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Review of methods for the analysis of triglycerides in milk fat: application for studies of milk quality and adulteration

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Various analytical methods are used for the determination and quantification of triglycerides in milk fat. This paper reviews the latest developments (1990–1994) analyzing triacylglycerols in milk fat with an emphasis on the use of chromatographic methods. Moreover, the different approaches for the detection of adulteration of milk fat will be compared.

INTRODUCTION

Adulteration of expensive oils and fats such as milk fat has always been a serious problem because of the economic advantages by partly replacing high-priced fats and oils by low-priced oils (e.g. sunflower oil) without labeling the product accordingly. This paper discusses methods for the detection of adulteration of milk fat with an emphasis on chromatographic methods. Of all milk fat products butter is the most important one for economic reasons. Until now butter should contain at least 82% pure milk fat, water and sometimes salt and should be made of milk or cream only. However a proposal submitted by the Commission of the European Union to the Council of the European Union would permit the addition of foreign fat to milk fat if it is necessary for its production and as long as it is not intended to replace the milk fat. This milk fat could be used for the production of the so-called 'recombinant' butter, which is made by recombining milk, cream, butter, anhydridous milk fat or butter oil. Until now the discussion about the necessity of labeling this product accordingly is not yet finished (Schlimme, 1993). If recombined butter has not to be labeled accordingly, this would underline the need of an analytical method for the determination of the amount of foreign fat added, thus ensuring that in fact milk fat is not replaced by other fats for economic reasons.

The composition of the milk fat may be altered for nutritional purposes by changing the diet of the cattle, but is also influenced by genetic factors and variations in feeding depending on season and region. Gibson (1991) stated that only minor changes in the milk fat compositions can be induced genetically. Palmquist *et al.* (1993) showed that the feeding conditions are the most influential factors for the changes of the milk fat compositions. Milk fat is composed mainly of saturated fatty acids, which are considered to be of lower nutritional value compared to unsaturated fatty acids. Therefore increasing the portion of unsaturated fatty acids in milk fat by an appropriate diet is of special interest (Sutton, 1989; Grummer 1990, 1991). In more detail the feeding of protected canola seed has been investigated by Ashes *et al.* (1992) and Khoransani *et al.* (1991) and that of a linoleic-rich diet by Jensen *et al.* (1990, 1991). The difference of feeding conditions due to seasons are thought to be of high influence for milk fat composition by Guyot (1977*a*,*b*). More recently this has been confirmed by others (Precht, 1990*a*, 1991, 1992*a*–*c*; Bornaz *et al.*, 1992; Hinrichs *et al.*, 1992). Underfeeding of cows, especially occurring in interseasonal period and winter, significantly alters the lipid composition of the milk. Frede (1986) states that specific triglycerides may be used as an indicator for underfed cows. More details about the changes in the composition of the triglycerides of underfed cows are given by Jensen *et al.* (1990, 1991) and Precht (1992*c*).

Analysis of triglycerides in natural samples is a very difficult task due to their overwhelming variety. Based on their fatty acid composition, the possible number of triglycerides in milk fat is calculated to be greater than 1300 (Barron *et al.*, 1990). Thus, the largest type represented by one specific triglyceride is only about 4·2% (Gresti *et al.*, 1993) and even less, for example, in fish oil, with only about 2·8% (Laakso & Christie, 1991). Hence, the identification of every compound in such complex edible oils has not yet been achieved (Kuksis *et al.*, 1991).

This review is most concerned with the analysis of triglycerides, therefore no references will be given considering the analytical parameters of the analysis of

fatty acids methyl esters (FAME). The interested reader is referred to Ackman (1991), Craske (1993), Lower (1990*a*,*b*) and Matter (1991).

