Z Ernährungswiss 35: 226–234 (1996)

ORIGINAL CONTRIBUTION

G. Kohn
P. van der Ploeg
M. Möbius
G. Sawatzki

Influence of the derivatization procedure on the results of the gaschromatographic fatty acid analysis of human milk and infant formulae

Der Einfluß verschiedener Derivatisierungsverfahren auf die Ergebnisse der gaschromatographischen Fettsäureanalyse von Frauenmilch und Säuglingsmilchnahrungen

Summary Many different analytical procedures for fatty acid analysis of infant formulae and human milk are described. The objective was to study possible pitfalls in the use of different acid-catalyzed procedures compared to a base-catalyzed procedure based on sodium-methoxide in methanol. The influence of the different methods on the relative fatty acid composition (wt% of total fatty acids) and the total fatty acid recovery rate (expressed as % of total lipids) was studied in two experimental LCP-containing formulae and a human milk sample. MeOH/HCl-procedures were found to result in an incomplete transesterification of triglycerides, if an additional unpolar solvent like toluene or hexane is not added and a water-free preparation is not guaranteed. In infant formulae the low transesterification of triglycerides (up to only 37 %) could result in an 100 %-overestimation of the relative amount of LCP, if these fatty acids primarily derive from phospholipids. This is the case in infant formulae containing egg lipids as raw materials. In formula containing fish oils and in human milk the efficacy of esterification results in incorrect absolute amounts of fatty acids, but has no remarkable effect on the relative fatty acid distribution. This is due to the fact that in these samples LCP are primarily bound to triglycerides. Furthermore, in formulae based on butterfat the derivatization procedure should be designed in such a way that losses of short-chain fatty acids due to evaporation steps can be avoided. The procedure based on sodium methoxide was found to result in a satisfactory (about 90 %) conversion of formula lipids and a reliable content of all individual fatty acids. Due to a possibly high amount of free fatty acids in human milk, which are not methylated by sodium-methoxide, caution is expressed about the use of this reagent for fatty acid analysis of mothers milk.

It is concluded that accurate fatty acid analysis of infant formulae and human milk requires a careful and quantitative derivatization of both polar and unpolar lipid classes. Sodium methoxide seems to be a reliable and time-saving method for routine fatty acid analysis of infant formulae, which should be validated by interlaboratory comparison. Anhydrous procedures based on methanolic hydrogen chloride including an additional unpolar solvent are also suitable for infant formulae but seem to be preferable for human milk samples.

Zusammenfassung Zur gaschromatographischen Bestimmung des Fettsäuremusters von Säuglingsnahrungen und von Humanmilch werden zahlreiche unterschiedliche Derivatisierungsverfahren benutzt. Potentielle Fehlerquellen unterschiedlicher säurekatalysierter Verfahren sollten mit denen eines basischen Verfahrens auf der Grundlage von Natriummethylat in Methanol verglichen werden. Untersucht wurde die relative Fettsäurezusammensetzung (in Gew.% der Gesamtfettsäuren) von zwei experimentellen Säuglingsmilchnahrungen, die langkettig-hochungesättigte Fettsäuren (LCP) in Form von Eilipiden bzw. Fischölen enthielten und von einer Frauenmilchprobe. Zusätzlich wurde die Wiederfindungsrate der Gesamtfettsäuren bezogen auf die Lipidein-

Received: 6 June 1995
Accepted: 21 May 1996

Dr. G. Kohn (✉) · P. van der Ploeg
M. Möbius · G. Sawatzki
Milupa GmbH & Co KG
Research Department
Bahnstraße 14–30
61381 Friedrichsdorf/Ts.

waage bestimmt. Es konnte gezeigt werden, daß die Derivatisierung mit methanolischer Salzsäure in einer unvollständigen Transesterifizierung der Triglyceride resultiert, wenn wasserfreie Reaktionsbedingungen und der Zusatz eines unpolaren Lösungsvermittlers (Toluol/Hexan) nicht gewährleistet waren. In einer Säuglingsmilchnahrung, die Eilipide als Rohstoff enthielt, resultierte die nur bis zu 37 %ige Umesterung der Triglyceride in bis zu 100 %-überhöhten relativen Anteilen der LCP-Fettsäuren, da diese sich in erster Linie aus den polaren Phospholipiden ableiten. In Frauenmilch und in der auf Fischöl basierenden Säuglingsmilchnahrung hatte die Effizienz der Transesterifizierung der Triglyceride nur einen geringfügigen Einfluß auf die relativen LCP-Gehalte, da die LCP hier überwiegend in Form der Triglyceride vorliegen. Bei der Fettsäureanalyse von Formelnahrungen die Butterfett enthalten, ist es weiterhin notwendig, das Derivatisierungsverfahren derart durchzuführen, daß der Verlust flüchtiger, kurzkettiger Fettsäuren vermieden wird.

