Research Final Report
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Project Title **Factors Affecting the Content of *Trans* Fats in Eggs**

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Executive Summary

The studies have been carried out to emphasize a proper methodology for determining *trans* fats, to determine natural levels of *trans* fats in egg without added *trans* fat in the hen's ration, and to ascertain the effect of feeding various types of fats on *trans* fats levels in eggs. Hens were fed with diets containing five different types of fats, varying in *trans*-fat levels. The diet and egg samples were analyzed for *trans*-fatty acids by two different methods, i.e, Gas Chromatograph (GC) system and Fourier Transform Infrared (FTIR) spectroscopy. The *trans*-fat contents of standard ration with 0% added fat/oil, and /or the ration containing added soybean oil, and the egg samples obtained from the hens fed with these diets were below the detection limit of the GC system. Thus, hens fed with *trans*-fat free diets produced eggs that did not contain detectable levels of *trans*-fats. The *trans*-fat levels in the other diets (standard laying ration + tallow, standard ration + shortening, and standard ration + tallow-shortening blend) were found to be 2.78%, 3.25% and 2.85% *trans*-fat/total fat, respectively, whereas the egg yolk from hens fed with these diets showed only 0.60%, 0.78% and 0.73% *trans*-fat/total fat, respectively. Therefore, about a quarter of *trans*-fats available in each ration were deposited in egg yolks of hens consuming these rations. The concentrations of *trans* fats determined by GC were below the limit of detection for FTIR method. A correlation coefficient of 0.988 was found between GC and FTIR results of diets containing tallow, shortening, and tallow-shortening blend and pure fat samples. No correlation could be made with the egg yolk data as the low levels of *trans*-fat in egg yolk could not be quantified by FTIR method.

Lay Summary

Hens were fed with five different experimental diets with and without added trans fats. The fats extracted from diets, and eggs obtained from the hens fed with these rations were analyzed for trans fats by two different methods, namely Gas Chromatography (GC) and Fourier Transform Infrared spectroscopy (FTIR). The trans fats were not detected in the rations containing no added fat and/or ration containing soybean oil. Trans fats levels in other rations containing tallow, shortening, or tallow-shortening blend were found to be 2.78%, 3.25% and 2.85% trans fat/total fat, respectively, whereas, the egg yolk from hens fed with these rations contained only 0.6%, 0.78%, and 0.73% trans fat/total fat, respectively. Therefore, about a quarter of trans-fats available in each ration were deposited in egg yolk of hens consuming these rations.

Technical Report

Objectives

The project objectives are summarized as follows: Development of GC-FID and FTIR methodologies for determination of *trans* fat levels in feed samples, pure lipid samples and hens' eggs as well as correlating GC-FID and FTIR methods; Determination of natural level of *trans* fat in eggs without added *trans* fat in ration; Ascertain the effect of feeding various types of fats on *trans* fat levels in eggs.

Materials

Soybean oil and shortening for feed trials were purchased from Kroger's (Athens, GA). Tallow was obtained from Welch, Holme & Clark Co., Inc. (Newark, NJ). ACS-grade anhydrous methanol, hexanes, chloroform, carbon disulfide, and sulfuric acid were purchased from Fisher Scientific Ltd. (Fair Lawn, NJ), whereas anhydrous sodium sulfate and sodium hydroxide were acquired from VWR International Inc. (Suwanee, GA). Hydroquinone, 14% (v/v) boron trifluoride in methanol, and a Supelco 37 component FAME mixture were purchased from Sigma Chemical Company (St. Louis, MO). Triheptadecanoin (>99%), methyl heptadecanoate (>99%), methyl oleate (>99%), and methyl eliadate (>99%) were acquired from Nu-Chek Prep (Elysian, MN). Industrial-grade nitrogen, scientific air, UHP helium, and UHP hydrogen were obtained from Airgas National Welders (Toccoa, GA).

Feeding Trials

A population of Single Comb White Leghorn (SCWL) laying hens was maintained on a diet free of animal by-products for a period of 30 days. After that time, hens were divided into five groups of ten each and were fed with diets containing different types of fats,

varying in *trans*-fat levels. The five diets were as follows: I Standard corn/soy laying ration with 0% added fat/oil; II Standard corn/soy laying ration with 4% (w/w) added soybean oil; III Standard corn/soy laying ration with 4% (w/w) added tallow; IV Standard corn/soy laying ration with 4% (w/w) added shortening; and V Standard corn/soy laying ration with 4% (w/w) added tallow-shortening blend. During the feeding period both feed consumption and egg production were monitored, as was any gain or loss in body weight. Each hen was individually leg banded so as to ensure proper identification. Eggs from each of the 5 groups of hens were collected on day 7, 14, and 21. Five egg yolks from each of the 5 groups were pooled and homogenized separately before analyzing for *trans*-fats.

