

## Quantitative aspects of triglyceride analysis by gas–liquid chromatography using a short metal capillary column

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### Abstract

The performance of a gas–liquid chromatographic system employing a short mega-bore metal capillary column for the analysis of the triglyceride (TG) profile of milk fat was studied. By utilising experimental design, significant effects of the composition of the flame ionization detection (FID) fuel gases on quantitation of the analytes were observed. This was attributed to the dynamically changing gas mixture composition reaching the detector as a result of a reduction in the flow-rate of the carrier gas due to temperature programming. Provided that FID fuel gases were optimised, theoretical response factors for a series of saturated TGs were obtained. Furthermore, it was shown that the “effective carbon number” of saturated TGs equals the total number of C atoms minus the three carboxyl C atoms. Analytical precision expressed as the relative standard deviation of repeated milk fat analyses was <1% for TG groups representing >1% of the total TG profile.

*Keywords:* Factorial design; Milk; Triglycerides; Glycerides; Lipids

### 1. Introduction

Analysis of triglycerides (Tgs) by gas–liquid chromatography (GLC) is an attractive technique for the determination of certain quality parameters of fats and oils. In particular, separation of TGs according to the combined chain lengths of the constituent fatty acid moieties (acyl-C-number) by packed column GLC provides the basis for an EU-wide reference method for the determination of milk fat purity [1]. A similar technique is also used by the chocolate industry to discriminate between pure cocoa butter and cocoa butter equivalents [2].

TGs may be analysed by packed or by capillary column GLC [3,4]. Traditionally, non-polar stationary phases (e.g. methylpolysiloxanes) have been used

for this purpose, primarily due to their excellent thermostability. In the first place, these phases separate TGs according to their molecular mass. By using non-polar phases in packed column GLC, isomeric TGs of a given molecular mass are grouped into one peak, while GLC using capillaries coated with similar phases give more detailed information about the isomers present within one TG group. Thermostable, polarizable columns allow the most detailed analysis of TGs, as they are capable of separating TGs according to the acyl-C-number and the number of double bonds present in the constituent fatty acids [5]. In some cases, as in the method laid down in Council Regulation (EC) 454/95 [1], the analyst is not interested in obtaining a high resolution of isomeric TGs, but in a reliable and precise acyl-C-number separation. Therefore, Council Regulation (EC) 454/95 [1] describes packed

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column technology for TG profiling of milk fat, but allows the optional use of capillary columns, provided equivalent results are obtained. Operating conditions have been devised for a short wide-bore (5 m×0.53 mm) metal capillary column [6,7] which gives separation results that compare favourably to those accomplished with a packed column. According to these and other authors [8,9], key factors governing the qualitative as well as the quantitative outcome of TG analysis by GLC are the injection technique, the type of carrier gas and its flow-rate, the temperature programme, column length, and the amounts and molecular mass(es) of the analyte(s). Cold on-column injection (OCI) proved to be the most suited sampling technique, capable of avoiding discrimination effects as observed with split or splitless injectors [8]. Use of highly inert fused-silica capillary columns minimise losses in the chromatographic process due to irreversible adsorption of the analytes, at least for saturated Tgs.

In general, quantitation in GLC is based on the relationship between the mass of an analyte and the detector response produced by it. Provided analyte losses during sampling and chromatography are excluded, the response for a substance in the flame ionization detection (FID) system is largely governed by the content of ionizable (“active”) carbon atoms of the analyte [10]. This concept is valid for quantitative GLC of fatty acid methyl esters (FAMES) [11], with the exception of short-chain FAMES [12]. Albertyn et al. [13] pointed out that the composition of the FID fuel gases affects the detector response of FAMES significantly. It is known that in temperature-programmed GLC, column flow-rate decreases with an increase in temperature, when applying a constant inlet pressure to the capillary column. A varying column flow inevitably leads to an alteration in the composition of FID fuel gases. This, in turn, may well cause a change in detector response. Only very modern GLC equipment offers electronic pressure control as an option, which allows one to keep column flow constant. Since no information is available regarding the extent to which dynamically changing FID fuel gases may influence the quantitation of TGs, we addressed this problem by using a short mega-bore capillary column (5 m×0.53 mm), as specified as an option to packed columns in Council Regulation (EC) 454/95

[1], as a model. Furthermore, to economise on the experimentation required, a factorial design was used to reveal influential factors.

