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Analysis

Improved Bligh and Dyer extraction procedure

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Summary

Fatty acids are specific compounds with well known chemical composition, but they exist in a great variety of molecules which differ in chemical and physical characteristics. Quantitative extraction of fatty acids is therefore a challenge. An acidic Bligh and Dyer method has been developed and compared with the traditional Stoldt fat extraction method. It is shown that depending on the matrix, the HCl-Bligh and Dyer extraction leads to 10–15% more total fatty acids and a 30–50% increase in polyunsaturated fatty acids compared with the official EU method. Besides being much more efficient for extraction of fatty acids, the HCl-Bligh and Dyer method is faster, and allows the inclusion of internal standard at the beginning of the sample treatment.

Introduction

Lipids have been simply defined as molecules soluble in an organic solvent. This broad and non-specific definition implies that the amount of lipids and the composition of lipids in the same source may differ with the way the sample is treated and the solvent used for extraction. Although fatty acids (FA) are specific compounds with well known chemical composition, they exist in a great variety of molecules which differ in chemical and physical characteristics. This implies a challenge with respect to the optimal pre-treatment and extraction procedure applied for quantitative extraction of the FA from different matrices.

A widely used method for lipid extraction and FA analysis in feed is the Stoldt fat method based on boiling the sample in 3M HCl followed by filtration, drying and finally Soxhlet extraction of the fat with petrol ether [1]. In the official EU method for the determination of fat and FA in feed, the isolated fat from this method is further used for quantitative determination of the FA present after conversion of the FA to their corresponding methyl esters followed by gas chromatography (GC) separation. However, this method is very time consuming and has several disadvantages, *e.g.* the filtration step may allow shorter, chain-free FA and phospholipids to be washed through the filter and escape the analysis. Further, during the drying step, oxidation of polyunsaturated fatty acids (PUFA) is a risk. Moreover, polar lipid compounds may escape extraction with lipophilic extraction solvents such as petrol ether or diethyl ether, while a more polar extraction solvent such as chloroform will extract the polar lipid compounds such as free FA and phospholipids as well.

The Bligh and Dyer extraction (water-methanol-chloroform) [2] has for many years been known as an efficient method for the extraction of triacylglycerols, free FA and phospholipids. However, for liberation of calcium soaps an acid treatment is very efficient, as known from the Stoldt fat method. It has previously been reported that the total amount of fat in feed is higher when extracted with Bligh and Dyer compared to the traditional Stoldt method [3], but the effect on the amount of FA has not been investigated.

The solution for this dilemma has been a combination of the two methods in such a way, that an acidic treatment has been introduced to the Bligh and Dyer method prior to the extraction procedure. In the present paper the quantitative amount of FA obtained by this "HCl-Bligh and Dyer extraction" method is compared with the traditional Stoldt fat extraction. A further advantage of the HCl-Bligh and Dyer extraction is that addition of internal standard occurs already with the addition of the first chloroform to the sample and not just to the extracted fat residue from the Stoldt fat extraction.

Experimental

The HCl-Bligh and Dyer method procedure has been tested with pig faeces rich in calcium soaps and herring/mackerel scrap rich in PUFA. Homogenate (0.6 g) was weighed out into a 10-ml-culture tube with screw cap, 1 ml of 3M HCl added and the sample heated for 1 h at 80°C in a water bath. Last, 1.50 ml methanol and 1.00 ml chloroform with 17:0 as internal standard were added. This mixture was shaken for 1 min. Afterwards 1.00 ml ELGA-purified water and 2.00 ml chloroform were added, the tube shaken for 1 min, and then centrifuged for 5 min at 3000 rpm. One 1 ml of the chloroform phase was taken for methylation. The chloroform was evaporated under a stream of nitrogen and 0.8 ml of NaOH/methanol solution were added. The tube was then filledwith nitrogen, sealed and placeed in an oven for 15 min at 100°C. After cooling 1 ml BF_3 solution was added and the tube filled with nitrogen again. The sample was then placed in an oven for 45 min at 100°C. This time after cooling the sample 2 ml heptane and 4 ml saturated NaCl solution were added, the tubes shaken on a vortex mixer, and centrifuged at 3000 rpm for 10 min. For GC 1 µl of the heptane phase was used.