Lipid Extraction

Total lipids were extracted by the Bligh & Dyer method [1] with slight modifications. Briefly, 25 g of separated egg yolks were weighed into a 250 mL Erlenmeyer flask. Thirty five milliliters of deionized water were added to adjust the moisture content to 80% and then contents were blended with a Polytron® PT 3100 homogenizer for 1 min at a speed of 15,000 rpm (Kinematica, Inc. Bohemia, NY). Fifty milliliters of methanol and 25 mL of chloroform were added creating a monophasic system. Another 25 mL chloroform followed by 25 mL deionized water were added to create a diphasic system with the lower phase comprising practically 100% chloroform and the upper phase methanol-water. Finally, an additional 35.5 mL of chloroform were added to aid in separation. The egg yolk mixture was homogenized between each addition of solvents for complete lipid extraction. A 2 min quiescent period facilitated separation before filtering through a Büchner funnel lined with Whatman No.1 filter paper under slight vacuum.

The filter paper and solids were returned to the Erlenmeyer flask. To this, 37.5 mL chloroform and ~10 mg of hydroquinone (as antioxidant) were added. The contents were blended for 1 min and again filtered as described above. The supernatant containing any residual non-polar lipids was transferred to a 250 mL separatory funnel and the phases were allowed to separate overnight. The bottom chloroform layer was collected through anhydrous sodium sulfate to remove any remaining moisture. The lipid extract was then transferred to a 250 mL round bottom flask and concentrated with a Büchi Rotovapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (BÜCHI Corporation, New Castle, DE) at 35°C and 250 mbar. Collected lipids were transferred to a 20 mL amber vial and the chloroform was evaporated off with an N-EVAP 111 nitrogen evaporator (Organomation Associates, Inc. Berlin, MA). Lipids were extracted in triplicates (n=3) and were stored under a nitrogen headspace at -80^{o}C to retard lipid oxidation.

Fatty Acid Methylation

Fatty acid profiles of egg lipids were determined by GC-FID after converting triacylglycerols [TAGs] to their respective fatty acid methyl esters [FAMEs] [2]. Approximately 80 mg of extracted lipids were weighed into a 5 mL Reacti-vial^{TM} (Pierce, Rockford, IL). One hundred microliters of 20% (w/v) triheptadecanoin in hexanes were added to each Reacti-vial^{TM} as an internal standard. The TAGs were then hydrolyzed and derivatized with 2 mL transmethylation reagent consisting of 6% (v/v) concentrated H_2SO_4 in anhydrous methanol with ~5 mg of hydroquinone. A magnetic stir bar was placed in each Reacti-vial^{TM}; the vials were incubated at 65°C overnight with stirring on a Pierce Reacti-Therm III incubating block (Pierce, Rockford, IL, USA). The next day,

samples were removed from the incubator and allowed to cool to room temperature. One milliliter of deionized water was added to each Reacti-vial™ and the contents were vortexed for ~30 s. FAMEs were extracted three times with 1.5 mL portions of hexane. The hexane layers were collected and then washed twice with 1.5 mL of deionized water. The hexane layer was collected in a clean test tube, and then dried under nitrogen gas using the N-EVAP nitrogen evaporator. The FAMEs were dissolved in 3 mL of carbon disulfide. A 100 µL aliquot of this was added to a 2-mL amber GC vial, diluted with 900 µL carbon disulfide, and the vial was then capped and crimped.

Gas Chromatographic Analysis

FAMEs from diet and egg samples were analyzed for *trans*-fatty acids using an Agilent Technologies 6890N Network GC System with capillary split/splitless inlet with electronic pneumatics control (EPC) and a flame ionization detector [FID] with EPC for packed and capillary columns (Agilent Technologies, Wilmington, DE). A 100 m, highly polar, biscyanopropyl column with an inner diameter of 0.25 mm and a film thickness of 2 µm (SP-2560, Supelco, Bellefonte, PA) was employed. The initial oven temperature was 140°C and was held for 5 min. Then, the temperature was ramped at 5°C/min to a final temperature or 240°C, which was maintained for 15 min resulting in a total runtime of 45 min. The injector was heated to 250°C. An Agilent 7683 autosampler tray module equipped with a 7683B auto-injector module and a 10 µL syringe was employed for injecting 1 µL samples through a pre-pierced 11 mm inlet septum (P/N: 5183-4761-100) into the GC inlet containing a split liner packed with glass wool (P/N: 5183-4647). The GC was operated in split mode at a split ratio of 50:1 with a carrier gas (*i.e.*, helium) flow of 59.2 mL/min. The column pressure was 40.12 psi. The detector was heated to 250°C

