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Combined thin layer chromatography and gas chromatography with mass spectrometric analysis of lipid classes and fatty acids in malnourished polar bears *(Ursus maritimus)* which swam to Iceland

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ABSTRACT

Between 2008 and 2011, four polar bears (*Ursus maritimus*) from the Greenland population swam and/or drifted on ice to Iceland where they arrived in very poor body condition. Body fat resources in these animals were only between 0% and 10% of the body weight (usually 25%). Here we studied the lipid composition in different tissues (adipose tissue if available, liver, kidney and muscle). Lipid classes were determined by thin layer chromatography (TLC) and on-column gas chromatography with mass spectrometry (GC/MS). The fatty acid pattern of total lipids and free fatty acids was analyzed by GC/MS in selected ion monitoring (SIM) mode. Additionally, cholesteryl esters and native fatty acid methyl esters, initially detected as zones in thin layer chromatograms, were enriched by solid phase extraction and quantified by GC/MS. The ratio of free fatty acids to native fatty acid methyl esters could be correlated with the remained body lipids in the polar bears and thus may also serve as a marker for other starving animals or even for humans.

1. Introduction

Polar bears (*Ursus maritimus*) are top predators within the Arctic Circle, including the ice-covered seas and land masses [1–3]. Although most maternity denning areas are on land, the primary habitat of polar bears is annual sea ice, where they hunt on seals, their primary prey [1–5]. When no sea ice is present and food is out of reach, they are living from their fat reserves [3,4,6]. Polar bears are no native species in Iceland, but 50–60 individuals have reached the island in the 20th century, usually traveling on packed ice [1]. The last four polar bears (at the time of writing this article a fifth polar bear was shot in Iceland in July 2016) have been sighted in Iceland between 2008 and 2011 [1]. These four individuals, originated from the East-Greenland populations, have swum to Iceland most likely within a few days [1]. They arrived extremely malnourished in Iceland, with lipid contents of 10 wt-% or less of the body weight [1]. At least two of the polar bears almost for sure have left their natural habitat already malnourished in a season after late-winter hunting and therefore should have been in a much better body condition [1]. The arriving of these animals had been associated with climate change, resulting in shorter hunting periods [1,2]. Earlier sea-ice breakup due to climate changes leading to less time for ringed seal consumption and a longer fasten-time on shore has been correlated with a decline in body fat, body size and reproduction rates of polar bears [2,7,8]. E.g. loss of 43% body mass (93% attributed to fat-decline) was observed in a female polar bear during prolonged fasting periods [9].

Because excesses of dietary fatty acids are stored (after some modifications) in adipose tissue, fatty acid profiles have been correlated with the polar bearís diet and prey species [3,6,10]. Additionally, differences in polar bear fat composition of free-ranging to captive animals was also mainly influenced by the feed [6,10]. Due to modifications of the fat before storage, Grahl-Nielsen et al. could not correlate the fatty acid pattern of different seal species and polar bear adipose tissue [4]. Nonetheless, body condition and

Abbreviations: 15:0-CE, pentadecanoic acid cholesteryl ester; *a*FA, *anteiso*-fatty acid; BSTFA, N,O-bis(trimethylsilyl)-trifluoroacetamide; GC/MS, gas chromatography with mass spectrometry; FAME, fatty acid methyl ester; FOV-MAE, focused open-vessel microwave-assisted extraction; FFA, free fatty acid; *i*FA, *iso*-fatty acid; LOD, limit of detection; nFAME, native fatty acid methyl ester; on-column GC/MS, gas chromatography with mass spectrometry with on-column injection; R_f, retention factor; SIM, selected ion monitoring; SPE, solid-phase extraction; TAG, triacylglycerol; TMCS, trimethylchlorosilane.

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diet seem to influence the fatty acids of polar bears adipose tissue [3,5–7,10].

The aim of this study therefore was to determine the lipid classes and fatty acid pattern of different organs and adipose tissue, which may differ to the normal pattern of polar bears due to the strong decrease of fat reservoirs of the starved polar bears. Both thin layer chromatography and gas chromatography with mass spectrometry were used for this purpose.

2. Materials and methods

2.1. Chemicals and standards

A Supelco 37 component fatty acid methyl ester (FAME) mix, petroleum ether (distilled prior use), tin (II) chloride dihydrate (>97% pure, Riedel-de Haën) and silica gel 60 were ordered from Sigma-Aldrich (Taufkirchen, Germany). Standards of *anteiso*-FAs (*a*FAs) (*a*13:0, *a*14:0, *a*15:0, *a*16:0, *a*17:0, *a*18:0, *a*19:0) and isoFAs (*i*FAs) (*i*14:0, *i*15:0, *i*16:0, *i*17:0, *i*18:0) were ordered from Larodan (Malmö, Sweden). Tripalmitin (95% pure), tristearin (>99% pure), triolein (>99% pure), hexanoic (caproic) acid methyl ester and *9Z,12Z,15Z*-octadecatrienoic (α-linolenic) acid methyl ester, squalene (all three purest), diethyl ether (>99.8% pure) and pyridine (>99.8% pure, distilled prior to use) were from Fluka (Buchs, Switzerland) while α-cholestane (min 98% pure) and 2′,7′-dichlorofluorescein were from Acros Organics (Geel, Belgium). Tetradecanoic (myristic) acid (>99% pure), hexadecanoic (palmitic) acid (>99% pure), *9Z*-octadecenoic (oleic) acid (purest), tetradecanoic (myristic) acid methyl ester (>99.5% pure), hexadecanoic (palmitic) acid methyl ester (>99% pure), *9Z*-octadecenoic (oleic) acid methyl ester (>96% pure), glycerol (>99% pure), acetic acid (purest), and cholesterol (purest) were from Merck (Darmstadt, Germany). Soy lecithin (>62%) was from Stern Lecithin und Soja (Hamburg, Germany). Ammonium heptamolybdate tetrahydrate was ordered from VWR chemicals (Leuven, Belgium). Tetradecanoic (myristic) acid ethyl ester and 10,11-dichloroundecanoic acid were synthesized by Thurnhofer et al.[11]. Pentadecanoic acid cholesteryl ester (15:0-CE) was obtained from Hammann et al.[12]. Ethyl acetate, methanol and cyclohexane (all purest) were from Th. Geyer (Renningen, Germany). Chloroform (>99.8% pure) was from Serva Feinbiochemica (Heidelberg, Germany). Ethanol, tricaprylin (>97% pure), NaCl (99.8%) and sulfuric acid (Rotipuran, 98%) were from Carl Roth (Karlsruhe, Germany). The silylating agent BSTFA/TMCS (N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS), 99:1 (v/v)) was from Supelco (Bellefonte, PA, USA). Helium (5.0 quality) was from Westfalen (Münster, Germany).

