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Investigation of the triacylglycerol composition of iceman's mummified tissue by high-temperature gas chromatography

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Abstract

The pattern of intact triacylglycerols of a skin sample from the 5300-year-old Iceman mummy (nicknamed Ötzi) was resolved on a diphenyl–dimethylpolysiloxane stationary phase by high-temperature gas chromatography. Adipocere from a 64-year-old glacier mummy as well as recent human subcutaneous fat served as a comparison in this study. Qualitatively, the results for mummy samples were similar with well-preserved saturated, but decomposed unsaturated, triacylglycerols, the latter being predominant in subcutaneous fat. Excellent preservation of triacylglycerols with odd carbon numbers and branched acyl chains was observed. The results presented here shed new light on the process of mummification.

Keywords: Mummified tissue; Iceman; Triacylglycerol; Fat, subcutaneous

1. Introduction

The mummified corpse of Iceman (Similaun Man, Man from Hauslabjoch) was discovered in September 1991 at 3210 m above sea level in a 20×6 m chamber-like depression, where it lay below a rocky ledge, sheltered from the shearing flow of glacial ice [1]. So trapped, the corpse was not expelled with the regular glacial turnover. Facilitating the corpse's preservation were the location, glaciological conditions and estimated time of death (between the end of summer and early autumn) as well as the circumstances leading to its discovery (the corpse was estimated to have emerged from the ice three days beforehand [1]). Calibrated radiocarbon dating of the body and artifacts specified his lifetime to between 3350 and 3100 BC [2]. This particularly well-preserved Late Neolithic 46-year-old male mummy [3], found together with its belongings provided a unique opportunity for inter-disciplinary research [4–6].

Transformation of body fat during mummification into its partial decomposition products has previously been investigated chromatographically and spectroscopically [7,8]. Natural mummification by adipocere build-up is normally associated with extensive glacial submersion of bodies [9]. Adipocere is a result of microbial activity converting body fat into a lipid mixture of grayish–white color and consists mainly of saturated free fatty acids with even-numbered carbon atoms such as myristic acid, the predominant palmitic acid, stearic acid and 10-hydroxystearic acid [10]. Calcium salts of certain fatty acids may also be found in adipocere [11]. The distribution of lipids

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following mummification by desiccation is different, with triacylglycerols and unsaturated fatty acids, like oleic acid, being more dominant. Mummification (especially in glacial ice) may result from a combination of the two transformation processes, with superficial desiccated skin often containing underlying adipocere)detectable using gas chromatography (GC) [8]).

We had previously characterized a sample of Iceman's skin using light microscopy, histology and attenuated total reflection infra-red (IR) spectroscopy [12] and reported that his mummification was due to adipocere formation. This finding was further supported by analysis of the free fatty acids by GC–MS [13]. The aforementioned IR technique had demonstrated the general preservation of triacylglycerols, but failed to provide detailed information regarding individual species. We monitored the fate of triacylglycerols within the mummification process, since the post-mortem action of enzymes on triacylglycerols is not well understood. The literature on adipocere is restricted to the consequences of mummification for free fatty acids [10,14].

In this study, we present new high-temperature GC results concerning the triacylglycerol composition of Iceman's skin and compare these to data obtained from a glacier-retrieved mummy with evident adipocere, as well as to fresh human subcutaneous fat. Based on our findings, we addressed some of the questions surrounding the mummification process that resulted in the excellent preservation of Iceman's corpse.

2. Experimental

2.1. Chemicals

Fused-silica capillaries (15 m×0.32 mm) with a high-temperature polyimide coating were obtained from Polymicro Technologies (Phoenix, AZ, USA). The high-temperature stable stationary phase, SOP-50, a symmetrically substituted and methoxy-terminated poly(50% dimethyl, 50% diphenyl)siloxane was provided by R. Aichholz (Novartis, Basel, Switzerland). Hydrogenation was catalyzed by palladium (10% Pd on activated carbon) obtained from Merck (Darmstadt, Germany).

The derivatizing reagent N,O-bis-(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+1% TMCS, for GC) was obtained from Fluka (Buchs, Switzerland). Ethereal diazomethane was prepared from N-nitroso-N-methylurea; both were synthesized according to the literature [15].