and the FID flame was generated from hydrogen at a flow of 40 mL/min, air at 450 mL/min, and nitrogen makeup gas flow of 23.9 mL/min. A Supelco 37 component FAME mixture was used to identify and quantify individual FAMEs by retention time mapping. A relative response factor was calculated for each FAME using methyl heptadecanoate as an internal standard. Response factors are the concentration of an analyte divided by the response to that analyte.

$R_i = Ws_i / Ps_i$

where, R_i = response factor for fatty acid methyl ester i; Ws_i = mg of FAME i injected into the inlet; Ps_i = peak area corresponding to FAME i.

A relative response factor is defined as the response factor of the internal standard divided by the response factor of the analyte.

$RRf_i = Rf_{is}/Rf_i$

where, RRf_i = relative response factor for fatty acid methyl ester i; Rf_{is} = response factor for the internal standard; and Rf_i = response factor for fatty acid methyl ester i. Each FAME will have a different response to the FID depending on chain length, saturation, and *cis/trans* configuration. For positively-identified compounds, employment of relative response factors improves the accuracy of the results. For unidentified compounds the relative response factor for the closest positively identified fatty acid was used.

Linearity

The linearity of the peak area response versus concentration for the standards triheptadecanoin were studied from 75-600 μg/mL. The calibration curve was generated with eight concentrations (75, 150, 225, 300, 375, 450, 525, 600) using least-squares

method. The correlation coefficient, *r*, was 0.999 (*n* = 8), slope 0.4033 and intercept was -13.441.

Precision and accuracy

Assay precision was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day was evaluated by assaying samples, at same concentration and during the same day. The inter-day was studied by comparing the assay on 3 different days [3]. The accuracy of this analytic method was evaluated by checking at three different concentration of triheptadecanoin. The intra-day relative standard deviation (RSD) was <0.006% for triheptadecanoin (n=3) and the inter-day relative standard deviation (RSD) was <0.04% (n=3). Table 1 presents precision and accuracy results.

Table 1. Precision and accuracy for intra-day and inter-day sampling

	Intra-day		
Added (µg/mL)	Measured	Precision (%RSD)	Accuracy
160	151.85±0.53	0.0035	-0.0178
320	309.47±1.72	0.0055	-0.0182
520	548.08±0.61	0.0011	0.0060
	Inter-day		
Added (µg/mL)	Measured	Precision (%RSD)	Accuracy
160	142.34 ± 4.96	0.0349	-0.3847
320	304.92 ± 7.56	0.0248	-0.1169
520	517.89±10.27	0.0198	-0.0081

Limit of Detection (LOD) and Limit of Quantification (LOD)

LOD and LOQ were determined by an empirical method that consisted of analyzing a series of standard solutions containing decreasing amounts of triheptadecanoin. LOD and LOQ are defined as having a signal to noise ratio of 3 and 10 to 1 respectively [4]. The LOQ, defined as the lowest concentration of measured value of standard solutions was 6 µg/mL and the LOD of the standard triheptadecanoin solutions was approximately 3 µg/mL.

Recovery

The analytical recovery of triheptadecanoin was assessed by direct comparison of concentrations of triheptadecanoin to methyl heptadecanoate using three replicates at four concentration levels (75, 150, 300, and 600 µg/mL). Analytical recoveries are shown in table 2. Recoveries of triheptadecanoin were 81.33% ± 1.5%.

Table 2. Recovery of triheptadecanion compared to methyl heptadecanoin

	Wt. injected (ng)	Wt. recovered (ng)	Recovery %
FAME	600	600.07	100.01
FAME	300	298.82	99.61
FAME	150	151.59	101.06
FAME	75	74.55	99.40
TAG	600	478.69	79.78
TAG	300	245.63	81.88
TAG	150	122.91	81.94
TAG	75	61.31	81.74

(n=3)