Regarding the authentication of plant oils, reviews are written by De Jong (1991), Kaufmann (1993) and Rossell (1991) including different analytical techniques and mathematical tools for authentication of various edible oils.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A general overview of the most common used reversed-phase high-performance liquid chromatography (RP-HPLC) equipment has been published by León (1993) and Camacho (1993):

- Column: μ-Bondapack C-18 (10 μm), RP-18 (Spheri 5), Zorbax X-ODS (6 μm), ODS-2 (5 μm)
- Mobile phase: acetonitrile mixed with acetone, isopropanols, dichloromethane or ethanol/hexanol
- Detector: refractive index detector, evaporative light-scattering detector

In the collaborative study of Wolff *et al.* (1991) the participating laboratories were obliged to use the RI-detector (refractive index) and acetone/acetonitrile as eluent. The most used RP-HPLC columns were

- Lichrospher, Lichrosorb (both Merck)
- Zorbax ODS (Du Pont)
- Nucleosil 5 C18 (Macherey-Nagel).

Bibliographic reviews of the separation of lipids by RP-HPLC covering the period until 1989 have been compiled by Christie (1987), Wojtusik *et al.* (1989), Lyapkov and Melamed (1990), Aitzetmüller (1986, 1991) and more generally by Sewell (1992). All authors included a list of references with examples for the successful separation of triglycerides of various origin. Table 1 summarizes all general aspects discussed in these papers.

The analytical parameters of recently developed methods for the analysis of triglyceride of milk fat are summarized in Table 2. In general the use of an ELSD leads to lower detection limits and allows, because of its gradient compatibility, a better separation.

Method evaluation

Purdon (1991) has given a guide for the selection of the stationary and mobile phase for lipid separation. The solvent characteristics based on Snyder's solvent triangle, the use of retention mapping for solvent optimization and in particular the need of an appropriate column equilibration after a gradient run are discussed in some detail. For the influence of temperature on the efficiency of the separation by HPLC see below. The

Table 1. General analytical aspects for the separation of triglycerides using non-aqueous reversed-phased HPLC as discussed in reviews covering the period up to 1989

Detector		
Flame-ionisation detector (FID)	Poor reproducibility.	Lyapkov & Melamed (1990)
	Good sensitivity, response factors has to be used.	Sewell (1992)
	Limited sensitivity, not commercially available	Wojtusik *et al.* (1989)
Evaporative light scatter detector (ELSD)	Too complex in design.	Lyapkov & Melamed (1990)
	High sensitivity, expensive	Wojtusik *et al.* (1989)
	very careful calibration necessary.	Sewell (1992)
Postcolumn reaction detector (PCRD)	Too complex in design.	Lyapkov & Melamed (1990) Wojtusik *et al.* (1989)
Infrared-detector (IR-detector)	Low sensitivity.	Lyapkov & Melamed (1990)
	Sensitivity can be as high as using a RI detector.	Sewell (1992)
	High detection limits, slow response time.	Wojtusik *et al.* (1989)
Ultraviolet-detector (UV-detector)	Quantification very difficult.	Lyapkov & Melamed (1990)
	Quantification is simple.	Sewell (1992)
	Accurate quantitative results not possible.	Wojtusik *et al.* (1989)
Refractive index detector (RI-detector)	Recommended for use.	Lyapkov & Melamed (1990)
	Widely used detector.	Sewell (1992)
	Extensively used, inexpensive.	Wojtusik *et al.* (1989)
Eluent		
Using propionitrile or acetonitrile, methylencloride, methyl tert.-butyl ether incomplete elution can occur; chloroform or tetrahydrofuran can eliminate this problem, but are also decreasing the selectivity.		Lyapkov & Melamed (1990)
Propiontrile shows for isocratic elution a better resolution than acetone/acetonitrile mixtures; an even better resolution has been reported for ternary mixtures like isopropanole/acetone/acetonitrile.		Wojtusik *et al.* (1989)
Propionitrile gives best separation.		Aitzetmüller (1991)
Solvents		
For chloroform, tetrahydrofuran, acetone peak form and resolution depends also on the amount of injected liquid.		Lyapkov & Melamed (1990)
No significant difference in chromatographic behavior.		Wojtusik *et al.* (1989)

Table 2. Recently published methods for the determination of triglycerides in milk fat using RP-HPLC with C_{18}-columns: methods using a FID or ELSD have been run in the gradient regime

