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METHODS

Quantification of Fatty Acids and their Regioisomeric Distribution in Triacylglycerols from Porcine and Bovine Sources Using ^{13}C NMR Spectroscopy

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Abstract The uptake of specific fatty acids in humans is dependent on their position on the glycerol backbone. There is a great interest in methods that can access this information fast and accurately. By way of high-resolution NMR, we have analyzed TAG extracted from pig and beef tissues and obtained quantitative data for the composition and regioisomeric distribution of all major unsaturated fatty acids usually found in these source materials, using a combination of manual integration and deconvolution of ^{13}C NMR spectra. In addition, we have developed a method for determining composition and regioisomeric distribution of the two main saturated fatty acids found in pork (16:0, 18:0). The results are discussed in relation to species-specific genetic characteristics of fatty acid and TAG biosynthesis. The developed method could support decisions related to breeding for desired fatty acid profiles, and stimulate further methodology developments using high field NMR.

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Abbreviations

DGAT	diacylglycerol acyltransferase
GC	gas–liquid chromatography
GLM	generalized linear model
GWAS	genome-wide association studies
HPLC	high-performance liquid chromatography
APCI	atmospheric pressure chemical ionization
LB	exponential line broadening
LC–MS	liquid chromatography-mass spectrometry
MAG	monoacylglycerol
MUFA	monounsaturated fatty acid
NIRS	near-infrared spectroscopy
NMR	nuclear magnetic resonance
PUFA	polyunsaturated fatty acid
QTL	quantitative trait loci
SCD	stearoyl-CoA desaturase
SFA	saturated fatty acid
sn	stereospecific numbering
SNP	single nucleotide polymorphism
TAG	triacylglycerol

Introduction

During human digestion and absorption of triacylglycerols in food, the distribution of individual fatty acids in the triacylglycerols, and especially the distribution in the *sn*-2 position, is of great importance (Hunter, 2001). Pancreatic

lipase removes fatty acids primarily in *sn*-1 and *sn*-3 positions of the glycerol backbone, leaving 2-monoacylglycerols (*sn*-2-MAG) and free fatty acids for absorption in the small intestine. *Sn*-2-MAG is resynthesized into TAG in the intestinal cells and enters the chylomicrons. Having 16:0 in the *sn*-2 position is presumed to be an advantage for growth of babies, due to their efficient absorption from the intestine. However, 16:0 in *sn*-2-MAG gives slower blood clearance and a prolonged postprandial lipemia response (Wang et al., 2016). In this context, it is of interest to analyze fatty acid composition, including regioisomerism, from various food sources by way of chemical or spectroscopic analysis. For instance, pork fat has a higher fraction of 16:0 in the *sn*-2 position of TAG than beef fat (Berry, 2009), and going down in more detail many of the differences between species can be traced down to genetic traits.

The standard protocol for investigation of the fatty acid configuration on the glycerol is by way of selective lipase-catalyzed hydrolysis of the fatty acids in the *sn*-1,3 position, followed by chemical hydrolysis of the fatty acids in the *sn*-2 position, and subsequent conversion to the corresponding methyl esters and GC analysis (Christie, 2003). More recently, several reports on the use of LC–MS techniques for establishing positional configuration of the fatty acids in triacylglycerols have emerged (Herrera et al., 2010; Judge et al., 2017; Malone and Evans, 2004; Mottram et al., 2001; Tarvainen et al., 2019).

For many lipid sources, similar information can be obtained from direct ^{13}C NMR-analysis of the triacylglycerols themselves. The carbonyl region of the ^{13}C NMR spectra is suited for distinguishing saturated from various unsaturated fats, including regioisomeric distribution on the glycerol. Fish oils can be thoroughly characterized in this manner (Standal et al., 2009; Suárez et al., 2010). Triacylglycerols from other sources, *e.g*. vegetable oils, may be characterized in this way, as well, and resonances from olefinic carbons have been put to good use (Wollenberg, 1990).

Our aim was to characterize triacylglycerol extracts from pork and beef using high-field ^{13}C NMR spectroscopy. The fatty acids normally present in triacylglycerols from pork in concentrations higher than 1 mol% are usually established as myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic acids (18:3), with contributions from vaccenic (18:1, 11-*cis*) and 11-*cis*-eicosenoic (20:1) acids sometimes also exceeding 1 mol% (Mattson et al., 1964; Morel et al., 2013; Morgan et al., 1992; Mottram et al., 2001). For beef, margaric (17:0) and other odd-numbered saturated fatty acids are present, as well as some elaidic acid (18:1, 9-*trans*). Spectra from these animal sources have been reported in the literature previously (Di Luccia et al., 2003). Of particular interest for us was trying to extract data for the individual saturated fatty acids directly from the spectra, including the positional distribution. Working to our advantage was the fact that there is a relatively low diversity in the fatty acids of triacylglycerols from these sources, especially compared to milk and fish oils (Andreotti et al., 2000; Suárez et al., 2010). Whereas unsaturated and most of the polyunsaturated fatty acids can be partly characterized using the carbonyl and olefinic regions of the spectra in a similar manner as in the examples above, the resonances for the long-chained saturated fatty acids appear as a pair of single carbonyl peaks in spectra at low resolution, representing the sum of all saturated fatty acids for *sn*-1,3 and *sn*-2, respectively. In a similar manner, the terminal methyl group of the saturated fatty acids are "blind" to the difference of *sn*-1,3 and *sn*-2 positions due to the long distance from the ester bonds. Thus, attempts to gain positional information of saturated fatty acids must be carried out using the most crowded region of the ^{13}C NMR spectra, between 29.0 and 29.8 ppm, the so-called "methylene envelope"(Gunstone, 1990).