Fourier Transform Infrared Spectroscopy [FTIR] Analysis

Extracted lipids from diet and egg samples were transmethylated according to AOCS official method, 1997 [5]. This method was used for FTIR analysis because it is completed in a shorter amount of time, thereby allowing the FTIR method to maintain its rapidity in analyzing *trans*-fats. Moreover, a greater quantity of sample can be transmethylated by this method at a time which is important because a larger quantity of sample is needed for FTIR analysis compared with GC analysis. One gram of egg lipids were transferred to a 250 mL flat bottom flask. Ten milliliters of 0.5 N sodium hydroxide in anhydrous methanol and several glass boiling beads were added to the flask and then connected to a condenser. The mixture was refluxed for 10 min, forming free fatty acids from triacylglycerols. A subsequent addition of 12 mL of 14% (v/v) boron trifluoride in methanol, the transmethylating reagent, was made through the top of the condenser. After 2 min, 5 mL hexane was introduced and the mixture was refluxed for an additional

minute. The contents were transferred to a 250 mL separatory funnel where FAMEs were extracted twice with 50 mL portions of hexane. The hexanes layer was passed through anhydrous sodium sulfate to remove any moisture and collected in a 100 mL round bottom flask. The hexanes were evaporated off using the Büchi Rotovapor system, and solvent residue was removed under a stream of nitrogen with the N-EVAP.

A 300 μL aliquot of FAMEs was pipetted onto a Thermo Smart Ark Horizontal Attenuated Total Reflectance (ATR) cell installed in a Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific, Rockford, IL). The ATR cell was kept at a temperature of 45°C – 65°C to ensure samples were in a liquid state. The ATR cell was cleaned with a dustless wipe prior to each sampling. Thirty two scans over a wave number range of 4000 to 400 cm^{-1} were taken at a resolution of 4 cm^{-1}. A new background was collected prior to each sample, and the results were expressed as absorbance. A series of standards were made by accurately weighing proportions of methyl elaidate and methyl oleate ranging from 0.5 to 10% methyl elaidate by weight. Using these standards, a calibration curve was generated using the TQ analyst software program. A calibration curve for standards was prepared using the partial least squares regression analysis of second derivative spectra in the wave number range of 978 cm^{-1} to 955 cm^{-1}. The Norris derivative filter was used to smooth the spectra, reducing random noise, thus decreasing the error in the calibration model. The correlation coefficient for the calibration was > 0.998.

Results and Discussion

Gas Chromatography Studies

Over 80% by weight of the lipids in the prepared extract comprises 6 fatty acids, namely myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and tricosanoic acid. Of these, oleic acid is the dominant fatty acid contributing approximately 40% of the fatty acids. Figure 1 depicts a typical chromatogram of egg yolk lipids (*i.e.,* FAMEs) from hens diet IV (*i.e.,* standard corn/soy-laying ration + 4% shortening). A Supelco 37 component FAME mixture was used to identify and quantify individual FAMEs by retention time mapping. Each of the 37 components in the standard was identified and their retention time recorded. Samples' FAMEs retention times were compared to those in the Supelco 37 component FAME mixture. Unidentified peaks can be tentatively identified based on their retention times compared to positively identified FAMEs. Peak 3 is not contained in the Supelco 37 component FAME mixture but lies between palmitic acid, C16:0 (peak 2), and palmitoelic acid, C16:1n-7 (peak 4), it is believed that this peak corresponds to an isomer of palmitoelic acid, C16:1n-9. *Trans* isomers of C18:1 appear before the oleic acid peak while other *cis* isomers appear after it [6]. Therefore, it can be assumed peak 9 corresponds to C18:1n-11, while peak 7 is a coelution of several *trans* isomers of C18:1. When oil is partially hydrogenated the *trans* double bond occurs at several positions on the fatty acid molecule, with carbons 8-12 being the most common [7].