Die schnelle und einfache Derivatisierung mit Natriummethylat führte zu zufriedenstellenden Umesterungsraten der Gesamtlipide und zu zuverlässigen Fettsäuremustern von Säuglingsmilchnahrungen. Es wird daher empfohlen, diese Methode in einem Ringversuch auf die Eignung als Routinemethode zu überprüfen. Da freie Fettsäuren mit der Natriummethylatmethode nicht derivatisiert werden, sind bei der Analyse von Frauenmilchproben säurekatalysierte Verfahren auf Basis methanolischer Salzsäure vorzuziehen. Derartige Verfahren sind auch für Säuglingsmilchnahrungen geeignet, wobei allerdings auf wasserfreie Reaktionsbedingungen und auf den Zusatz eines unpolaren Lösungsvermittlers zu achten ist.

Key words Derivatization – fatty acid methyl ester (FAME) – gas chromatography – human milk – infant formulae

Schlüsselwörter Derivatisierung – Fettsäuremethylester – Gaschromatographie – Humanmilch – Säuglingsmilchnahrung

Abbreviations *AA* = arachidonic acid (20:4 n-6) · *BF_3*/MeOH = boron trifluoride in methanol · *DHA* = docosahexaenoic acid (22:6 n-3) · *F* = infant formulae · *FAME* = fatty acid methyl ester *HM* = human milk · *LCP* = long-chain polyunsaturated fatty acids (C20/C22) · *MeOH/HCl* = methanolic hydrogen chloride · *Na-methoxide/MeOH* = sodium methoxide in methanol

Introduction

During recent years international research has focused on long-chain polyunsaturated fatty acids with 20 and 22 carbon atoms (LCP), because of their importance for functional characteristics of cellular membranes and their role as mother substances of eicosanoids. Evidence from animal studies and clinical trials has been accumulated that LCP like arachidonic and docosahexaenoic acid play an important role in the growth and functional development of the newborn infant (6, 8, 20, 28). Based on these results recently the ESPGAN-Committee on Nutrition (14), the British Nutrition Foundation (4) and the Commission of the European Community (11, 12) and the International Society for the Study of Fatty Acids and Lipids (18) have established recommendations concerning the content of LCP and other fatty acids in infant formulae.

In contrast to these detailed recommendations a generally accepted analytical method for the analysis of fatty acids in infant formulae and in human milk has not been established so far. Although gas chromatography is principally accepted as the method which most investigators use, still a lot of differences exist in the use of packed or capillary columns and especially in the use of different derivatization procedures. Recently proposed methods for quantitative determination of linoleic acid in infant formulae (1, 23) are still based on packed glass columns. But in view of the detailed recommendations on one hand and the very complex fatty acid composition of human milk and infant formulae on the other, most authors agree that modern fused-silica-capillary columns are the method of choice because of their superior resolution capacity (27).

Prior to capillary gas chromatography the total lipids extracted from human milk or infant formulae are usually converted to their corresponding fatty acid methyl ester (FAME) by acid- or base-catalyzed esterification. Methods based on boron trifluoride-methanol, methanolic hydrogen chloride and sodium methoxide are most frequently used in studies on human milk and infant formulae. The results of these fatty acid analyses are usually expressed as relative weight percentage of total fatty acids measured, although there is an increasing need also for absolute amounts of each fatty acid. The above-mentioned recommendations (ESPGAN, BNF, EC and ISSFAL) demand relative and absolute amounts (expressed as % of total fatty acids or % of energy or mg/l) concerning the content of LCP-fatty acids, erucic acid, linoleic and alpha-linolenic acid, lauric acid and trans-fatty acids in infant formulae. Furthermore, some authors have speculated that also in human milk absolute concentrations of fatty acids (mg/l) might be nutritionally more relevant than relative data (19).

In view of these recommendations, accurate data concerning the content of fatty acids in human milk and infant formula require a quantitative extraction of lipids

and an optimized derivatization procedure to form fatty acid methyl ester. Deficiencies in the derivatization step could directly influence the quality of the results. Problems during the esterification process are usually related to incomplete esterification of total lipids, an underestimation of short chain fatty acids, a loss of polyunsaturated fatty acids or the formation of artifacts (27). Especially in case of samples containing a complex composition of different lipid classes, care must be taken to guarantee that both polar (phospholipids) and unpolar lipids (triglycerides and cholesterol ester) are completely dissolved before transesterification will proceed (7). The objective of this study was to compare the influence of some most frequently cited derivatization procedures on the results of the gaschromatographic fatty acid analysis of human milk and infant formulae containing LCP. Acid-catalyzed procedures based on methanolic hydrogen chloride and boron trifluoride-methanol (modified from Morrison and Smith (24)) were compared to a simple base-catalyzed procedure based on sodium methoxide in methanol (according to Schulte and Weber (26)).