An additional complicating issue comes from the fact that chemical shifts along the fatty acid chain varies according to the fatty acids in the neighboring position (Gunstone, 1990; Lie Ken Jie and Lam, 1995; Simova et al., 2003). This effect is confirmed in our work, and reports suggesting independent chemical shifts for each fatty acid in triacylglycerols (Mannina et al., 2000), does not seem to hold up at high resolution. In this project, we thus needed to establish what kind of influence unsaturated neighbors could exert on the chemical shifts in the crowded 29.0–29.8 ppm region, and whether we could use any of the signals for individual quantification of 16:0 and 18:0, including position. Although NMR methods for quantification of these fatty acids in triacylglycerol mixtures exist (Merchak et al., 2017; Sachleben et al., 2014), collection of positional information of individual saturated species has so far been absent in the literature.

In a broader context, such information could be put to use for the study of breed and genetic differences in animals, as well as a probe for studying feeding differences in more details. Additionally, this type of analysis could influence decisions related to selection of animals for breeding based on the fatty acid expression of genetic traits.

Materials and Methods

Chemicals

Triacylglycerol standards, all with a stated purity of 99% or higher, were supplied by Larodan AB (Solna, Sweden).

The reference standard "BCR-163 Beef-Pork fat blend" was supplied by LGC ARMI (Manchester, NH, USA), and chloroform-*d* (99.8% D) supplied by Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were supplied by Sigma-Aldrich Norway AS (Oslo, Norway).

Extraction of Triacylglycerols from Biological Sources

Triacylglycerols were extracted using 2:1 chloroform/methanol (Folch et al., 1957), with a simplified washing procedure, as specified below. Backfat samples from pigs, 16 each from Duroc and Landrace (van Son et al., 2017), were homogenized from the frozen state in an IKA analytical grinder to a greasy paste. To approximately 0.5 g of each sample was added 10 mL chloroform/methanol and allowed to extract for 30 min in a vertical rotating mixer. After centrifuging for 3 min at 3000 rpm, the extract was separated from solids and washed with 2 mL water. Phase separation was facilitated with centrifuging the samples at 2000 rpm, after which the bottom layer containing the triacylglycerols was transferred to vials. Solvent was removed using a nitrogen blowdown and a heating block at 40 °C, followed by final drying at high vacuum.

Samples from beef were obtained from the ground meat of eight cows and further homogenized in the same manner as above, giving a product with a powdery consistence. Due to expected lower lipid content, approximately 2.0 g was taken from each sample and extracted with 40 mL chloroform/methanol, with agitation for 30 min using an orbital shaker. Samples were filtrated and washed with 8 mL water. Phases were separated in a separating funnel, and the solvent was removed from the organic phase using a rotary evaporator. Phospholipids were removed by repeated partitioning between 1:1 *n*-hexane / 87% ethanol (aq.), using a similar procedure, albeit scaled-down, as described in the literature (Galanos and Kapoulas, 1962). Drying of samples was performed with nitrogen blowdown and high vacuum, in a similar manner as described above.

^{13}C NMR Spectroscopy

Triacylglycerol samples were dissolved in 0.5 mL chloroform-*d*, in concentrations corresponding to 50–100 mg triacylglycerol in each sample. Samples of BCR-163 and samples from animal sources were weighed out from the liquid state after melting the sample at 55 °C. Samples from animal sources were filtered through glass wool after dissolution. From BCR-163, five different samples were prepared, whereas for the beef samples, three spectra were recorded from each sample. For the pork samples, one spectrum per sample was acquired. Additionally, another six samples from a single pork animal was prepared, spiked with tripalmitin, tristearin, 1,3-stearin-2-olein, 1,3-olein-2-stearin, 1,2-palmitin-3-olein, and 1,3-olein-2-palmitin, respectively.

All experiments were performed on a Bruker AVIII HD 800 spectrometer, operating at a 201.19 Hz resonance frequency for ^{13}C, and equipped with a TCI CryoProbe™. The spectrometer was controlled using TopSpin 3.5 software (Bruker Biospin, Rheinstetten, Germany), and was for routine samples run in automation mode with spinning samples and automatic gradient shimming. All samples were measured at 298 K. Spectra were recorded in inverse-gated decoupling mode, using a 90° pulse angle at 11.5 μs length. In order to reduce baseline-roll from probe ring-down, the spectra were recorded using the "baseopt" digital filter available in the software and with a pre-scan delay of 20 μs. The sweep width was set at 200 ppm with 95 ppm as center frequency, and the free induction decay was collected using a time domain of 256 kB complex data points, leading to an acquisition time of 3.3 s. A relaxation delay of 27 s was employed between scans, and thus with a repetition time of 30 s, which were judged to be sufficient for the purpose of this study (Gouk et al., 2013). For each sample, 128 transients were collected, except for the spiking experiments, where 32 transients were collected, and spectra recorded of synthetic mixtures, for which 256 transients were collected.

All routine experiments were processed with a small exponential multiplication (LB = 0.01 Hz, (Gouk et al., 2013)), and with zero-filling to 512 kB real data points. Reported chemical shift values were calibrated against C-9′ in *sn*-1,3 of triolein at $\delta_C = 129.7215$ ppm (Wollenberg, 1990). After baseline correction and phasing, deconvolution using TopSpin 3.5 software was applied. Peaks for deconvolution were picked manually as specified below, and were fitted using pure Lorentzian line-shapes. Integration of the carbonyl region was performed manually.

Prior to running routine experiments, the quantitative behavior of the instrument was checked with a triolein standard sample, for which 7 experiments were run and processed with LB = 1.0 Hz and zero-filling to 256 kB data points. Average values from the integration revealed no signal drop-off near the spectral edges, and no average integration result deviating more than 2% from the expected values.

