0.12	0.39	3.17	4.03
c9C18:1	0.00009036 x Sn	0.9999	387322	3.0–60.0	0.14	0.46	4.53	4.48
c15C24:1	0.00009911 x Sn	0.9985	353264	0.5–5.0	0.20	0.65	6.62	8.48
c9c12C18:2 (LA)	0.00007346 x Sn	0.9999	474061	10.0–200.0	0.17	0.56	4.48	3.58
c9c12c15C18:3 (αLNA)	0.00008411 x Sn	0.9998	414513	5.0–150.0	0.18	0.59	3.74	3.57
c5c8c11c14C20:4 (AA)	0.00007466 x Sn	0.9993	467109	10.0–100.0	0.22	0.73	3.76	4.04
c5c8c11c14c17C20:5 (EPA)	0.00005860 x Sn	0.9989	591133	2.0–20.0	0.23	0.75	2.58	4.84
c7c10c13c16c19C22:5 (DPA)	0.00007550 x Sn	0.9991	460817	1.0–20.0	0.21	0.68	2.73	4.94
c4c7c10c13c16c19C22:6 (DHA)	0.00007556 x Sn	0.9993	460556	1.0–25.0	0.27	0.88	2.97	3.69
c9c12c15c18c21C24:5 (C24:5n-3)	0.00007549 x Sn	0.9989	460842	0.5–5.0	0.54	1.77	4.32	4.86
c6c9c12c15c18c21C24:6 (C24:6n-3)	0.00007555 x Sn	0.9982	460580	0.5–5.0	0.63	2.07	5.73	6.34

Sn – peak area of the assayed fatty acid; linear regression forcing the intercept at point 0,0; r – regression coefficient; [1] Detector (MS) response to 1 ng of assayed fatty acids in processed FA standard solutions; [2] LOD was calculated with a signal (Sn) to noise (σ) ratio of 3 (LOD = 3 x Sn / σ) (Shrivastava and Gupta, 2022); [3] LOQ was defined as 10 times the noise under an analytical peak (LOQ = 10 x Sn/ σ) (Shrivastava and Gupta, 2022); FA analytical peak noise was calculated based on the noise on the left (σL) and right (σR) side of the analytical peak of FA, i.e., σ = ((σL + σR)/2) (Czauderna et al., 2011); C.V. was calculated as C.V., % = (SD/µ) x 100%, where: SD is the standard of deviation of FA measurements in erythrocyte samples, µ is the mean value of FA measurements; 2 min C.V., % – initial saponification of lipids in erythrocytes at 95°C – 2 min; 10 min C.V., % – initial saponification of lipids in erythrocytes at 95 °C – 10 min

C10:0 – capric acid; C14:0 – myristic acid; C16:0 – palmitic acid; C18:0 – stearic acid; C20:0 – arachidic acid; c9C14:1 – myristoleic acid; c9C16:1 – palmitoleic acid; c9C18:1 – oleic acid; c15C24:1 – nervonic acid; c9c12C18:2 (LA) – linoleic acid; c9c12c15C18:3 (αLNA) – αlinolenic acid; c5c8c11c14C20:4 (AA) – arachidonic acid; c5c8c11c14c17C20:5 (EPA) – eicosapentaenoic acid; c7c10c13c16c19C22:5 (DPA) – docosapentaenoic acid; c4c7c10c13c16c19C22:6 (DHA) – docosahexaenoic acid; c9c12c15c18c21C24:5 (C24:5n–3) – tetracosapentaenoic acid; c6c9c12c15c18c21C24:6 (C24:6n–3) – tetracosahexaenoic acid (nisinic acid)

Table 4. Effect of duration of the initial lipid saponification at 95 °C on fatty acids (FA) in three erythrocyte samples[1] from healthy piglet fed high-fat diet (HFD-20) supplemented with fish oil (FO)

FA, µg/g	Initial saponification of lipids in erythrocytes at 95 °C		
	2 min	10 min	20 min
C14:0	4.1	3.9	4.3
C16:0	32.5	34.3	33.6
C18:0	27.4	28.2	27.9
c9C16:1	1.35	1.29	1.38
c9C18:1	35.8	34.9	36.3
c15C24:1	0.871	0.883	0.879
c9c12C18:2 (LA)	0.679	0.673	0.682
c9c12c15C18:3 (αLNA)	33.8	33.1	34.3
c5c8c11c14C20:4 (AA)	1.142	0.254	0.166
c5c8c11c14c17C20:5 (EPA)	0.439	0.084	0.073
c7c10c13c16c19C22:5 (DPA)	0.645	0.119	0.071
c4c7c10c13c16c19C22:6 (DHA)	0.833	0.069	0.019
c9c12c15c18c21C24:5 (C24:5n-3)	1.057	0.594	0.247
c6c9c12c15c18c21C24:6 (C24:6n-3)	0.984	0.458	0.153