Material and methods

Derivatization experiments were performed with the following samples:

1) A pooled human milk sample (bank milk); human milk samples usually contain about 99 % of total lipids as triglycerides and about 1 % as phospholipids, but due to high lipolytic activity the present sample also contains considerable amounts (about 10 % of total fatty acids) as free fatty acids.
2) Infant formula X; the fat blend of this experimental product consists of butter fat, vegetable oils and egg lipids. LCP-fatty acids of this product primarily derive from egg phospholipids.
3) Infant formula Y; the fat blend of this experimental product consists of butter fat, vegetable oils and fish oil. In contrast to infant formula X LCP-fatty acids of this product derive exclusively from triglycerides.

The total lipids of both formulae and of the present human milk sample were extracted according to the method of Bligh and Dyer (3) and the total amount was determined gravimetrically. Each lipid sample was quantitatively subdivided into appropriate aliquots for the derivatization experiments and supplemented with pyrogallol to prevent autoxydation. Derivatization was performed with acid-catalyzed procedures based on methanolic hydrogen chloride and boron trifluoride-methanol (modified from Morrison and Smith (24)) and with a base-catalyzed procedure using sodium methoxide in methanol (according to Schulte and Weber (26)). A more detailed description of the different procedures is given below. The chromatographic analysis of the resulting fatty acid methyl ester was performed on a gaschromatograph (model 6500; Fa. Dani) equipped with a programmed temperature vaporizer (PTV-injector) and an autosampler (LS 32; Fa. Chrompack). The gas chromatographic conditions were the following:

Injector (PTV): 65 °C–260 °C; about 1 300 °C/min; split ratio: 19 to 1; carrier gas: Helium
Column oven: initial temp.: 50 °C for 0.1 min; from 50 °C to 185 °C at 9.5 °C/min; 185 °C for 5 min; from 185 °C to 240 °C at 2 °C/min; end temp.: 240 °C
Detector: flame ionization detector (FID) at 265 °C
Capillary column: fused-silica (DB-23; Fa. Fisons); length: 30 m; i.d.: 0.25 mm; film thickness: 0.25 µm)
Total run-time: 35 min.

All fatty acid methyl ester were identified with authentic standards and the content of individual fatty acids was calculated with a response factor of 1. This was possible due to the use of the cold injection system (PTV) and proven by quantitative standards. In case of hot-split injection systems the careful evaluation of response factors is clearly advised. The results are given as weight percentage of total fatty acids to allow direct comparison of each derivatization procedure. In addition, based on an internal standard, the absolute amount of all fatty acids found was calculated and correlated with the total amount of lipids used in each test. This value is considered as the recovery rate of each esterification process and expressed as % of total lipids. Is has to be taken into consideration that compounds like free cholesterol, fat-soluble-vitamins and pigments principally contribute to the total lipid extract but not to the total amount of FAME. Nevertheless, the recovery rate is used as an indicator for the efficacy of the different derivatization procedures.

The results for the human milk sample and for infant formula X (Tables 1 and 2) are expressed as mean values plus standard deviation. A statistical evaluation of differences between the tested derivatization procedures was performed for the total recovery rate and for the content of AA and DHA by using the SAS-procedure NPAR1WAY (Wilcoxon-two sample test).

Derivatization procedures

Methanolic hydrogen chloride-A (via acetylchloride)

Methanolic hydrogen chloride-A is prepared by adding acetylchloride to dry methanol to guarantee anhydrous conditions. Hexane is used to improve solubility of unpolar lipids (triglycerides/cholesterol ester).

For derivatization an aliquot of the total lipid extract (containing 3 mg lipids) is transferred into a screw-top tube. 2 ml of a mixture (4 to 1; v/v) of methanol and hexane is added, containing 300 µg of 21-0-methylester as an internal standard. Under gentle mixing 200 µl of acetylchloride is added. The tube is closed and refluxed

Table 1 Influence of the derivatization procedure on the results of the fatty acid analysis of human milk (data are given as wt % of total fatty acids; data represent mean values ± standard deviation; n = 10)

Fatty acids	MeOH/HCl-A	MeOH/HCl-B	MeOH/HCl-C	BF_3/MeOH	Na-methoxide/ MeOH
saturated fatty acids:					
Σ 4:0–10:0	1.09 (± 0.08)	0.96 (± 0.07)	1.14 (± 0.04)	0.93 (± 0.24)	1.01 (± 0.07)
Σ 12:0–14:0	11.54 (± 0.59)	11.41 (± 0.27)	11.88 (± 0.30)	11.12 (± 0.99)	11.74 (± 0.41)
16:0	22.65 (± 1.35)	23.52 (± 0.09)	21.43 (± 0.21)	22.77 (± 1.47)	23.85 (± 2.55)
unsaturated fatty acids:					
18:1 n-9	34.83 (± 0.87)	34.37 (± 0.57)	35.71 (± 0.23)	35.26 (± 0.47)	33.55 (± 2.13)
18:2 n-6	11.57 (± 0.72)	11.18 (± 0.09)	12.31 (± 0.08)	11.43 (± 0.86)	11.09 (± 1.20
18:3 n-6	0.09 (± 0.01)	0.08 (± 0.01)	0.10 (± 0.01)	0.08 (± 0.02)	0.08 (± 0.02)
18:3 n-3	1.34 (± 0.13)	1.25 (± 0.02)	1.46 (± 0.02)	1.29 (± 0.18)	1.25 (± 0.22)
LCP:					
20:4 n-6	0.40 (± 0.06)	0.36 (± 0.01)	0.45 (± 0.01)	0.39 (± 0.07)	0.36 (± 0.10)
20:5 n-3	0.08 (± 0.01)	0.06 (± 0.00)	0.08 ± 0.03)	0.07 (± 0.02)	0.05 (± 0.04)
22:6 n-3	0.30 (± 0.02)	0.28 (± 0.00)	0.31 (± 0.01)	0.29 (± 0.04)	0.29 (± 0.03)
recovery of total fatty acids (expressed as % of total lipids):	96.8 (± 1.7)	80.4 (± 2.4)	42.7[A] (± 0.5)	89.1 (± 1.7)	80.7[B] (± 1.8)