## 2. Experimental

### 2.1. Materials

High purity (99%) standard substances (tricaprylin, C<sub>24</sub>; tricaprin, C<sub>30</sub>; trilaurin, C<sub>36</sub>; trimyristin, C<sub>42</sub>; tripalmitin, C<sub>48</sub> and tristearin, C<sub>54</sub>) were obtained from Sigma (Sigma-Aldrich, Vienna, Austria). TG mixtures consisting of approximately equal proportions of each individual TG were prepared gravimetrically and dissolved in *n*-heptane (Merck, Vienna, Austria). The total TG concentration was 10 mg/ml. Theoretical response factors (TRFs) to convert area% for individual TGs into % (w/w) were calculated according to ref. [10] and were normalised to C<sub>42</sub>.

### 2.2. Methods

A 5 m×0.53 mm Ultimet HT-SimDist CB capillary column, film thickness 0.17 µm (Chrompack Analytika, Gerasdorf, Austria), operated in a Fisons/Carlo Erba 5300 gas chromatograph (Fisons, Vienna, Austria) equipped with OCI and FID systems was used for TG separations. The FID signal was processed by a DP 700 computing integrator in connection with the WINner software for chromatogram storage and data re-processing (Thermo Separation Products, Vienna, Austria). Samples were injected at a column temperature of 80°C. After an isothermal period of 1 min, the temperature was increased at a rate of 50°C/min to 190°C, held there for 1 min and then programmed at 6°C/min to a final temperature of 350°C (held there for 6 min). Secondary cooling was turned on 2 min before and turned off immediately after completion of sample injection (0.5 µl, 5 µl OCI syringe). The FID temperature was set at 360°C. Gas flow-rates were measured with a soap-bubble meter at the detector base.

Experiments were designed as outlined by Box et al. [14] and Scheffler [15]. The designs were evaluated by means of the Statgraphics plus for Windows,

Ver. 2.0 software package (Manugistics, Rockville, MA, USA).

### 3. Results

In the first “scouting” experiment, the effects of three variables, i.e. (a) nature of the carrier gas ( $N_2$  vs.  $H_2$ ), (b)  $H_2$  flow to the FID system and (c)  $N_2$  flow to the FID system, were explored with a  $2^3$  factorial design. The air flow-rate was held constant at 240 ml/min throughout the experiments. The design was blocked in factor (a) since it was impractical to change over the carrier gas line between runs. The factor levels along with the response variable are given in Table 1. Total error (TE), defined by Craske and Bannon [11] as  $\sum \text{abs}(C_i - c_i)$ , where  $C_i$  is the known % (w/w) of an individual TG in the calibration mixture, and  $c_i$  the % (w/w) found by converting GLC area% using only TRFs, was used as the response variable.

In the experimental region explored, the nature of the carrier gas ( $H_2$  vs.  $N_2$ ) had the greatest effect on TE (Table 1). In all experiments where  $N_2$  was used as the carrier, TE was lower compared to the runs employing  $H_2$  as the carrier gas. Further analysis of Table 1 revealed a strong interaction effect between the flow-rates of the  $N_2$  make-up and the  $H_2$  fuel gas to the FID system. Therefore, a second experiment, with the FID gases as experimental variables, was set-up for an in-depth study of the experimental domain. A  $2^2$  plan augmented with four axial points (star design) was chosen. The levels for  $N_2$  were (coded level values in brackets): 60 (–1) and 120

Table 2

Design matrix for  $2^2$  star design with centre point experiments (run 9)

Run	FID- $N_2$ (kPa)	FID- $H_2$ (kPa)
1	60	40
2	120	40
3	60	80
4	120	80
5	50	60
6	130	60
7	90	30
8	90	90
9	90	60

Analyses were performed in a random order.