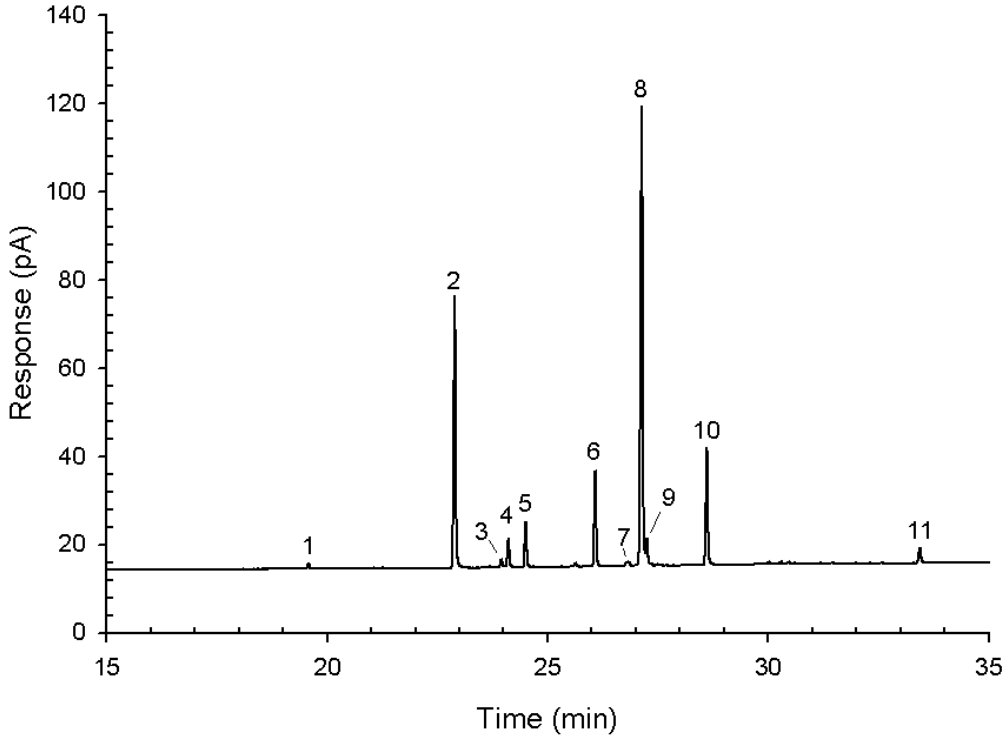

Fig 1. Typical chromatogram of egg lipids.
(Peaks: 2 Palmitic Acid, 6 Stearic Acid, 8 Oleic Acid, and 10 Linoleic Acid)

The *trans*-fat contents of diet I (*i.e.*, standard corn/soy-laying ration with 0% added fat/oil) and diet II (*i.e.*, standard corn/soy-laying ration + 4% (w/w) soybean oil) were below the detection limit for the GC-FID system (Table 3). The *trans*-fat contents of egg samples acquired from the hens fed with either diet I or II for 21 days were also below the limit of detection of the GC system. Hens fed with diets devoid of endogenous *trans*-fats will produce eggs that will contain no natural detectable levels of *trans*-fats. Diets III, IV, and V contained some level of *trans* fats known to come from partial hydrogenation. The *trans*-fat levels in diets III, IV, and V (*i.e.*, standard corn/soy-laying ration + 4% tallow, 4% shortening, and 4% tallow-shortening blend, respectively) were found to be

2.78%, 3.25% and 2.85% *trans*-fat/total fat respectively, whereas the egg yolk from hens fed with these diets showed only 0.60%, 0.78% and 0.73% *trans*-fat/total fat. Therefore, about a quarter of *trans*-fats available in each ration were deposited in egg yolks of hens consuming these diets.

Table 3. *Trans*-fat levels in rations and egg yolk samples of hens

	% *Trans* fat, as determined by GC-FID				
	Diet[†]	Day 7[*]	Day 14[*]	Day 21[*]	Day 21[††]
I	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
II	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
III	2.78 ± 0.08	0.65 ± 0.02	0.67 ± 0.06	0.60 ± 0.03	0.06 ± 0.01
IV	3.25 ± 0.03	0.71 ± 0.02	0.80 ± 0.04	0.78 ± 0.02	0.08 ± 0.01
V	2.85 ± 0.05	0.71 ± 0.01	0.71 ± 0.04	0.73 ± 0.01	0.07 ± 0.01

[†]Diet I – standard corn/soy-laying ration with 0% added fat/oil; Diet II – Standard corn/soy laying ration with 4% added soybean oil; Diet III – Standard corn/soy laying ration with 4% added tallow; Diet IV - Standard corn/soy laying ration with 4% added shortening; Diet V - Standard corn/soy laying ration with 4% A/V blend added. * % *Trans*-fat as g *trans*-fat/g total fat. [††] % *Trans*-fat as g *trans*-fat/g whole egg.

FT-IR Studies

Methods have been developed to determine *trans*-fat percent using neat oils mainly from edible oil processing industries [8]. These methods have been examined for use in identifying the *trans*-fat content in diet and egg samples. Initially, extracted egg lipids were used as such, which eliminated the need to derivatize them into FAMEs. The extracted lipids, however, showed an interference band at 967 cm^{-1} (Fig 2) which interfered with quantification of the *trans*-fats. Therefore, the extracted lipids were transmethylated in an effort to eliminate the interference band. Transmethylated samples showed no interference at the characteristic *trans* absorption band of 966 cm^{-1}.