[A] = $p \leq 0.02$; MeOH/HCl-C vs. all other methods
[B] = $p \leq 0.02$; Na-methoxide/MeOH vs. MeOH/HCl-A and BF_3/MeOH

for 1 h at 100 °C. After cooling 4 ml potassium carbonate (6 % K_2CO_3) is added and the tubes are mixed thoroughly and centrifuged for 10 min at 3 200 rpm (2 200 x g). The upper hexane phase containing FAME is removed into an autosampler vial and dried with sodiumsulfate. A reduction of the hexane phase under a stream of nitrogen is not necessary.

Methanolic hydrogen chloride-B (plus additional toluene)

In contrast to MeOH/HCl-A methanolic hydrogen chloride-B was prepared by adding 1 volume of 6N HCl to 6 volumes of methanol. This results in a water content of the preparation of about 110 µl/ml. Toluene is added to improve solubility of unpolar lipids.

For derivatization an aliquot of the total lipid extract (containing 5 mg lipids) is transferred into a screw-top tube. After addition of an appropriate amount of an internal standard (300 µg 21-0-methylester) the solution is evaporated to dryness under nitrogen. 500 µl toluene is added to effect solution of unpolar compounds like triacylglycerols and cholesterol ester. The lipid sample is then dissolved in 2 ml of methanolic hydrogen chloride and refluxed for about 4.5 h at 90 °C. After cooling to room temperature FAMEs are extracted with 5 ml hexane. After centrifugation at 3 200 rpm (2 200 x g) for 10 min the hexane layer is transferred into a reaction vial and evaporated under a gentle stream of nitrogen. For GC analysis the FAMEs were redissolved in about 500 µl of hexane and transferred into autosampler vials.

Methanolic hydrogen chloride-C
(without additional toluene)

Virtually the same procedure as described above, except that no additional toluene is added to improve solubility of unpolar lipids.

Table 2 Influence of the derivatization procedure on the results of the fatty acid analysis of infant formula X (data are given as wt % of total fatty acids; data represent mean values ± standard deviation; n = 10)

Fatty acids	MeOH/HCl-A	MeOH/HCl-B	MeOH/HCl-C	BF_3/MeOH	Na-methoxide/ MeOH
saturated fatty acids:					
Σ 4:0–10:0	2.70 (± 0.18)	1.13 (± 0.36)	2.66 (± 0.41)	1.02 (± 0.17)	3.44 (± 0.09)
Σ 12:0–14:0	11.05 (± 0.17)	12.03 (± 0.23)	14.92 (± 1.75)	10.26 (± 0.18)	10.85 (± 0.15)
16:0	25.66 (± 0.18)	26.61 (± 0.21)	26.03 (± 0.49)	26.31 (± 0.14)	25.50 (± 0.18)
unsaturated fatty acids:					
18:1 n-9	34.21 (± 0.28)	33.49 (± 0.23)	30.39 (± 0.86)	35.71 (± 0.19)	33.94 (± 0.35)
18:2 n-6	13.64 (± 0.11)	13.76 (± 0.09)	12.29 (± 0.33)	13.46 (± 0.11)	13.56 (± 0.13)
18:3 n-6	0.22 (± 0.00)	0.21 (± 0.02)	0.19 (± 0.02)	0.21 (± 0.00)	0.23 (± 0.01)
18:3 n-3	0.92 (± 0.01)	0.91 (± 0.02)	0.86 (± 0.04)	0.86 (± 0.01)	0.91 (± 0.02)
LCP:					
20:4 n-6	0.27 (± 0.01)	0.30 (± 0.01)	0.54[A] (± 0.01)	0.25 (± 0.00)	0.27 (± 0.02)
20:5 n-3	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.01)	0.00 (± 0.00)	0.00 (± 0.01)
22:6 n-3	0.17 (± 0.00)	0.18 (± 0.00)	0.35[A] (± 0.02)	0.15 (± 0.00)	0.15 (± 0.00)
recovery of total fatty acids (expressed as % of total lipids):	97.8 (± 1.1)	75.9 (± 2.3)	36.9[A] (± 1.2)	88.0 (± 2.1)	91.4[B] (± 1.4)