(+1) kPa head-pressure and for  $H_2$ : 40 (–1) and 80 (+1) kPa head-pressure. Star points were placed at level values (coded) of  $\sqrt{2}$ . The complete design matrix is given in Table 2. Experimental points were run in duplicate except for the centre point (four replicates). The resulting response surface had the form of a saddle (Fig. 1) with an optimum (TE < 1%) in the region defined by a FID- $N_2$  head pressure of 100–120 kPa and a FID- $H_2$  head pressure of 70–80 kPa. Some additional experiments with  $N_2/H_2$  (kPa) conditions of 100/70, 110/70, 110/80 and 120/80 were run and all of them resulted in TE values < 1. The smallest value, i.e. TE = 0.84, was obtained with 120 kPa  $N_2$ , corresponding to a flow-rate of 32 ml/min, 80 kPa  $H_2$  (= 37 ml/min) and an air-flow of 240 ml/min. Column flow-rate, measured at room-temperature, was 14 ml  $N_2$ /min (equivalent to 15 kPa head pressure) in these experiments, which decreased to 12.5 ml/min at 80°C (programme start) and 5.1 ml/min at 350°C (programme end).

Table 1

Factors and factor levels used in the  $2^3$  experimental design and resulting total errors (analyses were performed in a random order)

Run	FID- $N_2$ (kPa)	FID- $H_2$ (kPa)	Carrier gas	Total error
1	60	40	$N_2$	1.47
2	120	40	$N_2$	2.90
3	60	80	$N_2$	3.41
4	120	80	$N_2$	1.28
5	60	40	$H_2$	2.99
6	120	40	$H_2$	3.65
7	60	80	$H_2$	4.49
8	120	80	$H_2$	4.61

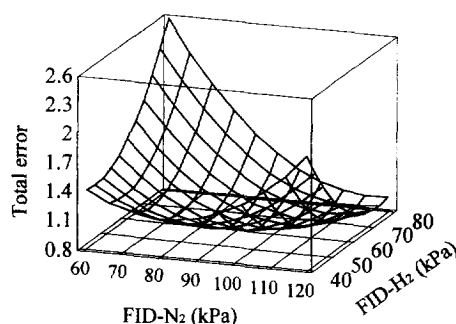


Fig. 1. Response surface of the fitted second-order equation of the star-design describing the interaction of FID fuel gases.

Table 3

Empirical response factors (mean of four replicates, given along with relative standard deviations; R.S.Ds.) of saturated triglycerides (normalised to C<sub>42</sub>)

Triglyceride	Response factor		R.S.D. (%)
	Theoretical	Empirical	
C <sub>24</sub>	1.139	1.140	0.301
C <sub>30</sub>	1.074	1.065	0.275
C <sub>36</sub>	1.031	1.051	0.233
C <sub>42</sub>	1.000	1.000	—
C <sub>48</sub>	0.977	0.992	0.235
C <sub>54</sub>	0.959	0.964	0.389

Empirical RFs obtained with the optimised parameter set were in excellent agreement with the TRFs (Table 3).

To test the appropriateness of these operating conditions for real samples, a bovine milk fat dissolved in *n*-heptane (10 mg/ml) was injected five times in series and the standard deviation of the peak area% was calculated as a measure of analytical precision. Standard deviation ranged between 0.002 area% for cholesterol and 0.053 area% for C<sub>52</sub>. Relative standard deviations varied between 0.11 for C<sub>48</sub> (at a concentration level of 9.22%) and 5.76 for C<sub>26</sub> (at a concentration level of 0.23%). Long-term stability of the separation system was checked by injecting the TG standard mixture twice a week over a six-week period. The mean value for TE was 0.776, with a standard deviation of 0.091.