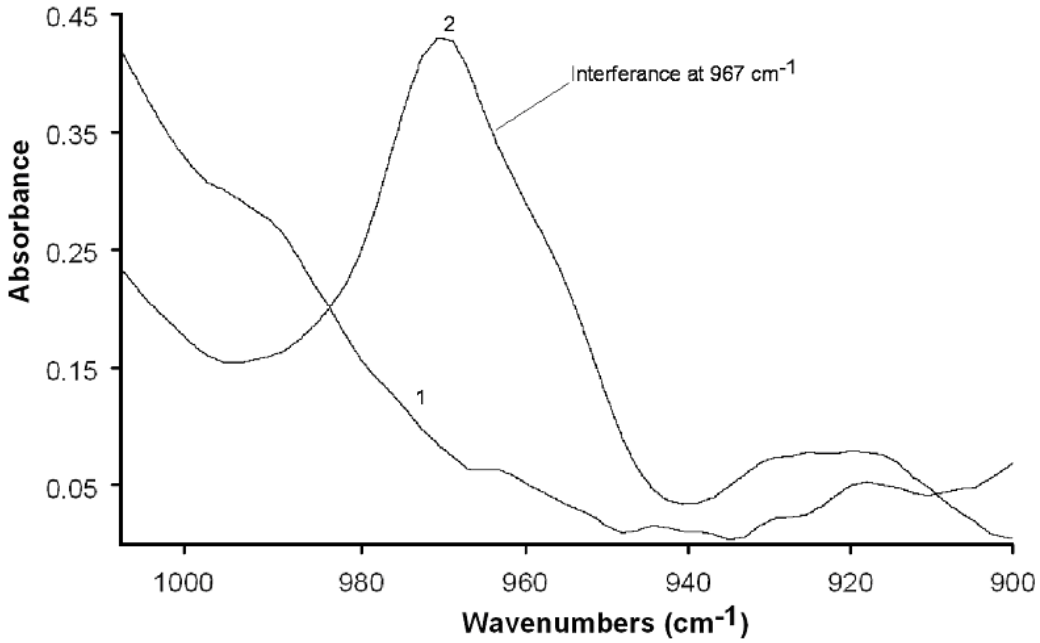

Fig 2. Egg lipids showing the interference band at 967 cm^{-1} in non-transmethylated sample
(1) Transmethylated egg lipids (2) Non-Transmethylated egg lipids

Figs 3 and 4 depict the *trans* band at 967 cm^{-1} of prepared samples as second derivative of absorbance versus wave number and absorbance versus wavenumber, respectively. It can be seen that by taking the second derivative of absorbance the sloping baseline of the IR spectra is removed, making area measurements more accurate at lower *trans* levels and thereby increasing the detection limit of the assays [9].

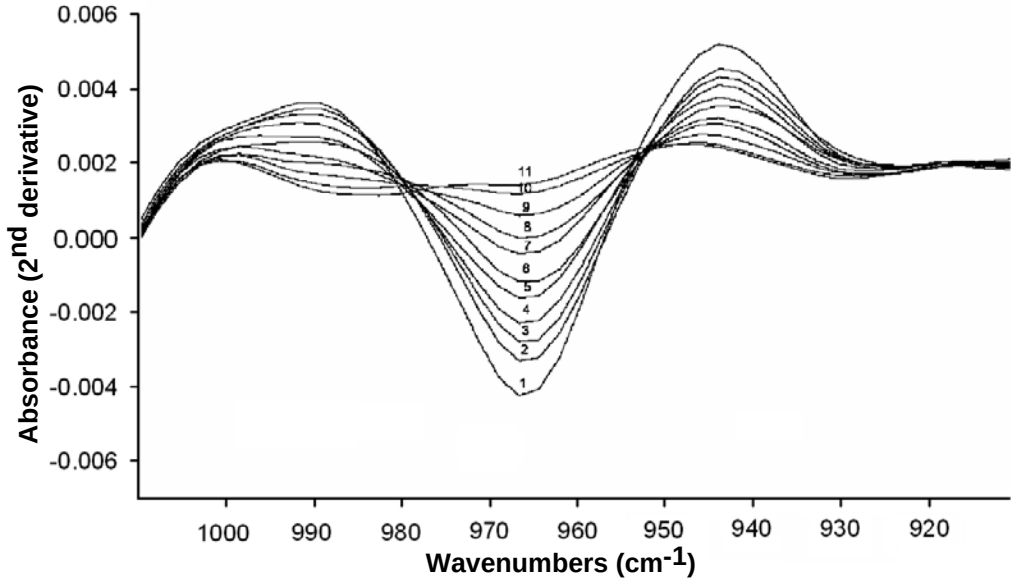

Fig 3. Second derivative spectra of absorbance of *trans* band at 967 cm^{-1} of methyl elaidate in methyl oleate at different concentrations

(1) 10%; (2) 9%; (3) 8%; (4) 7%; (5) 6%; (6) 5%; (7) 4%; (8) 3%; (9) 2%; (10) 1%; (11) 0.5% methyl elaidate.