[1] three blood samples were collected from the healthy piglet (without pancreatic exocrine insufficiency (EPI) fed for 16 days HFD-20 supplemented with 5 g of FO

C14:0 – myristic acid; C16:0 – palmitic acid; C18:0 – stearic acid; c9C16:1 – palmitoleic acid; c9C18:1 – oleic acid; c15C24:1 – nervonic acid; c9c12C18:2 (LA) – linoleic acid; c9c12c15C18:3 (αLNA) – αlinolenic acid; c5c8c11c14C20:4 (AA) – arachidonic acid; c5c8c11c14c17C20:5 (EPA) – eicosapentaenoic acid; c7c10c13c16c19C22:5 (DPA) – docosapentaenoic acid; c4c7c10c13c16c19C22:6 (DHA) – docosahexaenoic acid; c9c12c15c18c21C24:5 (C24:5n–3) – tetracosapentaenoic acid; c6c9c12c15c18c21C24:6 (C24:6n–3) – tetracosahexaenoic acid (nisinic acid)

Table 5. Effect of initial saponification time at 95 °C on the concentrations (μg/g)[1] of fatty acids (FA) in three erythrocyte samples from piglets with pancreatic exocrine insufficiency fed a high-fat diet (HFD-20) supplemented with pancreatic-like microbial enzymes[2] and fish oil (FO)[3]

FA, μg/g	Initial saponification of lipids in erythrocytes at 95 °C		
	2 min	10 min	*P*-value
C10:0	3.72 ± 0.14	3.91 ± 0.15	0.62
C14:0	3.69 ± 0.18	3.47 ± 0.17	0.48
C16:0	37.9 ± 2.1	40.1 ± 1.9	0.39
C18:0	33.7 ± 3.1	30.8 ± 2.8	0.54
C20:0	0.35 ± 0.13	0.29 ± 0.15	0.71
c9C14:1	0.32 ± 0.14	0.38 ± 0.12	0.81
c9C16:1	0.71 ± 0.22	0.83 ± 0.19	0.76
c9C18:1	38.9 ± 2.8	37.6 ± 3.2	0.89
c15C24:1	0.40 ± 0.10	0.43 ± 0.14	0.74
c9c12C18:2 (LA)	0.69 ± 0.14	0.73 ± 0.12	0.68
c9c12c15C18:3 (αLNA)	41.3 ± 2.8	43.1 ± 3.1	0.73
c5c8c11c14C20:4 (AA)	1.48 ± 0.25	0.35 ± 0.12	0.01
c5c8c11c14c17C20:5 (EPA)	0.57 ± 0.10	0.11 ± 0.04	0.01
c7c10c13c16c19C22:5 (DPA)	0.81 ± 0.17	0.11 ± 0.05	0.01
c4c7c10c13c16c19C22:6 (DHA)	0.95 ± 0.24	0.09 ± 0.04	0.01
c9c12c15c18c21C24:5 (C24:5n-3)	1.15 ± 0.19	0.61 ± 0.13	0.01
c6c9c12c15c18c21C24:6 (C24:6n-3)	1.09 ± 0.18	0.39 ± 0.09	0.01

[1] mean concentrations of fatty acids obtained from individually analysed three erythrocyte samples collected from eight piglets; data are presented as means and corresponding standard deviations (SE) of the means; [2] 0.8 g enzymes/kg HFD-20; [3] 5 g FO/piglets/day; 1 g FO contains 220 mg of EPA (C20:5n-3) and 330 mg of DHA (C22:6n-3)