[A] = $p \leq 0.001$; MeOH/HCl-C vs. all other methods
[B] = $p \leq 0.01$; Na-methoxide/MeOH vs. MeOH/HCl-A, MeOH/HCl-B and $BF_{3/MeOH}$

Boron trifluoride-methanol
(modified from Morrison and Smith (24))

For derivatization an aliquot of the total lipid extract (containing 5 mg lipids) is transferred into a screw-top tube. After adding an appropriate amount of an internal standard (300 µg 21-0-methylester) the solution is evaporated to dryness under a gentle stream of nitrogen. The lipid sample is saponified in 2 ml of methanolic potassium hydroxide (0.5 mol/l KOH in methanol) for 15 min at 90 °C. After cooling to room temperature 2 ml of a commercial boron trifluoride-methanol complex (20 % in methanol; Fa. Merck) is added and refluxed for 15 min at 90 °C for esterification of free fatty acids.

After cooling to room temperature the FAME's are extracted with 5 ml hexane. After centrifugation at 3 200 rpm (2 200 x g) for 10 min the hexane layer is transferred into a reaction vial and evaporated under a gentle stream of nitrogen. For GC analysis the fatty acid methyl ester were dissolved in 500 µl of hexane and transferred to an autosampler vial.

Sodium methoxide (according to Schulte and Weber (26))

The sodium methoxide-reagent is prepared by mixing 1 ml of a commercial sodium methoxide-solution (5.4 mol/l in methanol; Fa. Fluka) with 1.7 ml of methanol.

For derivatization an aliquot of the total lipid extract (containing 10 mg lipids) is transferred to a micro test-tube. After adding an appropriate amount of an internal standard (600 µg 21-0-methylester) the solution is dried under a gentle stream of nitrogen. Petroleum ether (1 ml) and sodium methoxide-solution (50 µl) are added and the mixture is shaken for about 30 s on a vortex-mixer. After a reaction period of 30 min at room temperature 100 mg calciumchloride is added and again shaken for about 30 s on a vortex-mixer. The solution is centrifuged for 5 min at about 12 000 rpm (10 000 x g) and transferred to an autosampler vial. The solution is ready for gas chromatography. A further reduction of the volume under a gentle stream of nitrogen is not necessary.

Results

The fatty acid spectrum of the human milk sample (Table 1) is characterized by more or less the same relative percentage distribution of each fatty acid independent of an acid- or base-catalyzed derivatization procedure. Saturated as well as mono- and polyunsaturated C18-fatty acids exhibit only small differences between the derivatization procedures used. Within the LCP-fatty acids the content of AA is slightly but not significantly elevated after derivatization with MeOH/HCl-C compared to all other procedures. The significantly lowest recovery rate of about 43 % of total lipids indicates an extremely incomplete transmethylation of human milk triglycerides with MeOH/HCl-C compared to 81 %, 89 % or 97 % in the other acid-catalyzed procedures. The base-catalyzed transmethylation step with sodium methoxide/methanol reveals a conversion rate of 81 %, which is significantly lower compared to MeOH/HCl-A and BF_3/MeOH.

In contrast to the analyzed human milk sample the results of the fatty acid analysis of infant formula X (Table 2) markedly differ according to the derivatization procedure used. Especially the relative amount of AA and DHA is significantly different and about 100 % higher after derivatization with MeOH/HCl-C compared to all other esterification procedures. Furthermore, this method seems to result in a lower relative percentage of oleic acid (18:1 n-9) and a higher amount of saturated medium-chain fatty acids (C12:0–C14:0) in the total fatty acid spectrum. The content of saturated short-chain fatty acids (C4:0–C10:0) in infant formula X seems to be highest after derivatization with MeOH/HCl-A and sodium methoxide. The recovery rates found in this test indicate the significantly lowest esterification of triglycerides with MeOH/HCl-C (37 %) compared to 88–98 % after derivatization with BF_3-methanol, sodium methoxide and MeOH/HCl-A.

In contrast to product X, based on egg lipids, the infant formula Y (Table 3) contains substantial amounts of fish oil. The total fatty acid pattern of this product therefore exhibits only small amounts of AA and similar amounts of EPA (20:5 n-3) and DHA. Although the conversion rate of triglycerides to FAME in this product is only about 49 % after derivatization with MeOH/HCl-C compared to about 76–93 % after derivatization with all other methods, the relative amount of LCP-fatty acids seems to be independent of the derivatization procedure. The results of the fatty acid analysis differ remarkably only after transmethylation with MeOH/HCl-C from all other procedures in their content of palmitic and linoleic acid. The relative amount of saturated short-chain fatty acids (C4:0–C10:0) in infant formula Y is highest in those samples derivatized with MeOH/HCl-A and sodium methoxide.