Statistical Analysis

The beef data were subjected to a t-test as comparison test (Microsoft Office Excel). Differences between the various groups of pork samples were analyzed using SAS Proc GLM (SAS Inst., Inc., Cary, NC, USA). Genetic factors, breed, and breed by genotype were used as fixed effects in the model and correlated with selected fatty acid parameters

obtained from NMR analyses. There were no other fixed effects to include, since animals were from same farm, and same period.

Results and Discussion

Choice of NMR Conditions

Quantification of fatty acids in triacylglycerols is often achieved by inspecting the carbonyl region alone. This allows for the use of narrow spectral widths and harvesting of sufficient number of data points to properly describe the peaks before complete T_2-relaxation. Often, relatively rapid pulses and short tip-angles are used, as well as power-gated decoupling. In order to enable quantitative measurements over the whole spectrum, we chose to obtain the full spectra under as quantitative conditions as practically possible, which would also simplify data analysis. Initially, three different inverse-gated experiments were evaluated, either by using (i) "standard" conditions with no digital filtering, (ii) Z-restored spin-echo (Xia et al., 2008), and (iii) "baseopt" digital filtering, as specified in the preceding section. Under conditions (i) baseline roll was countered with increased prescan delay (50 μs). The results were quite similar, with options (ii) and (iii) giving the best performance. In the end option (iii) was selected, due to more favorable peak shapes.

Due to the large spectral width, we chose to process the data with zero filling during Fourier transformation. For spectra of narrower spectral width, this would offer no improvement in resolution (Gouk et al., 2013). In well-separated spectra of pure compounds, we obtained better quantitative results using exponential multiplication values of LB = 0.2–0.5 Hz, but for mixtures and real samples this did not offer good enough separation. We settled for a recommended value of LB = 0.01 Hz (Gouk et al., 2013), but noted that some truncation artifacts still remained in the spectra. We will also mention that in the existing literature there is tradition to report chemical shifts of triacylglycerols with three decimals, since chemical shift differences observed in a single experiment often are reproducible (Gunstone, 1990). Detailed prediction of the absolute chemical shifts in complex triacylglycerol mixtures is difficult, however, and rather large variations are observed. This should always be kept in mind while studying the literature, and our reported values should be treated with similar scrutiny.

Quantification Strategy

As mentioned above, the carbonyl signals are often used for quantification purposes. Under our high-resolution spectral conditions, their appearance was complex, Fig. 1, especially for the pork extracts, Fig. 1c). We chose to use manual integration of the carbonyl signals for establishing ratios of saturated and unsaturated fatty acids for both *sn*-1,3 and *sn*-2 positions. This introduces some aspects of judgment from the analyst, especially when dealing with separation of 11-*cis* fatty acids from the saturated fats.

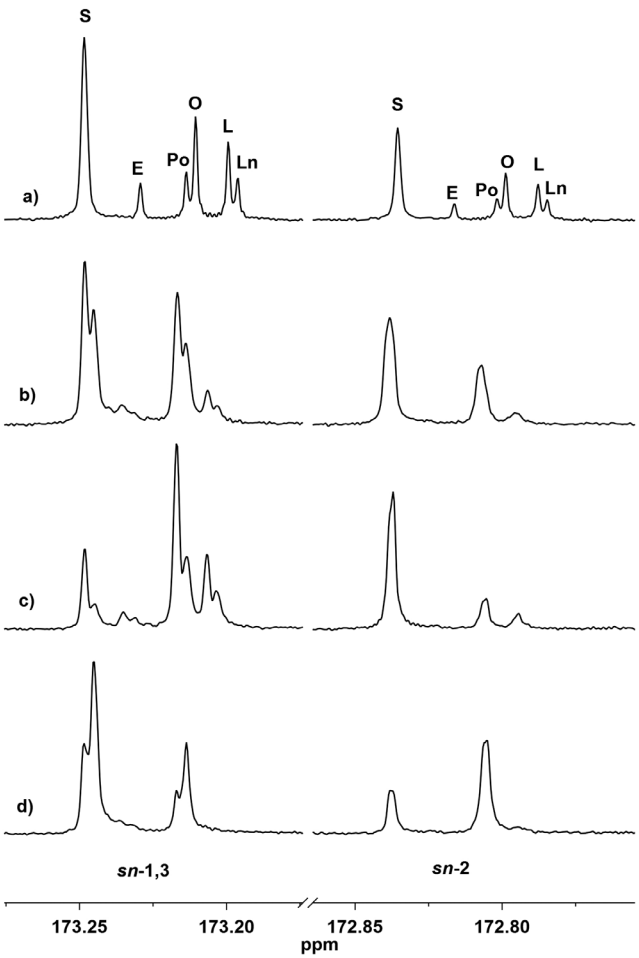

Fig 1 Excerpts from carbonyl region of ^{13}C NMR-spectra recorded at 201.19 MHz; (a) synthetic mixture of saturated fats (S, trimyristin, tripalmitin and tristearin, in total 44 mol%), tri-11-*cis*-eicosenoin (E, 6 mol%), tripalmitolein (Po, 7 mol%), triolein (O, 17 mol%), trilinolein (L, 15 mol%) and tri-α-linolenin (Ln, 11 mol%), (b) BCR-163 reference standard, (c) pork backfat extract (Norwegian Landrace), (d) beef meat lipid extract. Sample (b) was calibrated as indicated in "Materials And Methods," and remaining spectra aligned to (b) during figure preparation. Figure prepared using MestreNova 11.0.4.