C10:0 – capric acid; C14:0 – myristic acid; C16:0 – palmitic acid; C18:0 – stearic acid; C20:0 – arachidic acid; c9C14:1 – myristoleic acid; c9C16:1 – palmitoleic acid; c9C18:1 – oleic acid; c15C24:1 – nervonic acid; c9c12C18:2 (LA) – linoleic acid; c9c12c15C18:3 (αLNA) – αlinolenic acid; c5c8c11c14C20:4 (AA) – arachidonic acid; c5c8c11c14c17C20:5 (EPA) – eicosapentaenoic acid; c7c10c13c16c19C22:5 (DPA) – docosapentaenoic acid; c4c7c10c13c16c19C22:6 (DHA) – docosahexaenoic acid; c9c12c15c18c21C24:5 (C24:5n–3) – tetracosapentaenoic acid; c6c9c12c15c18c21C24:6 (C24:6n–3) – tetracosahexaenoic acid (nisinic acid)

FA profile analysis by a modified saponification method in erythrocytes from EPI piglets – Experiment 2

The improved initial saponification was used to quantify FA content in lipids extracted from erythrocyte samples collected from 8 piglets with EPI fed HFD-20 (Table 2) supplemented with pancreatic-like microbial enzymes and FO for 4 weeks (5 g/pig/day). FA levels in 24 red blood cell samples (3 RBC samples/piglet) were analysed by saponification at 95 °C for 2 and 10 min, followed by overnight saponification and methylation (Czauderna et al., 2007) (Table 5). The comparison of the results using both methods showed that the concentrations of AA, EPA, DPA, DHA, tetracosapentaenoic acid (c9c12c15c18c21C24:5) and tetracosahexaenoic acid (c6c9c12c15c18c21C24:6) in erythrocyte samples processed using the improved saponification at 95 °C for 2 min were higher (~77%, ~81%, ~83%, ~91%, ~43% and ~41%, respectively) than those in erythrocyte samples processed using the previously developed saponification (Czauderna et al., 2007) and base and acid-catalysed methylation methods (Czauderna et al., 2007). As expected, the concentrations of other FA (i.e. SFA and UFA containing 18 or fewer carbon atoms in UFA chains) in piglet erythrocytes were not affected ($P > 0.05$) by the duration of initial saponification at 95 °C (i.e. for 2 and 10 min).

Effect of feeding time of HFD-20 supplemented with FO on the concentrations of FA, tocopherols and MDA in erythrocytes of healthy piglet – Experiment 1

The improved initial saponification method, followed by overnight saponification and methylations were used to study the effect of HFD-20 (Table 2) supplemented with FO on FA levels in red blood cells of a piglet without EPI. No macroscopic lesions, adverse symptoms (like vomiting or diarrhoea) or pathological changes were observed in the internal organs, muscles or adipose tissues of the piglet. The results summarized in Figure 1 and 2 demonstrate that HFD-20 supplemented with FO led to a reduction in the concentrations of caprylic acid (C8:0), capric acid (C10:0), atherogenic SFA, i.e. lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0), myristoleic acid (c9c14:1), c9-palmitoleic acid (c9C16), c10-palmitoleic acid (c10C16:1), oleic acid c9C18:1, LA, αLNA, dihomo-gamma-linolenic acid (c8c11c14C20:3),