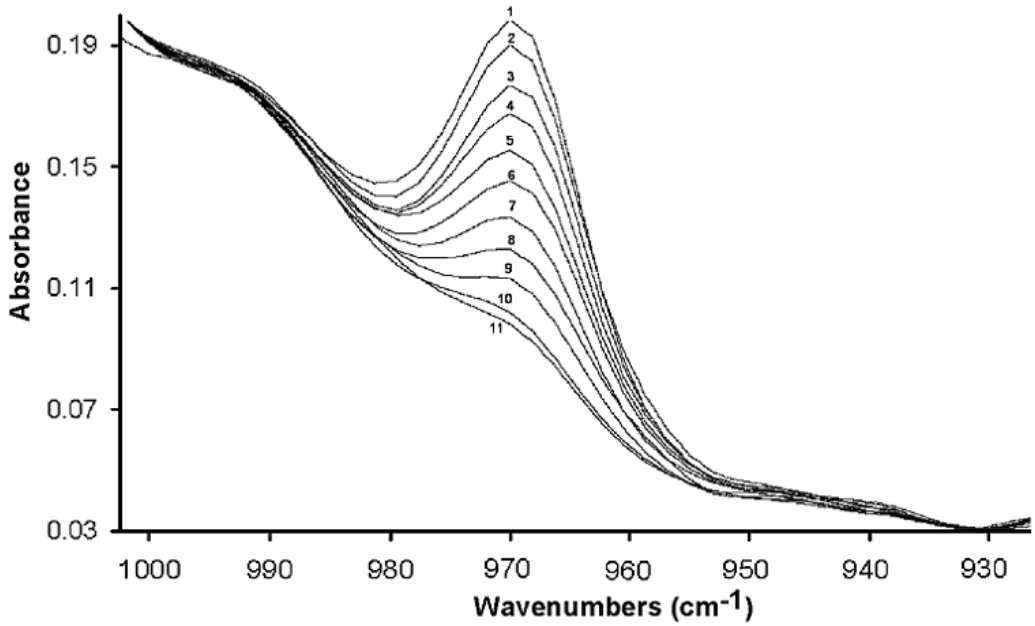

Fig 4. Spectra of absorbance of *trans* band at 967 cm^{-1} of methyl elaidate in methyl oleate

(1) 10%, (2) 9%, (3) 8%, (4) 7%, (5) 6%, (6) 5%, (7) 4%, (8) 3%, (9) 2%, (10) 1%, and (11) 0.5% methyl elaidate

Figure 5 shows the results of the TQ Analyst software for the partial least squares (PLS) regression with and without second derivative of absorbance readings. It can be seen that the PLS curve using the second derivative of absorbance affords slightly less variance below 2% *trans* level. This provides greater accuracy of the detection of *trans*-fat in samples that have trans-fat levels below 2%.

The *trans*-fat content of each diet as well as pure tallow and shortening was determined by FTIR spectroscopy. The results were compared to those obtained by GC-FID and an attempt was made to establish a correlation between these two analytical techniques. The FTIR results for egg yolk extracts fell below the detection limit for the method. FTIR methods have been cited of having a limit of detection of 1% *trans*-fat per gram total fat. The concentrations found by GC-FID fall below the limit of detection for the FTIR method. A correlation coefficient of 0.988 was found when comparing GC-FID results and FTIR results of diet and pure fat samples as depicted in Fig 6. However, no correlation could be made using the egg yolk data because the % *trans*-fats could not be quantified using the FTIR method.