Detector	Eluent	Reference
ELSD	Acetonitrile/ethanol/hexane.	Herslöf & Kindmark (1985)
	Acetonitrile/chloroform.	Kermasha *et al.* (1993)
	Dichloromethane/dichloroethane/acetonitrile.	(Laakso *et al.* 1992) Laakso & Kallio (1993*a,b*)
	Methylenechloride/acetonitrile.	Letter (1993)
	Propionitrile/acetonitrile.	Marai et al. (1994)
RI-detector	Acetone/acetonitrile.	Bornaz *et al.* (1992), Dotson *et al.* (1992), Gresti *et al.* (1993) Maniongui *et al.* (1991)
	Acetonitrile/propionitrile.	Hinrichs *et al.* (1992)
FID	Acetone/acetonitrile.	Nurmela & Satama (1988)

correlation of the effective carbon number (ECN†) or partition number (PN†) with the retention time has been examined in detail by Zeitoun *et al.* (1991) and Wolff *et al.* (1991). It is thought that the retention time is linear correlated with ECN. Nurmela and Satama (1988) confirmed this linearity even for a gradient elution. Also the strong correlation of the capacity factor‡ k' and the ECN investigated at various temperature is thought to be linear as stated by El-Hamdy and Perkins (1981) and Frede (1986). Goiffon *et al.* (1981*a,b*) has established the concept of partial retention times. This means that the retention time can be derived from retention times of uniform triglycerides like *XXX* in the form of partial retention time: $XYZ = \frac{1}{3}t_{XXX} + \frac{1}{3}t_{YYY} + \frac{1}{3}t_{ZZZ}$. This concept has been successfully applied by other authors (Perrin & Prévot, 1986; Fellat-Zarrouck *et al.*, 1988; Fabien *et al.*, 1993; Damiani *et al.*, 1994). Using a refractive index detector a similar relationship can be established for the response of the detector:

$$\frac{1}{F_{XYZ}} = \frac{1}{3F_{XXX}} + \frac{1}{3F_{YYY}} + \frac{1}{3F_{ZZZ}}$$

as shown by different authors (Perrin & Naudet, 1983; Fellat-Zarrouck *et al.*, 1988; Carelli & Cert, 1993).

Solvents and solubility problems

The choice of the solvent depends on the kind of detector used. The two most common detectors for the analysis of triglycerides are the RI-detector, which can only be used in isocratic elution, and the gradient compatible ELSD. For the RI-detector the two most used solvents are propionitrile and mixtures of acetone/acetonitrile. To accelerate the isocratic separation Aitzetmüller (1990) proposed the use of a flow gradient. For both eluents solubility problems have been reported. Lie Ken Jie (1980) reported a crystallization of triglycerides in the column using acetone/acetonitrile (2:1 v/v), while Geerart and Sandra (1987) found an incomplete solubility of three-fold unsaturated triglycerides in propionitrile. Nurmela and Satama (1988) reported that for acetone/acetonitrile as eluent, cholesterol is found to have an ECN value of 38. Using propionitrile, Aitzetmüller *et al.* (1988) did not succeed in the separation of the critical pair tripalmitylglycerole/stearyl-dioleylglycerole, while Frede (1986) using a temperature gradient did not experience such a problem. ELSD detectors are gradient compatible and have been used with a variety of different solvents, therefore avoiding solubility problems (Letter, 1993).

Detectors

Useful detectors for the RP-HPLC analysis of triglycerides are reviewed by Christie (1992) and Moreau (1994). This includes UV-, IR-, radioisotope-, flourescence-, RI-detector, FID, PCRD and ELSD. Both author stated the superiority of the FID and the ELSD. The latter is regarded to be the best detector. Three detectors for the detection of triglycerides are commonly used, namely UV-detector, ELSD and RI-detectors. They are compared by Aitzetmüller (1988) and Shukla (1991). Kuksis *et al.* (1991) compared UV- and RI-detectors, Herslöf and Kindmark (1985) ELSD and UV-detectors and Carelli and Cert (1993) ELSD and RI-detectors. All these authors again stated the superiority of the ELSD-detector, despite the problems with sigmoidal calibration curves and possible discrimination of volatile compounds due to vaporization. Contrary to the positive judgment of numerous authors, other experienced more problems such as poor reproducibility, the coefficient of variation ranges from 2·4% for tricaprylin to 16·92% for tributyrin (Robinson *et al.*, 1985), and inaccurate results at low concentrations with a relative standard deviation of up to 40% for triglycerides at concentrations lower 1% (Carelli & Cert, 1993).