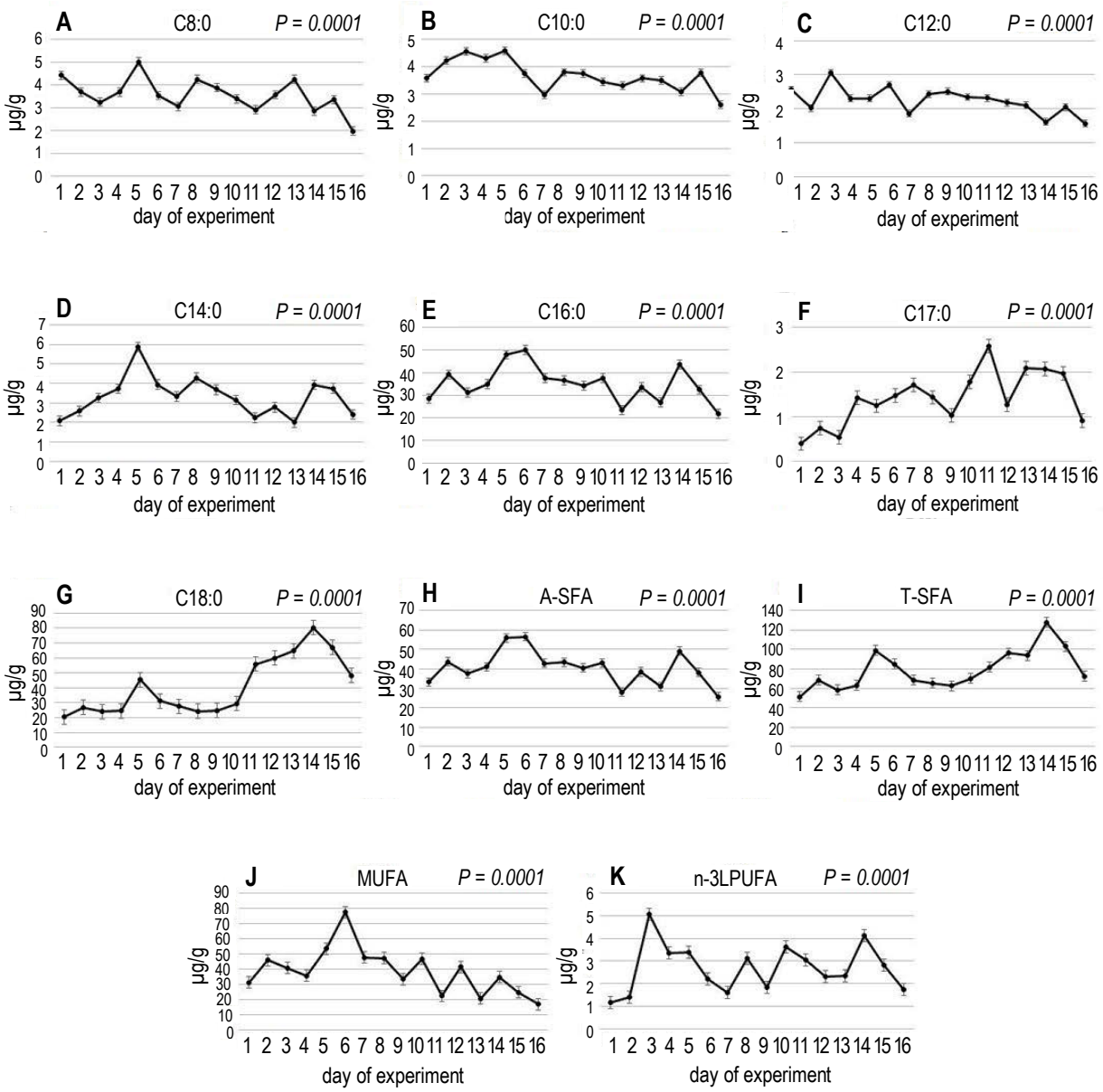

Figure 1. Effect of feeding duration of HFD-20 supplemented with FO (rich in EPA and DHA) on the concentrations (µg/g) of selected SFA, total concentration (µg/g) of MUFA and n-3LPUFA in erythrocytes of healthy piglet without EPI (A-SFA: atherogenic saturated fatty acids (C12:0, C14:0 and C16:0); T-SFA: thrombogenic saturated fatty acids (C14:0, C16:0 and C18:0)

HFD-20 – high-fat diet; FO – fish oil; EPA – eicosapentaenoic acid; DHA – docosahexaenoic acid; SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, n-3LPUFA – n-3 long-chain polyunsaturated fatty acids, EPI – pancreatic exocrine insufficiency; C8:0 – caprylic acid; C10:0 – capric acid; C12:0 – lauric acid; C14:0 – myristic acid; C16:0 – palmitic acid; C17:0 – margaric acid; C18:0 – stearic acid

arachidonic acid (AA), tetracosapentaenoic acid (c9c12c15c18c21C24:5)andtetracosahexaenoicacid (c6c9c12c15c18c21C24:6) in piglet erythrocytes. Conversely, FO added to HFD-20 increased the levels of heptadecanoic acid (C17:0), stearic acid (C18:0), c11c14-eicosadienoic acid (c11c14C20:2; C20:2n-6), DHA and the total concentration of thrombogenic SFA (i.e. C14:0, C16:0 and C18:0) in piglet erythrocytes.

Figure 3 shows the effect of FO-enriched HFD-20 on the concentrations of tocopherols (δ-T, γ-T and α-T) and total cholesterol (T-Ch) in piglet erythrocytes throughout the 16-day experimental period. It was found that the concentrations of δ-T, γ-T, α-T and T-Ch increased in erythrocytes of the piglet fed the experimental diet. Figure 4 shows the effect of HFD-20 supplemented with FO on the concentration of MDA in piglet erythrocytes. During the 16-day experimental period, MDA concentrations in erythrocytes showed a positive association (correlation coefficient: R = 0.6687) with the duration of the dietary experiment.