The FA methyl esters were analyzed by gas chromatography (HP 6890series GC system, Agilent Tecnologies, Palo Alto, USA) equipped with an automatic on-column injector (HP 7673) (Split ratio 4.325:1); a capillary column of 30 m × 320 µm inner diameter; 0.25 µm film thickness (Omegawax; Supelco 4-293-415), and a flame ionization detector. In total 8 mackerel and herring scrap from the filleting industry and 72 faecal samples from pigs were analysed by both methods. All FA were quantified on the same GC system.

Table 1. Comparison of the fatty acid (FA) content in herring and mackerel scrap, g/100 g sample depending on extraction method (n = 8).

FA, g/100 g sample	Stoldt	Bligh and Dyer	Relative value Stoldt = 100	*p*-Value *t*-test
14:0	1.37	1.39	101	0.37
16:0	2.51	2.53	101	0.41
18:1ω9	1.93	1.96	102	0.33
18:2ω6	0.327	0.343	105	0.17
18:3ω3	0.292	0.322	110	0.06
18:4ω6	0.671	0.793	118	0.08
20:1ω9	2.00	2.03	102	0.30
22:1ω9	3.16	3.21	102	0.59
20:5ω3	0.92	1.14	124	0.06
22:5ω3	0.165	0.203	123	0.07
22:6ω3	1.66	2.21	133	0.06
Sum FA	15.0	16.1	108	0.12

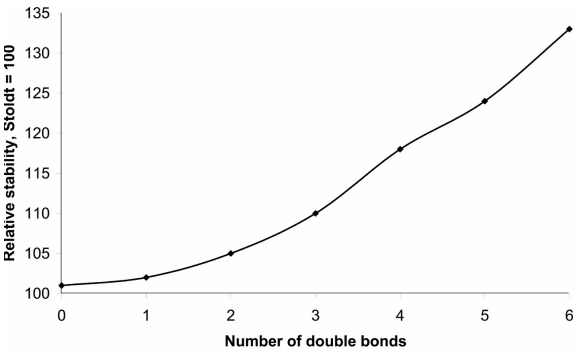

Figure 1. Relationship with the number of double bonds in the FA and the amount extracted by the HCl-Bligh & Dyer method compared to the Stoldt fat method.

Results

In mackerel and herring scrap, the lipid is easy to liberate and extract. The content of saturated and monounsaturated FA were the same by both methods, whereas the HCL-Bligh and Dyer method shows its superiority with increasing unsaturation of the FA (Table 1). As seen in Fig. 1 the efficiency of the HCl-Bligh and Dyer method increase progressively with increasing numbers of double bonds in the FA compared to the Stoldt fat method (Table 1).

Table 2. Comparison of the fat and fatty acid (FA) content in pig faeces depending on the extraction method, g/100 g sample (n = 72).

FA, g/100 g sample	Stoldt	Bligh and Dyer	Relative value Stoldt = 100	*p*-Value *t*-test
16:0	3.20	4.04	126	<0.001
18:0	2.56	2.68	104	0.20
18:1ω9	1.02	1.20	118	0.003
18:2ω6	0.25	0.32	126	<0.001
18:3ω3	0.029	0.045	157	<0.001
Sum FA	7.87	9.26	118	<0.001
Fat content	12.8	15.0	117	<0.001
FA in% of fat	61.2	63.1	103	0.22

In faecal samples part of the FA is located as free FA, some of which are tightly bound to calcium and thus difficult to liberate and extract. Apart from 18:0 all analysed FA were more efficiently extracted with the HCl-Bligh and Dyer method (Table 2), and here also the PUFA (18:2 and 18:3) showed the greatest difference.

Conclusion

The HCl-Bligh & Dyer method combines the HCl treatment of the sample with a Bligh and Dyer extraction of the lipid. Depending on the matrix, the HCl-Bligh and Dyer extraction lead to 10–15% more total FA and a 30–50% increase in some of the PUFA compared with the official EU method. Besides being more efficient for extraction of FA, the HCl-Bligh and Dyer method is faster, and allows inclusion of an internal standard from the beginning of the sample treatment.

References

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