#### 4. Discussion

TG profiling of fats and oils is often aimed at getting an in-depth knowledge about the occurrence and distribution pattern of individual TG species. Accordingly, the whole GLC system is tuned with a view to attaining maximum separation power. By using capillary columns coated with a polarizable stationary liquid, Geeraert and Sandra [5] opened up new perspectives in TG analysis. In the case of bovine milk, fat with its huge number of individual TG species, GLC is mostly supplemented by some pre-fractionation techniques to resolve this very complex mixture, at least partially [16,17]. If separation according to the acyl-C-number is all that is needed, e.g. to control purity of a fat, the accuracy

and precision of the analytical method are usually more important than high resolution. Operating conditions to achieve this goal are rarely dealt with in the literature. Molkentin and Precht [6,7] optimised a method employing a short metal capillary column to obtain TG separations resembling those obtained by using packed columns. Parameters optimised were injection temperature, shape of the temperature programme, carrier gas-flow and the amount of sample injected. As an optimum set of operational parameters for a 5-m mega-bore column, sample introduction by OCI, a temperature programme of 80°C (0.5 min isotherm)–50°C/min to 190°C–6°C/min–350°C (5 min isotherm), N<sub>2</sub> at 3 ml/min as the carrier gas, and a 0.5- $\mu$ l injection volume of a 1% milk fat solution in *n*-heptane was recommended. With these conditions, a low resolution, similar to that obtained by using 50 cm packed columns, and high repeatability of mass-fractions of milk fat TG were achieved. Most of the RF values reported were close to unity, though some of them deviated to a greater extent when sample mass was either low or high (0.5  $\mu$ l injections of 0.1 or 1.8% milk fat in *n*-heptane). By using 10–25 m capillary columns, other authors [18–21] found RF values close to unity for medium-molecular-mass TGs and more or less elevated values (around 1.1 to 1.5) for those with low and high molecular masses.

In a carefully conducted study, Mareš and Hušek [9] demonstrated how carrier gas flow-rate, shape of the temperature programme, sample composition and sample overload influenced the recovery of saturated TGs, and concluded that most of the factors influencing recovery are constant during a single analysis, except sample composition and concentration. Another factor that varies in a temperature-programmed capillary GLC run is column flow. Therefore, Mareš [22] speculated that changing column flow-rates may also affect recovery of TGs.

Though it is well known that the ionisation efficiency of a given compound and hence its FID response is influenced by the FID flame temperature and the flame geometry, most instrument optimisation strategies pay only little attention to the ratio of H<sub>2</sub>, air and make-up gases, which govern the aforementioned properties [23,24]. Albertyn et al. [13] demonstrated that the H<sub>2</sub> fuel gas flow exerted a remarkable effect on the accuracy of FAME analysis

on packed columns. Such strong effects of the gas flow-rates on the overall accuracy of TG analyses on short wide-bore capillary columns were also observed in this study. During temperature programming, column flow decreased considerably, since the gas chromatograph used was equipped with a pressure regulated pneumatic system. Consequently, we tried to adapt the FID fuel gases in such a way that the dynamic change of the composition of the gas mixture reaching the FID system and its effect on ionization efficiency was equalized as much as possible. The actual flow-rates found to be optimal may be peculiar to the instrument used. Nevertheless, the effects observed are general in nature and should hold true in all cases where a pressure-controlled system and short, wide-bore capillary columns are in use.

The response variable in the experimental designs is computed with the help of TRF values and, therefore, relies on the assumption that the concept of TRFs, as outlined by Ackman and Sipos [10], is generally true. With an optimised flow of fuel gases, the deviation of the empirically obtained values from the TRFs was negligible (less than 2% of the theoretical value) and was within experimental error. Thus, we conclude that the “effective carbon number”, calculated as described in ref. [25], of saturated triglycerides, is the number of carbon atoms minus three. The carboxyl C atoms do not contribute to the FID response, which has also been observed for methyl esters of medium- and long-chain fatty acids [10–12]. Bannon et al. [26] demonstrated that TRFs are also valid for the GLC analysis of unsaturated fatty acid esters. By analogy, the assumption that TRFs are also applicable to unsaturated TGs should, in principle, hold true, but thermal degradation reactions may lead to diminished recovery, especially with highly unsaturated TG species. Nevertheless, the ability to compute FID response factors on a theoretical basis is advantageous for several reasons. The most important is that a comparison of empirical and theoretical RF values is a simple but highly effective means to check whether mechanisms that

compromise recovery in the chromatographic system (irreversible adsorption, decomposition or discrimination) occur to a measurable extent.

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