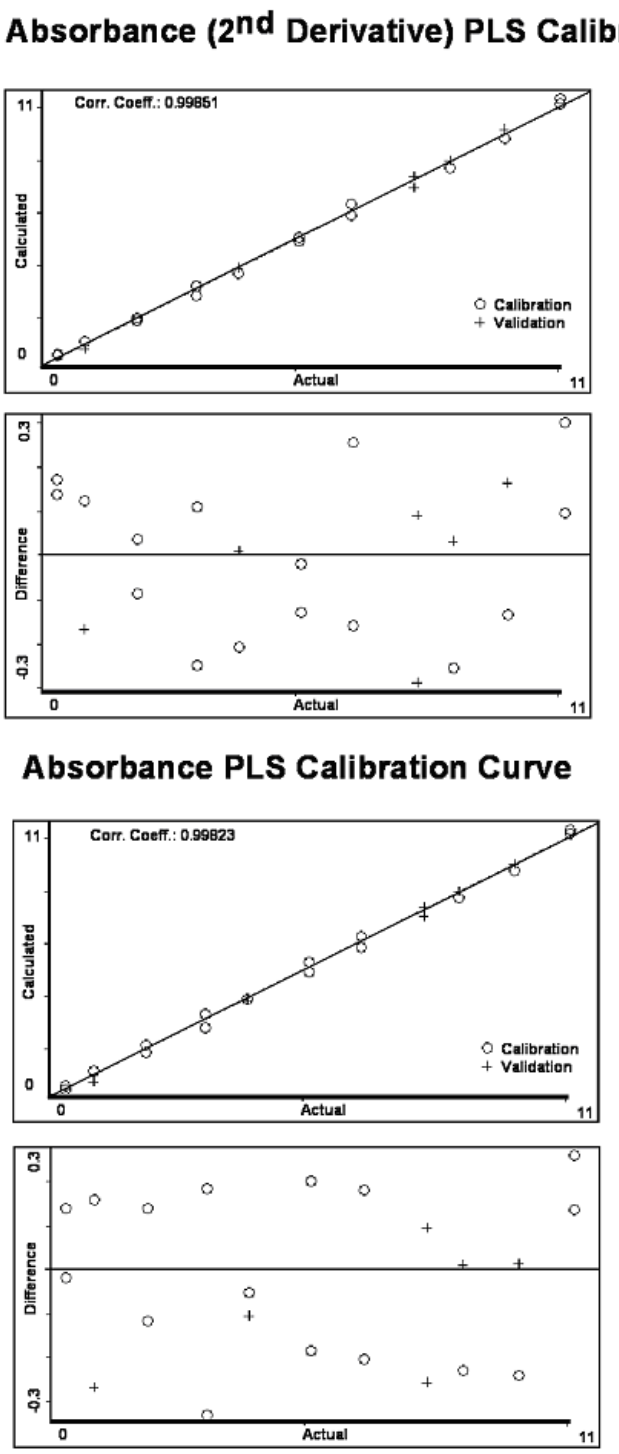

Fig 5. Comparison of calibration curve of *trans* standards using the second derivative of absorbance (above) versus absorbance (below)

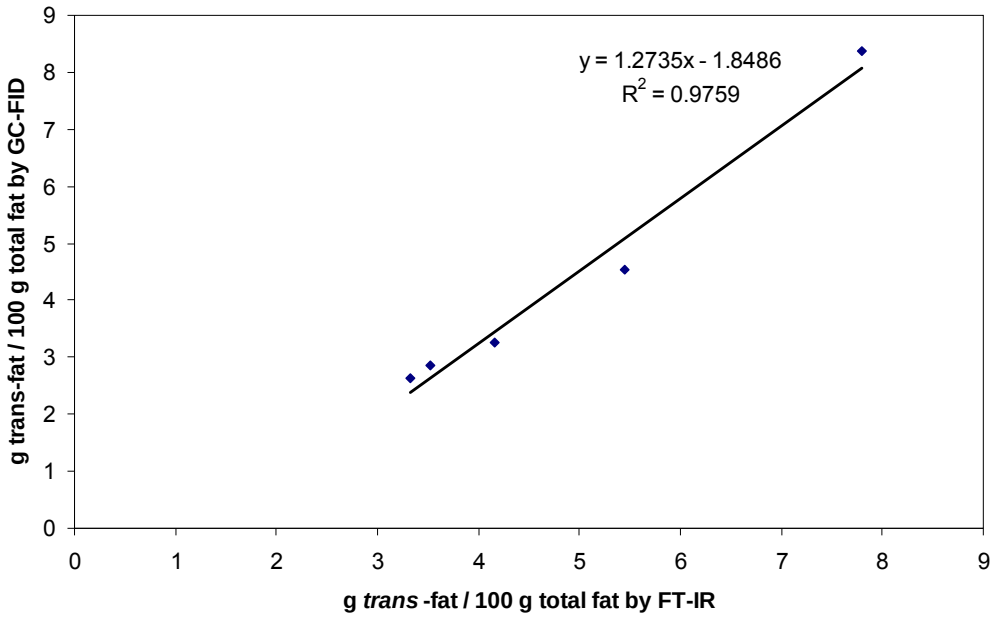

Fig 6. Trans-fat levels in rations and pure fats by GC-FID and FT-IR

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