The quantification was extended to include individual olefinic components following earlier published recommendations (Wollenberg, 1990), but with some modifications and with added components, as specified in Table 1. These components all have signals that exhibit some separation from interference, and for this reason deconvolution was chosen as integration method for the unsaturated fatty acids. Partly overlapping peaks were added to the

Table 1 Peaks selected for quantification by deconvolution

Peak (ppm)	Assignment			Reference
	Component	Position	Carbon	
130.410	18:1 9-*trans*	*sn*-1,3	C-10’	130.48 ppm (Gunstone, 1993)
129.842	18:1 11-c*is*	*sn*-1,3	C-11′	129.777 ppm (Vlahov et al., 2002)
129.837	20:1 11-c*is*	*sn*-1,3	C-11′	129.773 ppm (Vlahov et al., 2002)
129.727	16:1 9-*cis*	*sn*-1,3	C-9′	129.691 (Retief et al., 2009)
129.722	18:1 9-*cis*	*sn*-1,3	C-9′	129.721 (Wollenberg, 1990)
129.701	16:1 9-*cis*	*sn*-2	C-9′	129.665 (Retief et al., 2009)
129.695	18:1 9-*cis*	*sn*-2	C-9′	129.695 (Wollenberg, 1990)
128.260	18:3 (α isomer)	*sn*-1,3	C-12′	128.262 (Wollenberg, 1990)
128.247	18:3 (α isomer)	*sn*-2	C-12′	128.250 (Wollenberg, 1990)
127.929	18:2	*sn*-1,3	C-12′	127.939 (Wollenberg, 1990)
127.917	18:2	*sn*-2	C-12’	127.927 (Wollenberg, 1990)

Shift values as detected in BCR-163 reference material and calibrated as specified in “Materials And Methods”.

deconvolution on a case-to-case basis in order to increase integration accuracy. It has been recommended to use the olefinic signal from the carbon closest to the carbonyl for quantification (Wollenberg, 1990). This proved less than optimal for 18:2 and 18:3, as C-9′ from these fatty acids overlaps with other signals, and the C-10′ signal is unsymmetrical for 18:2, and broad and indistinct for 18:3. Thus, C-12′ was chosen for both these components. There have been attempts at simultaneous quantification of palmitoleic acid and oleic acid previously (Vlahov et al., 2002). They have overlapping signals for C-10′, but for C-9′ there is a separation of approximately 0.006 ppm for *sn*-1,3 (Vlahov et al., 2002), and we have assumed a similar difference for the *sn*-2 signals, allowing for independent quantification of these fatty acids as well. Reports for the 11-*cis* isomers of 18:1 and 20:1 are found in the same source. In our samples, signals for these components in the *sn*-2 position were barely detectable and were routinely excluded from the quantification. The assignment of 18:1 9-*trans* must be considered as tentative at this point. We registered two signals at 130.410 and 130.311 ppm which seemed to correlate with concentrations of this component determined by other means, but with a chemical shift difference not consistent with C-9′ and C-10′ for 18:1 9-*trans* reported elsewhere (Gunstone, 1993).

As a means for reconciling integration and deconvolution results, we made the conscious choice to renormalize all deconvolution results in order to make the sum of all unsaturated components in *sn*-2 equal to the amount of unsaturated fatty acids in *sn*-2 determined by manual integration of carbonyl signals. The amount of the individual unsaturated components in *sn*-1,3 would then follow from the renormalization. Only in very few cases did this lead to the contradictory result being that the sum of individual components in *sn*-1,3 from deconvolution was larger than the result from manual integration of carbonyl signals. The more complicated issue of using signals in the “methylene envelope” for quantification of the individual saturated fatty acids will be dealt with in later sections.

Quantification of a Pork/Beef Reference Material

In order to confirm the suitability of our method for quantitative purposes, we examined its performance against a known reference standard, a beef-pork fat blend (BCR-163). This standard has been used for validation of fatty acid analysis as methyl esters with GC. Unfortunately, certified values for the unsaturated fats are given as sum of all isomers within each class, and reference values for the principal isomers within each class are not given. We have therefore chosen to subtract the values for the minor components in order to get estimates for the principal isomers. The dataset was also renormalized to 100 mol%, being given originally as g/100 g of fatty acid methyl esters. For the positional distribution, there is no breakdown into individual isomers in the reference values for fatty acids in *sn*-2 position. We have chosen to quantify these using the main isomers only. The results obtained for five different parallel samples are given in Table 2. We note that the accuracy is in line with earlier similar efforts (Vlahov et al., 2002; Wollenberg, 1990), and decent results for 16:1 in the presence of large amounts of 18:1 were obtained. The biggest relative deviations were observed for the minor components, unsurprisingly; deviations for Σ18:3 and Σ20:1, both with sub-1% reference values, were especially high.

Table 2 Quantification results obtained by way of ^{13}C NMR for "BCR-163 Beef-Pork fat blend" using a combination of manual integration of carbonyl signals and deconvolution of olefinic signals, as specified in the main text

	Selected signal	Composition (mol%)	
		NMR (±SEM, $n = 5$)	Reference value
ΣSFA	C-1'	50.2 ± 0.4	50.4
16:1	C-9'	2.1 ± 0.09	2.5
18:1	C-9'	35.2 ± 0.3	34.7
18:1 9-*trans*	C-10'	1.5 ± 0.02	1.5
18:1 11-*cis*	C-11'	1.7 ± 0.07	1.3
Σ18:2	C-12′	6.7 ± 0.03[a]	6.1
Σ18:3	C-12′	0.9 ± 0.05[a]	0.4
Σ20:1	C-11'	1.3 ± 0.4[a]	0.7
ΣSFA *sn-2*	C-1'	59.5 ± 0.4	60.3
Σ16:1 *sn*-2	C-9'	3.1 ± 0.15[a]	3.6
Σ18:1 *sn*-2	C-9'	30.9 ± 0.4[a]	30.1
Σ18:2 *sn*-2	C-12′	5.3 ± 0.08[a]	5.0
Σ18:3 *sn*-2	C-12′	1.2 ± 0.13[a]	1.1

[a]Quantified only by using the main isomer, *e.g*. palmitoleic acid, oleic acid, linoleic acid, α-linolenic acid, and 11-*cis*-eicosenoic acid.