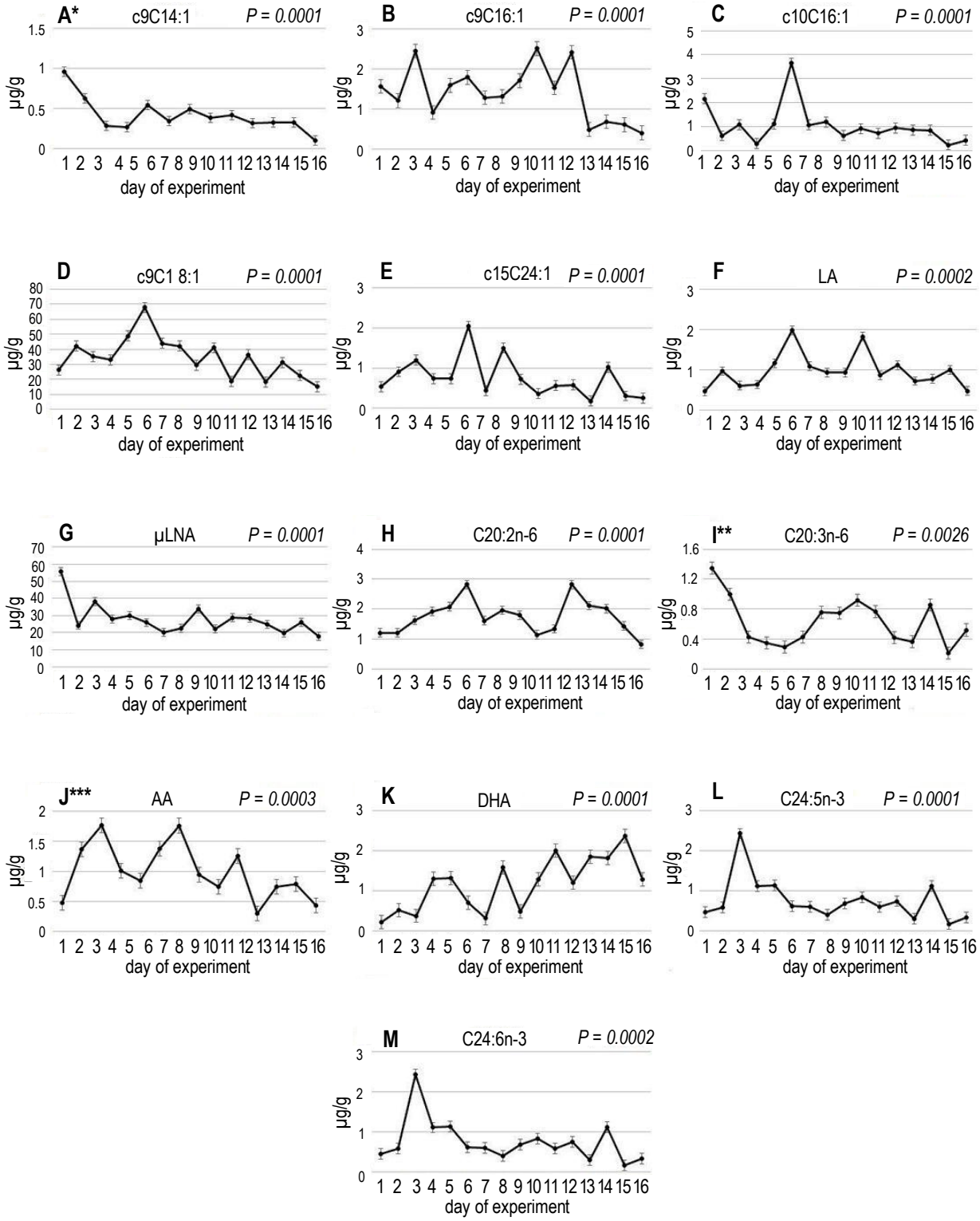

Figure 2. Effect of feeding time of HFD-20 supplemented with FO (rich in EPA and DHA) on the concentrations (µg/g) of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in erythrocytes of health piglet without EPI.