Temperature of the column

It has been shown that the temperature of the column is a very important parameter of the separation characteristic, even so it has not been mentioned by various authors. Aitzetmüller (1990), Defense (1984), Fiebig

†The partition number (PN) or equivalent carbon number (ECN) is defined by PN = CN − 2 × db (CN: carbon number, sum of carbon atoms of fatty acids, db: number of double bonds).

‡$k' = \frac{t_R - t_M}{t_R}$ retention time: t_R, column hold-up time: t_M.

(1985), Frede (1986), Geerart and De Schepper (1983), Guillaume and Guinchard (1993) and Jensen (1981) evaluated the influence of the temperature on the response factors. Clearly, there is an optimum in sensitivity and resolution for the separation of triglycerides, which is not necessarily close to room temperature (e.g. 14·5°C (Jensen, 1981)), but it depends on column and solvent. Moreover, there are a few attempts to achieve better separation by using a temperature gradient (Frede, 1986; Aitzetmüller, 1990; Hinrichs *et al.*, 1992), which revealed a clear improvement for the separation of triglycerides using isocratic conditions. However, this can cause a decrease in the resolution (Barron & Santa-Maria, 1989).

Nevertheless, most authors used constant temperature even under isocratic conditions:

25°C	Kermasha *et al.*, 1993.
30°C	Aitzetmüller & Grönheim, 1993; Barron *et al.*, 1990; Gresti *et al.*, 1993; Maniongui *et al.*, 1991; Schulte, 1981.
32°C	Bornaz *et al.*, 1992.
35°C	Letter, 1993.
40°C	Fabien *et al.*, 1993; Gresti *et al.*, 1993; Maniongui *et al.*, 1991; Nurmela & Satama, 1988; Vigneron *et al.*, 1986.
50°C	Bergqvist & Kaufmann 1993.
Room or ambient temperature	Flor *et al.*, 1993; Laakso *et al.*, 1992.

GAS CHROMATOGRAPHY

The application of gas chromatography for the analysis of triacylglycerols was reviewed in detail covering the period up to 1989 by Christie (1989) very comprehensively and also by Guyot (1977*a*,*b*), Lyapkov and Melamed (1990) and Schaller (1991). More recently, but not so comprehensive overviews, were compiled by Evershed (1992) and Trvzická and Mareš (1994).

All recently published GC-methods for the analysis of triglycerides of milk fat used a FID as detector. Kalo and Kemppinen (1993) and Lund (1988) used on-column injection, a programmable temperature vaporizer (PTV) as injector was used by Alonso (1993) and Kalo and Kemppinen (1993), while Lercker *et al.* (1992) used the split injection technique. However, Gresti *et al.* (1993) and Maniongui *et al.* (1991) did not state their kind of injector. Precht (1990*a*,*b*, 1991, 1992*a*–*c*) and Molkentin and Precht (1993) used the hot-injection technique or hot-needle technique, and packed columns. The hot-needle technique was first developed by Grob (1979). For this injection technique the syringe has to remain for about 3s in the injector block prior to the injection. Thus it is intended to avoid fractionation within the syringe or the injector block.

Method evaluation

Most of the work in the development of gas chromatographic analysis of lipids has been done prior to 1990, the reader is referred to the references given above.

The most critical steps in the development of the method comprises the selection of an appropriate injector system. For a detailed discussion on the importance of the injection technique, especially for triglyceride analysis, see the work of Grob (1979), Grob *et al.* (1985) and Hinshaw (1987). They support the cold on-column injection techniques for capillary columns. According to Schaller (1991) comparably good results could also be achieved using a partially optimized split injector. Precht (1990*a*) recommended the *hot-needle technique*, which he used extensively for his work.