Nomenclature [16] of the reference substances: Fatty acids: Myristic acid (C14:0)=M, palmitic acid (C16:0)=P, palmitoleic acid (C16:1)=Po, margaric acid (C17:0)=Mg, stearic acid (C18:0)=S, oleic acid (C18:1)=O, linoleic acid (C18:2)=L. Triacylglycerols: e.g., Tripalmitin=PPP, stearo–linoleo–stearin=SLS. The carbon number (CN) is the number of carbon atoms in the fatty acid moieties of the triacylglycerol, e.g., the CN of SSS and of SOO is 54.

The reference substances MMM, SSM, PPP, MgMgMg, PSS, POO, SSS, SOS, SOO, OOO and LLL (>98%) were obtained from Sigma-Aldrich (Vienna, Austria).

2.2. Samples

Recent subcutaneous fat was taken from the hip region of a 50-year-old male 3 h following his death as a result of cardiac arrest. The adipocere sample was taken at the lower arm from the corpse of a 35-year-old man found in a glacier (Stubai Alps, Tyrol, Austria), 64 years after his disappearance. The skin sample of the Iceman was from his left hip. With the epidermis lost, the outer side of the sample appeared leathery, whereas the inner side contained traces of subcutaneous fat.

2.3. Sample preparation

Extraction procedure: About 0.5 mg of each sample was extracted using *n*-hexane (200 μl) with sonication (3 min), shaking (1 min) and agitation (10 min).

Derivatization procedure: In order to reduce degradation of triacylglycerols eluting at higher temperatures and to prolong the lifetime of the capillary, free fatty acids (some of which are hydroxy fatty acids) were derivatized [17]. The extract was dried under a flow of nitrogen and the carboxyl groups were methylated at room temperature with ethereal diazo-

methane (15 min reaction time) [18]. The solution was then dried under a flow of nitrogen and the hydroxyl groups were silylated at 60°C in 100 μl of pyridine with 50 μl of BSTFA containing 1% TMCS (20 min reaction time). The solution was subsequently dried under a flow of nitrogen and finally dissolved in 20 μl of *n*-hexane prior to analysis by GC.

Hydrogenation: The solution of derivatized lipids was diluted with 500 μl of *n*-hexane and 0.5 mg of palladium–carbon was added. Hydrogen was introduced using a Pasteur pipette at 25°C under agitation and the flow was adjusted to 65 bubbles per minute (60 min reaction time). The catalyst was removed by centrifugation, the solution was evaporated to dryness under a flow of nitrogen and the lipids were dissolved in 20 μl of *n*-hexane prior to GC analysis.

2.4. Gas chromatography

All analyses were performed on a Carlo Erba SFE gas chromatograph 3060 equipped with a cold on-column injector, a flame ionization detector (FID), with a ceramic flame jet, and a constant pressure-constant flow module 516 (Fisons, Rodano, Italy). The fused-silica capillaries were statically coated with the stationary phase, SOP-50 (0.15 μm film thickness) [19]. A 1-μl sample was injected manually with a 10-μl syringe. Data acquisition was done using a PE Nelson Interface 900 Series (Perkin-Elmer, Cupertino, CA, USA). Hydrogen (>99.999%) was used as the carrier gas at a constant flow-rate of 1.65 ml/min. The temperature program used was as follows: from 60 to 280°C at 20 C°/min and from 280 to 380°C at 4 C°/min, while the FID was maintained at 400°C.

3. Results and discussion

3.1. Subcutaneous fat

The complex mixture of human subcutaneous fat was examined by high-temperature GC on the stationary phase, SOP-50, a dimethyl–diphenylsiloxane copolymer. The resolution of the sample into the 37 peaks seen in Fig. 1 was based not only on the different carbon numbers but was also due to the different polarities of the triacylglycerols.