Quantification of Fatty Acid Components from Beef Samples

The eight beef samples were selected from a larger collection of 72 animals, taken from a project looking at fatty acid distributions from a country-wide selection of animals. From this project, GC data for individual animals were available (Egelandsdal et al., 2020), and samples displaying large variations in fatty acid composition were selected for NMR experiments. The results are presented in Table 3, and for the major components the data from GC and NMR show insignificant differences with regards to sample mean and relative deviations within the population. For two of the minor components, 18:2 and 18:1 9-*trans*, there are some discrepancies. It must be pointed out that the NMR analyses are based on a separate extraction experiment, and additionally, the GC data were converted from mass basis to a mol% basis by disregarding approximately 2–5 g/100 g which was specified as "other" in the GC analysis.

One of the main advantages of NMR is the ability to extract positional information for the unsaturated components in a straightforward manner. The estimates obtained for 16:1, 18:1, and 18:2 in the *sn*-2 position are in good agreement with previously reported results obtained by the lipase/GC-method on adipose tissue

Table 3 Average fatty acid composition of triacylglycerols extracted from meat from eight cows, including regioisomeric distribution of unsaturated components, compared with corresponding results from GC (Egelandsdal et al., 2020), and GC values for beef reported in other sources (Mattson et al., 1964)

	Composition (mol%)		
	NMR (±SD, $n = 8$)	GC (±SD, $n = 8$)	GC, reported
ΣSFA	51.8 ± 5.2[a]	50.2 ± 4.7[a]	59
16:1	4.3 ± 1.6[a]	5.5 ± 1.8[a]	5
18:1	38.4 ± 4.1[a]	35.5 ± 3.7[a]	36
18:1, 9-*trans*	1.3 ± 0.5[a]	2.5 ± 0.7[b]	n.a.
18:1, 11-*cis*	1.3 ± 0.5[a]	1.3 ± 0.5[a]	n.a.
18:2	1.1 ± 0.3[a]	1.8 ± 0.5[b]	1
ΣSFA *sn-2*	29.4 ± 5.5	n.a.	30
16:1 *sn*-2	7.0 ± 2.3	n.a.	6
18:1 *sn*-2	61.3 ± 3.7	n.a.	61
18:2 *sn*-2	2.2 ± 0.5	n.a.	3

a,b: *t*-test, double-sided; when different $p < 0.05$.

from beef (Mattson et al., 1964), Table 3, whereas there are some discrepancies with results from HPLC-APCI (Mottram et al., 2001). We agree with the original authors that their anomalous high response for 17:1 may have skewed their data, and have thus not shown the comparison in the table.

The fatty acid composition of triacylglycerols of beef is known to be susceptible to dietary as well as genetic manipulation (Mir et al., 2003). Increase in 18:1 content is considered a desirable trait from a public health point of view. Likewise, the intramuscular fat content (*i.e.* marbling) of beef is also significantly associated with amount of 18:1, and an increase in product quality and value might be perceived for meat with the desired marbling pattern (Kazala et al., 2006). NMR could thus represent an alternative means for obtaining valuable information of this kind.

Quantification of Fatty Acid Components from Pork Samples and their Correlation with Genomic Traits

An overview of the results obtained for the samples extracted from pork backfat is shown in Table 4, separated on the different breeds. Where available, we have included average values obtained for a different and larger group of animals selected from the same population, using NIRS (van Son et al., 2017). The results from the two different methods are in good agreement, and despite the use of only 16 animals from each race for the NMR experiments, the SD were small.

For the pigs in this study, genotypes from the Illumina porcine 60K SNP chip (Illumina) were available. Genome-wide association studies (GWAS) have previously detected a major quantitative trait loci (QTL) affecting fatty acid composition on chromosome 14 (*i.e.* SSC14) in Duroc,

Table 4 Quantitative results obtained from landrace pigs (L) and Duroc (D)