For a comparison of HPLC versus capillary gas chromatography (c-GC) see Carelli and Cert (1993) and Kuksis (1994). The authors stated that both methods provides complementary information. While c-GC yields its most extensive resolution of diradylglycerols, HPLC provides the single most effective method of resolution of polyunsaturated triacylglycerols. A comparison of the application of HPLC and c-GC on the analysis of olive oil has shown that using c-GC trilinolein is always discriminated compared to HPLC (Fascioli *et al.*, 1992), but in a collaborative study published by Carelli and Cert (1993) this effect could not be observed.

Several authors developed models for the quantitative analysis of the adulteration of milk fat using gas chromatography. Schneller and Wullschleger (1992) used the C_{34}-peak† for the quantification of milk fat. He stated a imprecision of ±16·5% for the amount of added triglycerides, mostly due to seasonal variations in the milk fat composition. Lercker *et al.* (1992) developed a method which allows a very good detection of foreign fat in butter using c-GC and evaluating four different C_{54}-peaks, and the total area of C_{32}– C_{42} and C_{44}-peaks and diacylglycerides. Using GC with packed columns Precht (1990*a*, 1992*a*, 1992*b*, 1992*c*) built up equations by multiple linear regression which allows the detection of foreign fat in butter with a detection limit of <5%.

Only few authors have established a model for the quantitative of qualitative evaluation of adulterated milk fat using FAME-profiles. Muuse and Martens (1993) developed a method for the detection of milk fat in fat mixtures within the range of 25–75% using multiple peaks of the FAME analysis. Also Matter *et al.* (1989) showed the application of FAME patterns as a fingerprint for the identification of animal fats and the detection of their falsification. Analyzing the fatty acid methyl esters C_{14}/C_{16}, C_{18}, $C_{18'}$, a method for the identification of mixtures of milk fat, cocoa butter, nut oil and cocoa fat was presented by Sacca *et al.* (1991). Computing the solution for a linear equation system they quantified mixtures in the range of 30–70% of the respective components.

†The subscribed number stands for the carbon number (CN), the total number of acyl-C atoms within the triglyceride.

Other chromatographic methods

Recently published detailed reviews are available for other kinds of liquid chromatography applied on for the separation of triacylglycerols of milk fat. A detailed review of AG^+-HPLC was compiled by Christie (1994)†. For a discussion of RP-HPLC, AG^+-HPLC and GS-MS see Christie (1991); a comparison of gel-permeation, AG^+-HPLC, RP-HPLC and GC was compiled by Aitzetmüller (1988). While HPLC separates the triglycerides according to their ECN (effective carbon number), GC separates triglycerides according to their CN (carbon number), and Ag^+-HPLC according to the number of double bonds. Thus all methods give complementary results and are necessary to achieve a separation of the individual triglycerides in complex natural oils and fats. Hopia *et al.* (1993) explored the feasibility of size exclusion chromatography for the analysis of lipids from milk fat. It is very useful to separate different classes of lipids but does not allow a separation of the individual triglycerides. A comparison of c-GC, RP-HPLC and direct chemical ionisation/mass spectrometry was published by Řezanka and Mareš (1991). The authors emphasized that the correct identification and quantification of triglycerides of complex oils such as plant oils is only possible by combining at least the three methods. Optimizing the analytical parameters, it was possible to compare the data of the three methods without the use of correction factors.

Other methods

Collomb and Spahni (1991) compared different methods for their suitability for the detection of foreign fat in milk fat. This included:

- physico-chemical indices such as Reichert–Meissl, Polenske (both titrimetric determination of the non-water-soluble fatty acids), iodine value, semimicrobutyrique value
- scanning differential calorimetry
- IR-spectroscopy
- amount and properties of the unsaponifiables (sterols, hydrocarbons, aliphatic alcohols, tocopherol, tocotrienol)
- GC analysis of fatty acids
- analysis of mono-, di- or triglycerides
- $^{12}C/^{13}C$-isotopic measurements
- gravimetric detection of crystals of triglycerides, disterolethers, ketocompounds.

The authors summarized that the most sensitive detection of foreign fat is found to be performed with the analyses of triglycerides according to Precht (1992*b*,*c*), the detection of sterols reveals better results for the detection of added vegetable oils but fails naturally for the analysis of animal fat, which does not contain any phytosterol.

†Silver ion thin-layer chromatography shows the same resolution power as Ag^+-HPLC (Nikolova-Damyanova *et al.*, 1993).