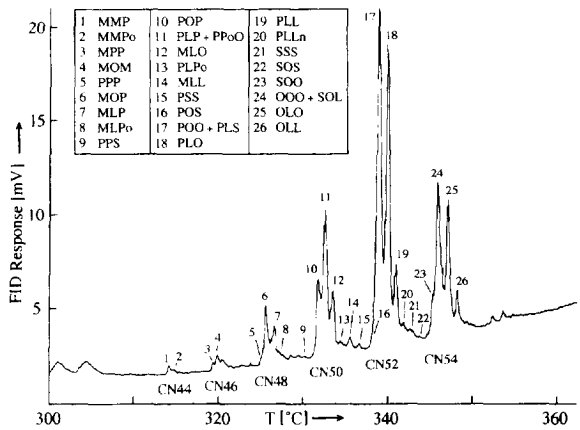

Fig. 1. High-temperature GC separation of recent human subcutaneous fat.

The peak identities were established on the basis of the following criteria: The relative retention times of reference standards showed stronger retardation for the triacylglycerols with a higher degree of unsaturation; re-chromatography of the hydrogenated samples revealed the carbon numbers; co-chromatography with reference standards; re-chromatography after lipase-catalyzed hydrolysis [18] unveiled the fatty acid constitution; predominant *sn*-2 position of the highest unsaturated fatty acids in the triacylglycerols of human subcutaneous fat [20]. Neither the on-column injection [21], nor the highly inert capillary [22] and the FID-detector [23] significantly discriminated between the investigated triacylglycerols. Therefore, we assumed for the quantification within this comparative study that the individual response factors of the triacylglycerols were nearly equal.

The quantitative constitution of subcutaneous fat from an individual is not only genetically determined but also depends on diet [24], age [25] and gender [26]. We identified 26 of the signals obtained for the sample investigated (Fig. 1). The main triacylglycerol was POO, followed by PLO and OOO, which together make up 60% of the total. About 92% of the triacylglycerols had more than one double bond, 7% had a single double bond and only 1% was saturated. Our results correlated well with those of Ruiz-Gutierrez et al. [20], allowing this sample to serve as a suitable reference for subsequent comparative studies using mummified samples.

3.2. Adipocere

We investigated the lipids in adipocere samples from several mummies retrieved from glaciers. Free fatty acids were predominant in all cases, but the proportion of triacylglycerols varied from mummy to mummy. The conditions of adipocere formation in each of these cases determined the extent to which triacylglycerols had decomposed.

The pattern of triacylglycerols obtained from the adipocere sample from the 64-year-old mummy (presented in Fig. 2) was compared with that of subcutaneous fat (Fig. 1). In addition to a considerable reduction in the number of peaks, we observed a shift of the maximal distribution of the triacylglycerols from carbon number 52 (subcutaneous fat) to 48 (adipocere sample). The highest carbon number of the triacylglycerols in both cases was 54.

Proportionally, 21% of the triacylglycerols were unsaturated (MOP, POP, POS and SOS) and 70% were saturated, the remaining 9% requiring further investigation (see below). All triacylglycerols with more than one double bond were completely decomposed.

Hydrogenation showed that the small peaks between the main ones in Fig. 2 were essentially saturated triacylglycerols, constituting up to 9% of the total triacylglycerols. The characteristic pattern of the hydrogenated sample was nearly identical to that obtained for hydrogenated butter fat by Geeraert and Sandra [16]. As in butter fat [27] and in human milk [28], these triacylglycerols contained fatty acids with an odd number of carbon atoms as well as with branched alkyl chains. We found also two triacylglycerols with odd-numbered and unsaturated fatty acids.

3.3. Iceman

As the Iceman sample was dominated by dermis and contained only parts of the former subcutaneous fat, the overall lipid concentration was lower than that of the more recent mummy sample described above. Nevertheless, the pattern of triacylglycerols obtained for the skin sample of the Iceman (Fig. 3) was almost identical to that of the adipocere sample in Fig. 2.