*– content of c9C14:1 at day 2, 10 and 14 was below the limit of quantification (<LOQ);

**– content of C20:3n-6 at day 2 was below the limit of quantification (<LOQ);

***– content of AA at day 8 and 11 was below the limit of quantification (<LOQ);

HFD-20 – high-fat diet; FO – fish oil; EPI – pancreatic exocrine insufficiency; EPA – eicosapentaenoic acid; DHA – docosahexaenoic acid; c9C14:1 – myristoleic acid; c9C16:1 – palmitoleic acid; c9C18:1 – oleic acid; c15C24:1 – nervonic acid; LA – linoleic acid; αLNA – α linolenic acid; C20:2n–6 – c11c14eicosedienoic acid; C20:3n–6 – c8c11c14eicosatrienoic acid; AA – arachidonic acid; C24:5n–3 – tetracosapentaenoic acid; C24:6n–3 – tetracosahexaenoic acid (nisinic acid)

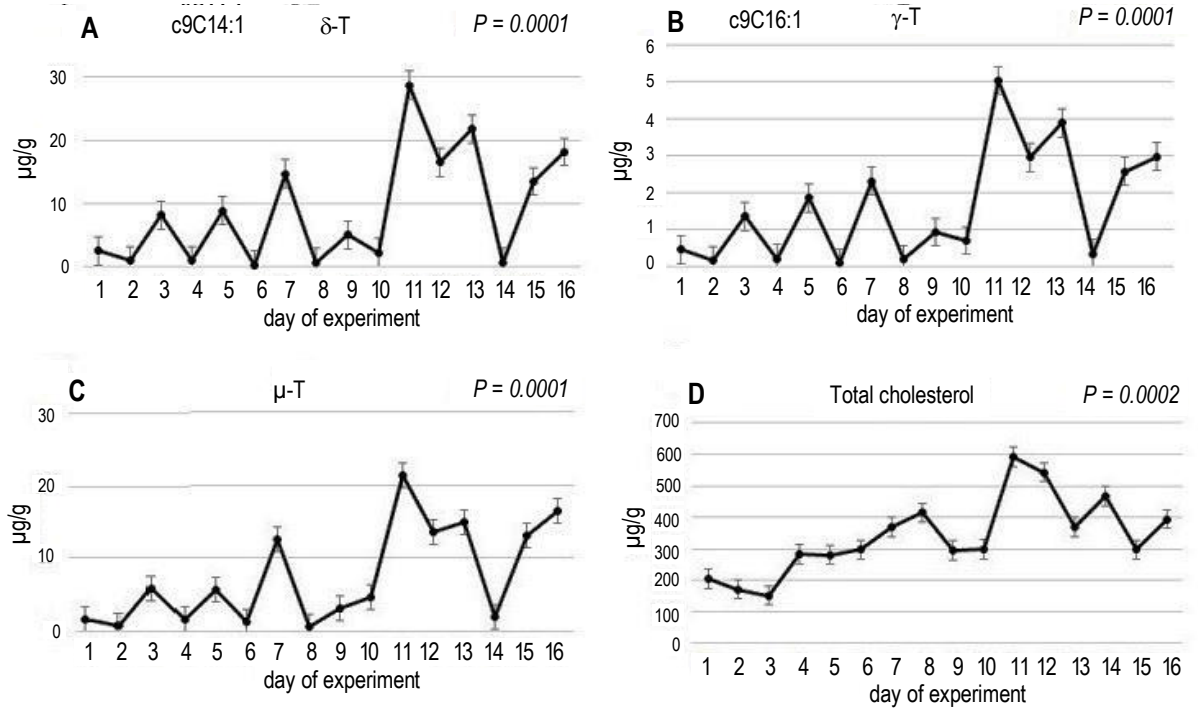

Figure 3. Effect of feeding time of HFD-20 supplemented with FO (rich in EPA and DHA) on the concentrations (μg/g) of δ-tocopherol (δ-T), γ-tocopherol (γ-T), α-tocopherol (α-T) and total cholesterol (T-Ch) in erythrocytes of piglet without pancreatic exocrine insufficiency (EPI).