Fatty acid	Breed	Composition (mol%)			
		sn-1,3	*sn*-2	Total	
		NMR (±SD, $n = 16$)	NMR (±SD, $n = 16$)	NMR (±SD, $n = 16$)	NIRS
ΣSFA	L	18.2 ± 1.4	68.8 ± 2.7	35.0 ± 1.7	34.4
	D	22.2 ± 1.5	71.0 ± 1.9	38.3 ± 1.5	38.3
	D (CT)[a]	20.0 ± 1.1	69.2 ± 1.8	36.2 ± 1.3	—
	D (CC)[a]	22.7 ± 0.9	71.4 ± 1.7	39.3 ± 1.9	—
ΣMUFA	L	58.0 ± 2.1	20.1 ± 1.7	45.4 ± 1.8	47.0
	D	53.4 ± 3.3	18.0 ± 1.2	41.6 ± 2.5	43.7
	D (CT)[a]	55.5 ± 2.8	19.1 ± 0.6	43.4 ± 1.9	—
	D (CC)[a]	52.9 ± 3.3	17.8 ± 1.1	41.2 ± 2.5	—
ΣPUFA	L	23.2 ± 2.2	11.2 ± 1.3	19.2 ± 1.9	18.6
	D	24.0 ± 2.7	11.0 ± 1.5	19.6 ± 2.3	18.1
16:0	L	4.2 ± 1.2	63.2 ± 4.3	23.9 ± 2.0	22.4
	D	4.7 ± 1.1	65.4 ± 3.0	24.9 ± 1.5	23.4
18:0	L	14.0 ± 1.2	5.6 ± 2.4	11.2 ± 1.1	12.0
	D	17.6 ± 1.7	5.6 ± 1.7	13.6 ± 1.3	14.9
16:1	L	0.5 ± 0.2	4.1 ± 0.5	1.7 ± 0.2	2.8
	D	0.4 ± 0.1	3.2 ± 0.3	1.3 ± 0.1	2.3
18:1	L	52.3 ± 1.9	15.9 ± 1.4	40.2 ± 1.6	44.2
	D	48.2 ± 3.1	14.8 ± 1.0	37.1 ± 2.3	41.3
18:1, 11-cis	L	3.6 ± 0.2	—	2.4 ± 0.1	—
	D	3.2 ± 0.2	—	2.2 ± 0.1	—
18:2	L	21.1 ± 2.0	9.4 ± 1.2	17.2 ± 1.7	16.7
	D	21.8 ± 2.4	9.2 ± 1.1	17.6 ± 2.0	16.3
18:3	L	2.1 ± 0.2	1.8 ± 0.2	2.0 ± 0.2	1.9
	D	2.1 ± 0.3	1.8 ± 0.3	2.0 ± 0.3	1.7
20:1, 11-cis	L	1.7 ± 0.2	—	1.1 ± 0.1	—
	D	1.6 ± 0.2	—	1.0 ± 0.1	—

Results from NIRS (van Son et al., 2017) have been converted to mol% using only the known reported individual components.

[a]For Duroc with genotype CT: $n = 3$; for genotype CC, $n = 13$.

Table 5 Hypothesis test of fixed effects of the models for the analyzed traits in landrace and Duroc with a GLM-analysis

Trait	Breed	Breed*SNP-genotype	R^2
ΣSFA (total)	***	*	0.65
ΣSFA (*sn*-1,3)	***	*	0.78
ΣMUFA (total)	*	ns	0.46
ΣMUFA (*sn*-2)	*	ns	0.38

*** = significant effect ($p < 0.0001$), * = significant effect ($p < 0.05$), ns = non-significant ($p > 0.05$).

whereas the SSC14 QTL was not segregating in the Landrace population (van Son et al., 2017). The QTL region on SSC14 is connected to the promoter region of the Stearoyl-CoA desaturase (SCD) gene, involved in desaturation of SFA to monounsaturated fatty acid (MUFA), with insertion of one omega-9 double bond in the fatty acids. For SSC14 in Duroc, the most significant single nucleotide polymorphism (SNP) was shown to explain between 55% and 76% of the genetic variance and between 27% and 54% of the phenotypic variance for SFA and MUFA fatty acids. The Landrace has only one variant of this SNP, which is the SNP variant (nucleotide T) which gives high levels of MUFA, while Duroc has an allele frequency of 70% for the SNP variant (nucleotide C) which gives lower levels of MUFA. This causes the two breeds to differ with respect to fatty acid composition, with Landrace typically having higher levels of total MUFA, whereas Duroc on average have higher levels of total SFA. The pigs randomly chosen for the NMR in our study were all homozygote TT for Landrace, while for Duroc, 13 animals were homozygote CC, and three were heterozygote CT.

In an analysis using a generalized linear model (GLM), both breed and genotype by breed were significant as fixed effects for several of the fatty acid traits in our study, Table 5. These results support previous finding of a large genetic effect on SFA and MUFA. It also shows that the heterozygotic CT pigs are intermediate (for the phenotype) and between the homozygotic TT and CC pigs, Table 6, indicating that the gene variant SSC14 has an additive effect on the phenotype, here fatty acid composition. For SFA in *sn*-1,3, this effect was significant even with as few as 16, 3, and 13 animals from each group.

Individual Quantification of Saturated Fatty Acids in Triacylglycerols from Pork

Careful examination of the methylene envelope region of ^{13}C NMR spectra of trimyristin, tripalmitin, and tristearin, and mixtures thereof, revealed different chemical shifts for each of the fatty acids in the regions C-5'-C-8′, both in *sn*-1,3 and *sn*-2 positions, Fig. 2a).[1] We expected the highest accuracy for quantification purposes by the use of the C-6′ chemical shifts, and were positively surprised when we discovered, after performing mixing experiments, that these signals does not overlap with any signals from either 16:1, 18:1, 18:2, 18:3, and 20:1, making them suited for quantification purposes. Unfortunately, after the preparation of a mixture of tristearin and 1,2-stearin-3-olein, it became apparent that the presence of an unsaturated fatty acid in *sn*-3 position, shifted the C-6′ resonance of stearin in *sn*-2 position just enough to create overlap with palmitic resonances, Fig. 2b). Thus, when observing this region in the BCR-163 reference sample, the resonances for C-6′ of the saturated fatty acids appear as a complex cluster of peaks, Fig. 2c), not directly corresponding to the reported concentration values for 14:0, 16:0, and 18:0 (for *sn*-2 the reported values are 6, 46 and 31 mol%, respectively). However, when observing the same peaks in the samples from pork, we saw that their appearance was somewhat simpler, Fig. 2d). For the peaks from the *sn*-2, or β, position, we realized that due to the high amount of unsaturated fats in the *sn*-1,3, or α, position, the likelihood of having two saturated neighbors, given a random distribution of the fatty acids in *sn*-1,3, was very small. Following on with this thought, and under the assumption of random distribution in *sn*-1,3, we realized that we could assign probabilities P (n,α) of having n unsaturated neighbors in the α positions, based on our manual integration of the carbonyl region. Furthermore, we made the initial assumption that the area A_A of peak A (Fig. 2c) comprised contributions from 18:0 with two unsaturated neighbors and 16:0 with one unsaturated neighbor, whereas the area A_B of peak B only had contributions from 16:0 with two unsaturated neighbors. Spiking of a pork sample with 1,3-olein-2-stearin and 1,2-palmitin-3-olein both gave an increase of peak A, as expected, whereas spiking with 1,3-olein-2-palmitin gave an increase of peak B, thus confirming our assumption.