Discussion

Recently, the ESPGAN-Committee on Nutrition (14), the British Nutrition Foundation (4), the Commission of the

Table 3 Influence of the derivatization procedure on the results of the fatty acid analysis of infant formula Y (data are given as wt % of total fatty acids; n = 2)

Fatty acids	MeOH/HCl-A	MeOH/HCl-B	MeOH/HCl-C	BF_3/MeOH	Na-methoxide/ MeOH
saturated fatty acids:					
Σ 4:0–10:0	3.82/3.98	1.44/1.88	2.02/2.20	1.30/0.86	4.62/4.72
Σ 12:0–14:0	8.22/8.28	8.90/8.87	10.34/10.78	8.06/8.04	8.16/8.30
16:0	19.99/19.94	20.87/21.08	22.59/23.21	20.87/20.96	19.70/19.58
unsaturated fatty acids:					
18:1 n-9	24.56/24.52	24.96/24.85	24.07/24.09	25.89/26.06	24.36/24.63
18:2 n-6	27.21/27.13	27.74/27.47	24.80/23.93	27.38/27.56	27.34/27.21
18:3 n-6	0.00/0.00	0.00/0.00	0.00/0.00	0.00/0.00	0.00/0.00
18:3 n-3	0.85/0.84	0.90/0.88	0.96/0.95	0.81/0.82	0.83/0.85
LCP:					
20:4 n-6	0.06/0.06	0.06/0.06	0.06/0.06	0.06/0.06	0.07/0.07
20:5 n-3	0.24/0.24	0.25/0.23	0.24/0.23	0.23/0.23	0.25/0.25
22:6 n-3	0.28/0.29	0.26/0.26	0.27/0.22	0.23/0.23	0.28/0.25
recovery of total fatty acids (expressed as % of total lipids):	91.4/93.3	76.9/76.5	48.0/49.1	76.0/76.0	89.1/88.4

European Community (11, 12) and the International Society for the Study of Fatty Acids and Lipids (18) have established recommendations concerning the content of several fatty acids in infant formulae. Quantitative amounts, ranges and upper limits have been recommended for LCP-fatty acids, for erucic acid (22:1 n-9), for linoleic (18:2 n-6) and alpha-linoleic acid (18:3 n-3), for trans-fatty acids, for lauric acid (12:0) and for MCT-fatty acids (8:0/10:0). In view of these detailed recommendations the analytical procedures used for fatty acid analysis should guarantee an accurate determination of volatile short-chain fatty acids as well as the precise determination of high-boiling long-chain saturated and polyunsaturated fatty acids within one analytical run. Provided that a complete extraction of lipids from infant formula is achieved, the derivatization method used should guarantee that all lipid classes are quantitatively methylated to their corresponding methyl ester.

So far, published studies on the quantitative fatty acid analysis of infant formulae have primarily focused on the content of single fatty acids (1, 23). Studies performed by Bellomonte et al. (1) concerning the content of linoleic acid and by Koletzko and Bremer (21) regarding the total fatty acid spectrum of infant formulae were based on methanolic hydrogen chloride as the derivatization agent. Although MeOH/HCl is one of the most frequently cited reagents for preparation of FAMEs, details of the method are usually not described. Several authors (reviewed by Christie (7)) have described the influence of the water content in the preparation on the completeness of the esterification process and the necessity of adding an unpolar solvent to the MeOH/HCl-preparation to ensure that also unpolar lipids like triglycerides are effectively solubilized and esterified. Results of this study clearly underline that a considerable amount of water in the MeOH/HCl-preparation leads to an inefficient triglyceride transesterification in both formulae. In case of human milk and infant formula Y, which contain LCP primarily in form of triglycerides, the incomplete transesterification of the unpolar lipids has only minor influences on the relative fatty acid composition. But it is obvious that results concerning the absolute amount of specific fatty acids in human milk and infant formula cannot be drawn from such an incomplete triglyceride transmethylation. In case of infant formula X the ineffective triglyceride transesterification leads to a dramatic overestimation of LCP-fatty acids in this product. This is due to the fact that LCP-fatty acids of infant formula X are primarily derived from polar phospholipids, which are effectively transmethylated in MeOH/HCl even in absence of an additional unpolar solvent. In the tested preparation MeOH/HCl-A anhydrous conditions were guaranteed by preparing the reagent with acetyl chloride, which is added to an excess of dry methanol. In contrast to Christie (7), who favors toluene as an additional solvent, hexane was used in this preparation, because of possible interference of toluene with short-chain fatty acids during gaschromatography on polar columns. In contrast to toluene, which has to be removed prior to GC, the small amount of hexane in this preparation acts as a solubilizer of unpolar lipids and it is furthermore used as an extracting solvent, so that FAMEs can be directly injected into GC without prior evaporation. In contrast to all other acid-catalyzed procedures tested, this method thereby circumvents the risk of a substantial loss of volatile short-chain fatty acids. Especially in case of products like infant formula X and Y, which contain substantial amounts of butter fat in their fat blend, but also in products based on MCT-triglycerides, the content of butyric (4:0), caproic (6:0), caprylic (8:0) and capric acid (10:0) will be underestimated if an evaporation step of the extracting solvent is not avoided. It is concluded that anhydrous procedures based on methanolic hydrogen chloride containing an unpolar solvent (like MeOH/HCl-A) are reliable and suitable methods for the fatty acid analysis of human milk and infant formulae.