In Table 1, we compared the decomposed and preserved triacylglycerols. Those containing unsaturated fatty acids (essentially oleic acid) were mostly degraded and represented only 5% of the total triacylglycerol content, although in recent subcutaneous fat, 99% have been unsaturated. About 79% of the triacylglycerols preserved in the sample of the Iceman had saturated fatty acids with even-numbered carbon atoms. The remaining 16% were triacylglycerols containing fatty acids with odd-numbered carbon atoms as well as with branched alkyl chains. As observed for the adipocere sample, all triacylglycerols with more than one double bond complete-

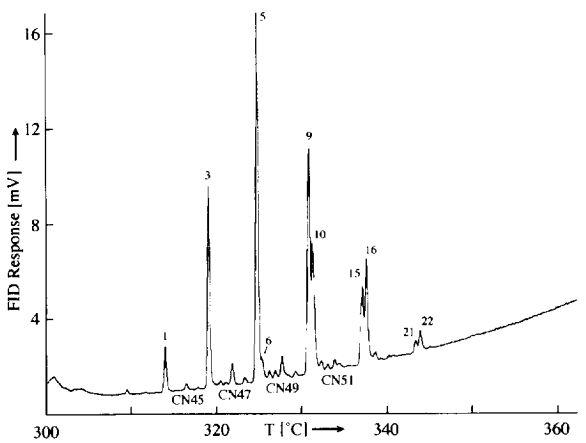

Fig. 2. High-temperature GC separation of triacylglycerols of adipocere from a 64-year-old mummy. The peak assignment is the same as in Fig. 1.

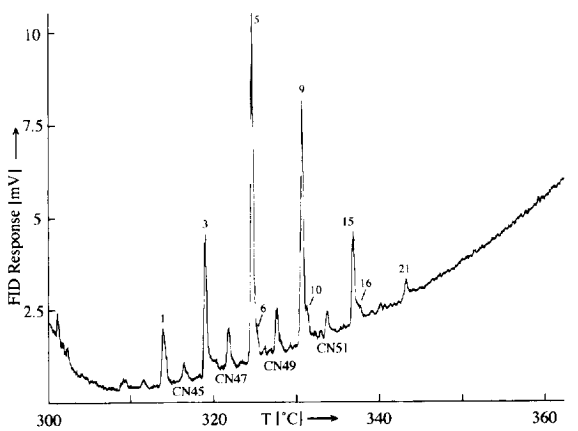

Fig. 3. High-temperature GC separation of triacylglycerols of adipocere from the 5300-year-old Iceman mummy. The assignment is the same as in Fig. 1.

Table 1
Preserved and decomposed triacylglycerols (TAG) with the number of double bonds (Δ)

Preserved			Decomposed		
Number	TAG	Δ	Number	TAG	Δ
1	MMP	0	11	PLP/PPO	2
3	MPP	0	12	MLO	3
5	PPP	0	17	POO	2
6	*MOP*	1	18	PLO	3
9	PPS	0	19	PLL	4
10	*POP*	1	23	SOO	2
15	PSS	0	24	OOO/SOL	3
16	*POS*	1	25	OLO	4
21	SSS	0	26	OLL	5
22	*SOS*[a]	1			

Italicised TAGs were preserved in lower concentrations and have oleic acid in position 2.
[a]SOS was only preserved in the adipocere sample.

ly decomposed. This corresponds to our previous findings [13], obtained when studying decomposition of triacylglycerols during adipocere formation under laboratory conditions, where we observed that oleic acid had been released at a high rate in an early stage of adipocere formation. All unsaturated triacylglycerols preserved in the samples of adipocere and of the Iceman contained oleic acid at position 2, but only saturated fatty acids at positions 1 and 3 (Table 1).

The specificity of human lipase to 1,3-positions and its preference for unsaturated fatty acids has been described previously [29,30] and Mant and Furbank [31] had earlier assumed that lipase-catalyzed hydrolysis occurred during adipocere formation. This specificity could explain why triacylglycerols with saturated fatty acids in the 1,3-position, such as MOP, POP and POS (SOS was only preserved in the adipocere sample described above), were more resistant to lipase-catalyzed hydrolysis than their unsaturated counterparts, such as POO or OOO. Being stable enzymes with post-mortem activity, lipases [32] could further explain the differences in the relative amounts of triacylglycerols preserved in the mummy samples. The Iceman was mummified under more favorable conditions than the other mummy, resulting in a prolonged activity of the lipases.

Based on the data presented here, we propose that, during mummification, triacylglycerols decompose as follows: Saturated species survive better than unsaturated ones, those with odd carbon numbers or branched acyl chains persist longer than others and all triacylglycerols with more than one double bond are completely degraded.

Acknowledgments

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