The ratio between the areas is expressed analytically in Eq. 1, using molar fractions $\chi_{n:0,\beta}$ for expressing the amount of fatty acid n:0 in position β. After assuming contributions only from 16:0 and 18:0, Eq. 2, we could solve analytically the distribution of 16:0 and 18:0 in the *sn*-2 position, shown for 16:0 in Eq. 3.

$$\frac{A_A}{A_B} = \frac{\chi_{18:0,\beta} \cdot P(2,\alpha) + \chi_{16:0,\beta} \cdot P(1,\alpha)}{\chi_{16:0,\beta} \cdot P(2,\alpha)} \quad (1)$$

$$\chi_{18:0,\beta} + \chi_{16:0,\beta} = 1 \quad (2)$$

[1] As a sidenote, we observed that there are differences in assignments for C-5′ and C-6′ in various sources (Mannina et al., 2000; Retief et al., 2009; Simova et al., 2003), and have chosen to assign C-5′ at higher field than C-6′, in line with the majority of the literature.

Table 6 Least square means and SEM (in brackets) of the breed*SNP-genotype effects from the model in Table 5 for the analyzed traits

Trait	CC	CT	TT
ΣSFA (total)	0.388[a] (0.0040)	0.363[b] (0.0083)	0.3494[b] (0.0036)
ΣSFA (*sn*-1,3)	0.227[a] (0.0033)	0.200[b] (0.0068)	0.181[c] (0.0029)
ΣMUFA (total)	0.412[a] (0.0061)	0.430[ab] (0.012)	0.453[b] (0.0054)
ΣMUFA (*sn*-2)	0.1777[a] (0.0041)	0.190[ab] (0.0085)	0.201[b] (0.0037)

[a,b,c]Different letters within row indicates significant differences at $p < 0.05$.

$$\chi_{16:0,\beta} = \frac{P(2,\alpha)}{P(2,\alpha)\cdot\left(\frac{A_A}{A_B}+1\right) - P(1,\alpha)} \quad (3)$$

In a similar fashion, the peaks C and D (Fig. 2c) for the *sn*-1,3 position were initially assumed to represent the following: Area A_C with contributions only from 18:0 with no unsaturated neighbor, and Area A_D comprising 18:0 with an unsaturated neighbor and 16:0 with no unsaturated neighbor. Spiking experiments with tristearin gave the expected increase of peak C, whereas spiking with tripalmitin and 1,3-stearin-2-olein gave an increase of peak D. Also here, probabilities based on random distributions in *sn*-2 could be calculated from manual integration of the carbonyl region, and an analytical expression for the ratio of the areas constructed, *i.e*. Eq. 4. Again, only contributions from 18:0 and 16:0 were considered, Eq. 5, which allowed us to solve analytically distribution of 16:0 and 18:0 in the *sn*-1,3 position, as shown for the latter in Eq. 6.

$$\frac{A_C}{A_D} = \frac{\chi_{18:0,\alpha}\cdot P(0,\beta)}{\chi_{18:0,\alpha}\cdot P(1,\beta) + \chi_{16:0,\alpha}\cdot P(0,\beta)} \quad (4)$$

$$\chi_{18:0,\alpha} + \chi_{16:0,\alpha} = 1 \quad (5)$$

$$\chi_{18:0,\alpha} = \frac{P(0,\beta)}{P(0,\beta)\cdot\left(\frac{A_D}{A_C}+1\right) - P(1,\beta)} \quad (6)$$

These expressions were employed on areas A_A, A_B, A_C, and A_D obtained through integration by way of deconvolution, and used for the calculation of data for 16:0 and 18:0 in Table 4.

We see from the values in Table 4 that the agreement with results from NIRS was very good for these two fatty acids, and seemed to provide reasonable estimates. Comparisons with relevant positional distributions available from other sources using the lipase degradation method (Berry, 2009; Mattson et al., 1964), Table 7, are also favorable, albeit with slightly elevated relative deviations compared to many of the polyunsaturated fatty acid (PUFA) and MUFA components. Although the underlying assumption of randomly distributed neighbors seems to be sound based on reported HPLC data on intact triacylglycerols