Lee (23) and Permanyer et al. (25) have performed their infant formula-studies by using BF_3/methanol as the derivatizing agent. Although BF_3/methanol has found an extremely wide application, caution should be expressed about the routine use of this agent. In this study BF_3/methanol-derivatization (modified from Morrison and Smith (24)) was found to result in a satisfactory conversion rate, but the evaporation step results in a loss of short and medium chain fatty acids as mentioned above. Furthermore, Christie (7) has pointed out that BF_3/methanol is known to form artifacts and that the use of old or concentrated reagents could result in a loss of polyunsaturated fatty acids.

Similar to the procedure already used by Jensen et al. (19) for different infant formulae, Schulte and Weber (26) published a quick and simple base-catalyzed transesterification method based on sodium methoxide in methanol. This method is rapid and it can be easily performed even at room temperature. Under such conditions the risk of isomerization of polyunsaturated fatty acids is remarkably reduced. As with the acidic procedures, an inert solvent must be added to sodium methoxide to dissolve unpolar lipids before the methanolysis can proceed. The procedure published by Schulte and Weber (26) uses petroleum ether as a solvent for the lipid sample. To prevent loss of volatile short-chain fatty acids, the method is designed in the way that extraction of FAMEs and evaporation prior to GC can be avoided. In contrast to the acid-catalyzed procedures sodium methoxide is not useful if large amounts of free fatty acids are present in the sample, because these are not converted to FAMEs. Usually, infant formulae do not contain substantial amounts of free fatty acids, so that sodium methoxide seems to be a time-saving and reliable method for routine fatty acid analysis of infant formulae. Due to the simplicity of the procedure, we propose that this method should be vali-

dated as a reference method for routine fatty acid analysis of infant formulae by interlaboratory comparisons and compared to acidic procedures like MeOH/HCl-A.

In contrast to infant formulae human milk samples may contain considerable amounts of free fatty acids. Owing to improper sampling or storage conditions free fatty acids are liberated by the activity of human milk lipases (2, 16). The human bank milk sample used in this study contains about 10 % of total fatty acids as free fatty acids. This is reflected by a significantly reduced conversion rate after base-catalyzed derivatization with sodium methoxide. Despite the lower conversion rate the relative fatty acid distribution of human milk is not different compared to MeOH/HCl-A or BF_3/MeOH. Such base-catalyzed transesterification procedures using sodium methoxide were already applied to human milk samples by several authors (5, 9, 10, 13, 15, 19, 22). Most studies reported results on the relative fatty acid distribution of total lipids without indicating possible problems caused by the content of free fatty acids. We conclude from our results that analytical results including available literature values on human milk fatty acids based on sodium methoxide or methanolic hydrogen chloride (like MeOH/HCl-A) should be comparable, if the authors are dealing with the relative fatty acid distribution. This will also refer to a comparison of the relative fatty acid pattern from human milk and infant formulae based on the two above-mentioned procedures.

In view of the recommendations to express results of human milk analysis, not only in relative data, but also in absolute terms (19), a quantitative transesterification of all lipid classes of human milk samples has to be achieved. Therefore, we think that acid-catalyzed procedures based on methanolic hydrogen chloride seem to be preferable. As discussed for infant formulae, the acid-catalyzed procedure applied to human milk samples should be performed under waterfree conditions and an additional unpolar solvent like toluene or hexane should be added to methanolic hydrogen chloride. Although BF_3/methanol is cited extensively in the literature on human milk fatty acids, we do not favor this reagent, because of possible disadvantages mentioned above (7).

In conclusion, the results underline the necessity of a careful and quantitative transmethylation of all lipid classes for accurate fatty acid analysis of human milk samples and infant formulae products. In view of the recent recommendations for specific fatty acids, sodium methoxide seems to be a reliable and time-sparing method for routine fatty acid analysis of infant formulae. This method should be proven by interlaboratory comparison. Acid-catalyzed procedures based on methanolic hydrogen chloride including an additional unpolar solvent like toluene or hexane are also suitable for infant formula analysis, but due to the problem of free fatty acids these procedures seem to be preferable for human milk samples.