2.2. Standard mixtures and solutions

The following six lipid class standards were prepared in *n*-hexane (c ~4 mg/mL): (i) triacylglycerol standard (tricaprylin, tripalmitin, tristearin and triolein), (ii) free fatty acids (myristic acid, palmitic acid and oleic acid), (iii) fatty acid methyl esters (myristic acid methyl ester, palmitic acid methyl ester, oleic acid methyl ester, caproic acid methyl ester and α-linolenic acid methyl ester), (iv) sterols (cholesterol), (v) phospholipids (soy lecithin) as well as (vi) hydrocarbons (squalene). For lipid class quantification, the six lipid class standards in *n*-hexane were combined and diluted/concentrated to the following concentrations: c ~8, 4, 2.8, 2, 1.6, 1, 0.5 and 0.12 mg lipid class/mL *n*-hexane. The external standard for cholesteryl ester quantification (15:0-CE), obtained from Hammann et al.[12], was dissolved to gain concentrations of 26, 20, 15, 10, 5 and 1 ng/μL *n*-hexane.

Table 1
Information and overview over the polar bears, whose lipids of different organs and adipose tissue were analyzed in this study [1].

	Bear 1	Bear 2	Bear 3	Bear 4
Gender	Female	Male	Female	Female
Age [years]	14.5	22.5	4.5	3.5
Body mass [kg]	142	220	138	95
Fat in relation to body mass [%]	no fat left	10	6	5
Body length [cm]	194	209	173	193
Arrival in Iceland	June 2008	June 2008	Jan 2010	May 2011

2.3. Samples

Four polar bears (*Ursus maritimus*) swam malnourished in 2008, 2010, and 2011 for unknown reasons to Iceland and were shot soon after arrival [1]. Age, gender, body mass and length, fat reservoir (Table 1) and potential reasons for their migration to Iceland and their bad condition were summarized by Vetter et al. [1]. Samples of liver, muscle, kidney and subcutaneous and (if present) mesenteric fat were dissected at the Institute for Experimental Pathology, Reykjavík, Iceland, wrapped in aluminum foil and kept frozen at −18 °C until analysis [1].

2.4. Sample preparation

Lipid extracts of lyophilized samples were previously gained by focused open-vessel microwave-assisted extraction (FOV-MAE) [1]. In brief, samples were lyophilized for at least 24 h (Lyovac GT2, Leybold-Heraeus, Cologne, Germany) and aliquots were extracted with 90 mL cyclohexane/ethyl acetate (46:54, w/w) in a modified Star 2 system (CEM, Kamp-Lintfort, Germany) [1]. The FOV-MAE system, equipped with a water trap and a refluxer, was programmed with a ramp heating within 10 min to 88 °C (held 10 min), heating within 5 min to 95 °C (held 30 min). The filtered extracts were concentrated by rotary evaporation (volume less than 5 mL), transferred into calibrated flasks and adjusted to a volume of exactly 5 mL [1]. One milliliter of the extracts was subjected to gravimetric lipid quantification. Delays in sampling and storage of lipids from mammals may influence the fatty acid profile [3]. To minimize such artefacts, samples of adipose tissue as well as organs were taken from inner parts if any possible. Samples were stored frozen until usage since adipocytes reportedly remained unchanged at −15 °C for months and cells remained intact when thawed [3,7]. Additionally, triacylglycerols, stored in large droplets, are – except at the edges – not accessible for enzymes [3].

2.5. Separation and quantification of lipid classes by thin layer chromatography (TLC)

Lipid classes were separated and qualified by thin layer chromatography (TLC) using the method of Malins and Mangold [13–15]. One microliter of standard solutions (lipid classes: c ~4 mg/mL) and samples (c ~20–30 mg/mL in *n*-hexane) were sprayed in form of zones onto glass plates (20 × 10 cm or 10 × 10 cm in size) coated with silica 60 (TLC silica gel 60, F254, MS-grade, Merck, Darmstadt, Germany) by means of a Linomat 5 (Camag, Berlin, Germany). TLC plates were developed about 8 cm with petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v) in chambers saturated with the solvent [13,14]. Lipid classes were detected with a TLC Visualizer (Camag, Berlin, Germany) after derivatization with 10% sulfuric methanol (immersion device, Camag, Berlin, Germany) and heating the plate to 150 °C with a TLC plate heater (Camag, Berlin, Germany) [15]. Densitometric quantification of spots on the TLC plates was performed with a TLC scanner (Camag, Berlin, Germany) at 530 nm. Zones of lipid classes were identified