HFD-20 – high-fat diet; FO – fish oil; EPA – eicosapentaenoic acid; DHA – docosahexaenoic acid

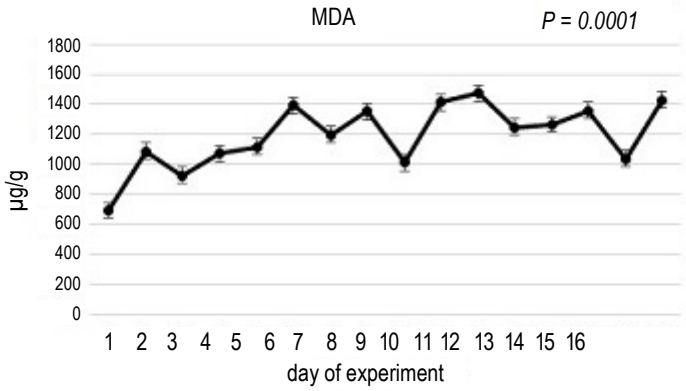

Figure 4. Effect of HFD-20 supplemented with FO (rich in EPA and DHA) on the concentration (μg MDA/g RBC) of MDA in erythrocyte cells of piglet without EPL; correlation coefficient (R) between Experiment 1 and MDA concentrations in RBC: R = 0.6687.

HFD-20 – high-fat diet; FO – fish oil; EPA – eicosapentaenoic acid; EPI – pancreatic exocrine insufficiency; DHA – docosahexaenoic acid; MDA – malondialdehyde, RBC – red blood cells

Discussion

Erythrocyte samples obtained from a single healthy piglet (without EPI) were obtained in similar pre-prandial conditions to eliminate individual variations and ensure homogeneity of biological components, which could affect the pre-column procedure. The findings summarised in Table 3 have demonstrated that the improved initial saponification at 95 °C for 2 min, followed by overnight saponification and base- and acid-methylations (Czauderna et al., 2007) were better analytical procedures for GC analysis of highly unsaturated LPUFA prone to peroxidation in assayed erythrocytes. The duration of initial saponification did not affect SFA, MUFA and PUFA levels containing 18 or shorter carbon chains. In fact, these FA are more chemically stable than highly unsaturated LPUFA (i.e., AA, EPA, DPA, DHA, C24:5n-3 and C24:6n-3). Based on these results,

it can be concluded that lipid saponification in erythrocytes occurs faster than lipid saponification in muscles, liver, kidneys, spleen or adipose tissues (Czauderna et al., 2007). Moreover, it is suggested that the current improved initial saponification followed by overnight saponification and FA methylation can be used to assess the fatty acid profiles, as well as concentrations of fatty acids, tocopherols, and malondialdehyde (MDA) in other tissues of monogastric mammals by utilising erythrocytes as a suitable model.

Effect of FO-enriched HFD-20 on FA, tocopherol and MDA contents in erythrocytes

A previous study conducted by Øverland et al. (1999) demonstrated that during the growing, finishing as well as all periods combined, a high-fat die (HFD) resulted in a higher average daily gain (ADG) of pigs and improved feed efficiency (expressed as kg of feed intake/kg weight gain) and a shorter time to market of 6.3 days in comparison to a low-fat diet (LFD). Notably, there were no significant differences ($P > 0.05$) between the HFD and LFD regarding backfat thickness, lean percentage, carcass meatiness. However, the HFD showed a tendency to reduce the fat area in the cutlet. In conclusion, the application of the HFD improved growth performance with only minor adverse effects on carcass quality of pigs (Øverland, et al., 1999). When using pigs as a large animal model, it is necessary to adjust the diet composition to match the standards employed in human nutrition, which typically includes a fat content of over 20%. It is generally accepted that a minimum of one week is required for dietary adaptation in monogastric animals. However, in our study focusing on the suitability of pigs as a large animal model for human studies, we extended this adaptation period to three weeks. The graphs presented in Figures 1–4 confirm this methodological principle. The fat content in erythrocytes during the first few days of the experiment increased, followed by a subsequent stabilisation.