References

1. Bellomonte G, Giammarioli S, Terilli R (1991) Quantitative determination of linoleic acid in infant formulas. Int J Vit Nutr Res 61:91–97
2. Berkow SE, Freed LM, Hamosh M, Bitman J, Wood DL, Happ B, Hamosh P (1984) Lipases and lipids in human milk: effect of freeze-thawing and storage. Peditar Res 18:1257–1262
3. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
4. British Nutrition Foundation (1992) Unsaturated Fatty Acids. Nutritional and Physiological Significance. The Report of the British Nutrition Foundation's Task Force. Chapman & Hall, London, pp 152–163
5. Businco L, Loppi M, Morse NL, Nisini R, Wright S (1993) Breast milk from mothers of children with newly developed atopic eczema has low levels of long-chain polyunsaturated fatty acids. J Allergy & Clinical Immunology 16:1134–1139
6. Carlson SE (1989) Dietary fatty acids in relation to neural development in humans. In: Galli C, Simopoulos AP (eds) Dietary w3 and w6 fatty acids. Biological effects and Nutritional essentiality. Plenum Press, New York, pp 135–146
7. Christie WW (1993) Preparation of ester derivatives of fatty acids for chromatographic analysis. In: Christie WW (ed) Advances in lipid methodology – two:. The Oily Press, Dundee, pp 69–111
8. Clandinin MT, Chappell JE, van Aerde JEE (1989) Requirements of newborn infants for long-chain polyunsaturated fatty acids. Acta Paediatr Scand 351 (Suppl):63–71
9. Clark RM, Ferris AM, Fey M, Brown PB, Hundrieser KE, Jensen RG (1982) Changes in the lipids of human milk from 2 to 16 weeks postpartum. J Pediatr Gastroenterol & Nutr 1:311–315
10. Clark RM, Hundrieser KE (1989) Changes in cholesteryl esters of human milk with total milk lipid. J Pediatr Gastroenterol & Nutr 9:347–350
11. Commission of the European Communities (1991) Commission Directive 91/321/EEC on infant formulae and follow-on formulae. Amtsblatt der Europäischen Gemeinschaften L 175:35–49
12. Commission of the European Communities (1994) Preliminary Draft of a Commission Directive amending Directive 91/321/EEC on infant formulae and follow-on formulae
13. Craig-Schmidt MC, Weete JD, Faircloth SA, Wick-Wire MA, Livant EJ (1984) The effect of hydrogenated fat in the diet of nursing mothers on lipid composition and prostaglandin content of human milk. Am J Clin Nutr 39:778–786
14. ESPGAN-Committee on Nutrition (1991) Committee Report: Comment on the Content and Composition of Lipids in Infant Formulas. Acta Paediatr Scand 80:887–896
15. Gibson RA, Kneebone GM (1981) Fatty acid composition of human colostrum and mature breast milk. Am J Clin Nutr 34:252–257
16. Hamosh M, Bitman J, Wood DL, Hamosh P, Mehta NR (1984) Lipids in milk and the first steps in their digestion. Pediatr 75 (Suppl):146–150
17. Hundrieser KE, Clark RM, Brown PB (1983) Distribution of trans-octadecenoic acid in the major glycerolipids of human milk. J Pediatr Gastroenterol & Nutr 2:635–639

18. ISSFAL-Board (1994) Recommendations for the essential fatty acid requirement for infant formulas. ISSFAL-Newsletter 1/1:4–5
19. Jensen RG, Bitman J, Wood L, Hamosh M, Clandinin MT, Clark RM (1984) Methods for the sampling and analysis of human milk lipids. In: Jensen RG, Neville MC (eds) Human lactation: milk components and lactation. Plenum Press, New York, pp 97–112
20. Koletzko B (1992) Fats for brains. Eur J Clin Nutr 46 (Suppl 1):S51–S62
21. Koletzko B, Bremer HJ (1989) Fat content and fatty acid composition of infant formulas. Acta Paediatr Scand 78:513–521
22. Lammi-Keefe CJ, Ferris AM, Jensen RG (1990) Changes in human milk at 06.00, 10.00, 14.00, 18.00 and 22.00 h. J Pediatr Gastroenterol & Nutr 11:83–88
23. Lee TW (1987) Quantitative determination of linoleic acid in infant formulas by gas chromatography. J Assoc Off Anal Chem 70:702–705
24. Morrison WR, Smith LM (1964) Preparation of fatty acid methyl esters and dimethylacetats from lipids with boron fluoride-methanol. J Lipid Res 5:600–608
25. Permanyer JJ, Pinto BA, Hernandez N, Boatella J (1990) Trans isomer content of infant milk formulas. Lait 70:307–311
26. Schulte E, Weber K (1989) Schnelle Herstellung der Fettsäuremethylester aus Fetten mit Trimethylsulfoniumhydroxid oder Natriummethylat. Fat Sci Technol 91:181–183
27. Shanta NC, Napolitano GE (1992) Gas chromatography of fatty acids. J Chromatogr 624:37–51
28. Uauy R (1990) Are w-3 fatty acids required for normal eye and brain development in the human? J Pediatr Gastroenterol Nutr 11:296–302