Safar *et al.* (1994) applied attenuated total reflection mid infrared spectroscopy (ATR-MIRS) which allowed a identification of different edible oils by multivariate data analysis. The application of Fourier-transform infrared (FTIR)-spectroscopy for the authentication of olive oils was shown by Lai *et al.* (1994).

Currie and Kallio (1993) showed the application of Tandem-MS for the identification of triglycerides in human milk.

Kirst *et al.* (1991) summarized the use of various less sophisticated methods for the characterization of milk fat (e.g. softening point, iodine value, determination of butyrique acid, tocopherol or carotene), but for the detection of adulteration they are not considered to be sensitive enough. In general the best detection limit for the addition of foreign oils to butter is about 5%, but only if a sample of unadulterated butter of the same area and season is available. Bindal and Wadhwa (1993) characterized goat, cows and buffalo milk using such methods.

The development of the classical Babcock test for the determination of the total fat content is summarized by Rosenthal and Rosen (1993). The application of near-infrared spectroscopy (NIR) in the detection of foreign fat in milk fat was shown by Sato *et al.* (1990). Using absorptions at specific wavelengths, the author achieved a method for the quantification of foreign fat in milk fat with a detection limit of 3%. Applying NIR and a sophisticated data evaluation using the PLS-algorithm (partial least square) for the determination of the total fat content of milk is shown by Carl (1991). The author stated a relative standard deviation of 2% for this analysis. Luinge *et al.* (1993) and Van de Voort *et al.* (1992) established a FTIR method for an easy and fast determination of the total fat content in milk.

No efforts will be made here to include the publication for the application of NMR on the analysis of triglycerides. The interested reader is referred to comprehensive and recently published reviews (Eads, 1991; Neff *et al.*, 1992; Henderson *et al.*, 1994) and literature cited therein.

IDENTIFICATION OF TRIGLYCERIDES

This section contains also some references regarding oils of various plants as the most probable source of foreign fat fraudulently added to milk fat. Efforts have been made by several authors to identify as most as possible of individual triacylglycerols of the huge variety in milk fat (Barron *et al.*, 1990; Jensen *et al.*, 1991; Maniongui *et al.*, 1991; Bornaz *et al.*, 1992; Gresti *et al.* 1993; Kermasha *et al.*, 1993), animal fat (Perrin & Naudet, 1983) and vegetable oils (Damiani *et al.*, 1994; Maurin *et al.*, 1992). Laakso and Kallio (1993*a*,*b*) investigated in detail the occurrence of *cis* and *trans* isomers of monoenoic and dienoic triacylglycerols. All these authors applied a combination of RP-HPLC and/or Ag^+-HPLC and/or c-GC for the identification of triacylglycerols. The potential using RP-HPLC-NMR

for the identification of triglycerides was shown by Neff *et al.* (1993) and that of GC-MS by Kalo and Kemppinen (1993).

Hilditch (1956) proposed a 1,3-random-2-random distribution of the fatty acids within the triacylglycerols. This means that for a given fatty acid profile of a fat sample the occurrence of the corresponding triacylglycerols can be calculated by statistically distributing the fatty acids on the three position within the triacylglycerol, assuming that the fatty acid occupying the 1 position always equals the fatty acid in the 3 position. This statistically most probable composition of triglycerides and its real occurrences has been compared for milk fat by Gresti *et al.* (1993) and for triglycerides of edible oils by Cortesi *et al.* (1990), Carelli and Cert (1993), Zeitoun *et al.* (1991) and Santinelli *et al.* (1992). The actual distribution of the fatty acids within the triglycerides follows only in general terms the path of the random distribution, there are always some differences due to biochemical reasons.

There has been some efforts to identify these triglycerides, which shows the greatest variation in concentration due to feeding conditions. Hinrichs *et al.* (1992) showed that the difference between summer and winter butter fat could be found mainly in two HPLC fractions with the partition number 48. Bornaz *et al.* (1992) applied HPLC on French butter fat of different seasons and identified seven peaks out of 46 identified which reflects the differences in the triglyceride composition due to seasonal variations in feeding.

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