The present study, along with previous research by Takkunen et al. (2013), has demonstrated that the concentrations of FA and tocopherols in mammalian erythrocytes can be used as biomarkers of dietary FA and tocopherol concentrations. We were particularly interested in analysing n-3LPUFA (especially EPA and DHA), as the consumption of diets enriched with these fatty acids has been consistently associated with various health-promoting physiological effects. Fortunately, our current work, as well as previous studies (Takkunen et al., 2013) have documented that DHA concentration in erythrocytes is a reliable marker for diets enriched with fish oil (FO), which is known to be particularly rich in n-3LPUFA. The endogenous biosynthesis of EPA and DHA in mammals from precursors such as αLNA is negligible compared to the amount of EPA or DHA present in a diet enriched with FO (Niedźwiedzka et al., 2008; Skiba et al., 2011). Moreover, our current research has revealed that short-term feeding (for 16 days) with HFD-20 supplemented with FO (enriched in EPA and DHA) resulted in an increase in the concentration of DHA in erythrocytes of a healthy and growing piglet (without EPI). Interestingly, a recent study indicated that FA composition in erythrocytes of humans (weighing 70–80 kg) stabilised at a new level 4 to 5 weeks after starting new diet (Kawabata et al., 2011). In fact, dietary FA are incorporated as phospholipids into erythrocyte membranes (Takkunen et al., 2013). Therefore, red blood cells are widely used in assessing dietary fat quality, as well as in validating dietary questionnaires. Considering the above, we argued that the concentrations of FA, tocopherols and T-Ch in erythrocytes were better biomarkers of dietary lipid quality compared to nutritional questionnaires.

In our current research, we have observed that the addition of FO to HFD-20 stimulated the removal of A-SFA, as well as C8:0 and C10:0 from erythrocytes. This findings were consistent with our previous results (Skiba at al., 2011; Krajewska-Bienias et al., 2017), showing that a diet rich in n-3PUFA, derived from linseed oil or FO, reduced the concentration of A-SFA in animal tissues. We were particularly interested in A-SFA, C8:0 and C10:0, since high levels of these SFA in diets has been shown to be closely associated with numerous disease conditions in humans and animals. On the other hand, the HFD-20 diet supplemented with FO had a beneficial effect on the concentration of tocopherols in erythrocytes. Indeed, tocopherols (mainly α-T) are present in FO, along with other vegetable oils (Ahmed et al., 2011). As expected, HFD-20 supplemented with FO (rich in EPA and DHA) stimulated the formation of MDA, i.e. the final product of PUFA peroxidation (Czauderna et al., 2011) in erythrocytes. Thus, our current study was in line with previous research in which dietary oils rich in PUFA (like FO or linseed oil) stimulated

the accumulation of MDA in animal tissues (Rozbicka-Wieczorek et al., 2014; Krajewska-Bienias et al., 2017). Highly unsaturated long-chain fatty acids (derived from dietary FO) are particularly susceptible to oxidation and produce potentially harmful oxidation products, such as lipid aldehydes, 4-hydroxy-2-alkenals and MD (Czauderna et al., 2011). Therefore, dietary FO (rich in EPA or DHA) significantly increased MDA concentrations and MDA_{index} values in mammalian tissues compared to diets without PUFA- or LPUFA-enriched supplements (Krajewska-Bienias et al., 2017). The increase in MDA concentration and MDA_{index} values was influenced by the duration of feeding with a diet supplemented with FO, as well as the levels of FO added to the diet. Based on these observations, we propose that erythrocytes can serve as reliable indicators for assessing the formation of MDA not only in erythrocytes themselves but also in other animal tissues.

Conclusions

The analysis of FA, particularly LPUFA, in erythrocytes requires the use of a modified gentle saponification method. Our study have demonstrated that the current improved saponification method, involving a brief treatment at 95 °C for 2 min, followed by a mild overnight saponification at 22–25 °C, and subsequent gentle base and acid-catalysed methylations of free FA, is a suitable analytical procedure for accurate and precise GC-MS analysis of highly unsaturated LPUFA, which are prone to peroxidation in erythrocytes. Short-term changes in dietary composition (like a FA profile) have a lesser impact on the chemical composition of erythrocytes compared to blood plasma. We propose that the impact of dietary FA on the profile of FA and tocopherols and MDA levels in erythrocytes and other animal tissues is similar. Notably, the DHA content in erythrocytes can serve as a marker for the bioaccumulation yield of DHA in other animal tissues, as well as reflect the levels of n-3LPUFA, particularly DHA, in the diets. Based on these findings, we suggest that the profiles of FA and tocopherols in erythrocytes can be employed as indicators of the FA and tocopherol content in the diet, as well as the effectiveness of their bioaccumulation in animal tissues. This highlights the potential utility of erythrocyte analysis in assessing dietary quality and the bioavailability of essential nutrients.

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Conflicts of interest

The Authors declare that there is no conflict of interest.

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