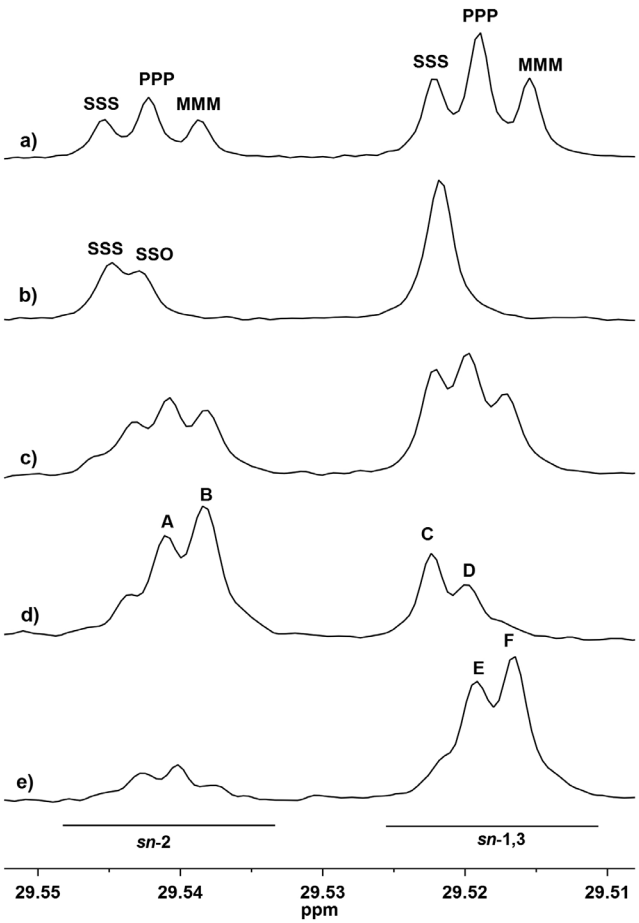

Fig 2 Excerpts from 29.51 ppm to 29.55 ppm of ^{13}C NMR-spectra recorded at 201.19 MHz, covering C-6′ region for long-chain saturated fatty acids; (a) synthetic mixture of triolein (27 mol%), tristearin (SSS, 21 mol%), tripalmitin (PPP, 33 mol%) and trimyristin (MMM, 19 mol%), (b) synthetic mixture of tristearin (S) and 1,2-stearin-3-olein (SSO), (c) BCR-163 reference standard, (d) pork backfat extract (Norwegian Landrace), (e) beef meat lipid extract. Sample (c) was calibrated as indicated in "Materials And Methods," and remaining spectra aligned to (c) during figure preparation. For explanation of A-F, see the main text. Figure prepared using MestreNova 11.0.4

(Mottram et al., 2001), some of the deviations could arise from the assumption not holding up completely. However, this aspect is also closely related to the working mechanism

Table 7 Estimated proportions (mol%) of saturated and unsaturated fatty acids in *sn*-2 position for backfat extracted from Norwegian Landrace and Duroc compared to values reported for "domestic pig" (Mattson et al., 1964) and "lard" (Berry, 2009)

Fatty acid	Norwegian Landrace	Duroc	Domestic pig	Lard
16:0	63.2	65.4	72	58
18:0	5.6	5.6	4	1
16:1	4.1	3.2	4	n.a.
18:1	15.9	14.8	12	15
18:2	9.4	9.2	3	8

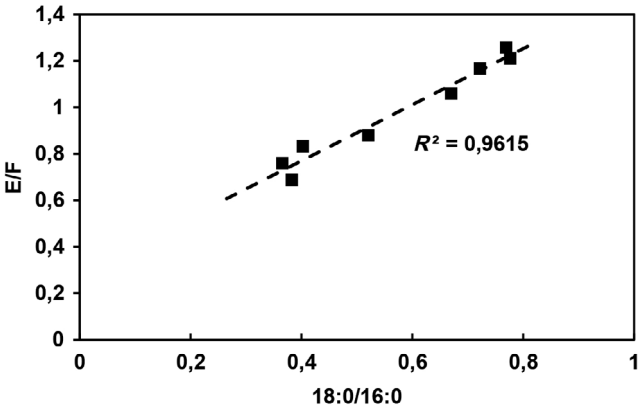

Fig 3 Ratios 18:0/16:0, determined from GC, plotted against ratios between areas of peaks E and F (Fig. 2e) from ^{13}C NMR experiments, determined by way of deconvolution. Data obtained from one NMR spectrum per animal, in total eight different animals

and specificities of the diacylglycerol transferase (DGAT) enzymes in pig, which catalyzes the rate-limiting step in triacylglycerol synthesis. Experiments where porcine DGAT2 was expressed in a murine adipocytes cell line and incubated with an isotope-labeled fatty acid mixture, indicated low substrate discrimination of this enzyme (Zhang et al., 2014). From the literature, it also seems that DGAT2 is of most importance for controlling fat deposition in pig (Hu et al., 2010). In contrast to this, studies on DGAT enzymes found in beef indicate that these enzymes are working in a more specific manner (Lozeman et al., 2001). This is also evident from the LC–MS quantification of individual triacylglycerol species in beef (Mottram et al., 2001), which deviates dramatically from what would be expected if *sn*-2 and *sn*-1,3 composition was independent events. As a consequence, our underlying assumption of *randomness* does not hold up here and using the analytical method outlined above on beef samples resulted in physically impossible results. We noted a positive correlation between ratios of the peak intensity of E and F, representing C-6′ at *sn*-1,3, *versus* experimentally determined 18:0/16:0-ratios from the whole extract using GC, Figs. 2e and 3. As the majority of the saturated fats in beef are found in *sn*-1,3, anyway (*cf*. Fig. 1 and Table 3), this is not surprising. However, we were unable to make any further progress with regards to positional information for 18:0 and 16:0 in beef. An additional message from the studies in beef is that DGAT in adipose tissue and muscle could have different substrate specificities (Lozeman et al., 2001), probably related to DGAT1/DGAT2 ratio. Variation in fatty acid composition in different tissues is reported for both beef and pork (Bosch et al., 2012; Sturdivant et al., 1992), and whereas extracted TAG from ground meat from beef may comprise contributions from intramuscular, abdominal, and subcutaneous fat, our pork samples were all extracted from backfat, and care must therefore be exercised when extrapolating to other pork tissues.

In conclusion, the developed NMR method quantifies individual unsaturated fatty acids, as well as the sum of the saturated fatty acids, present in triacylglycerol extracts from beef and pork. For the unsaturated fatty acids, a breakdown of their regioisomeric distribution on the glycerol has been provided. In addition, for the pork samples we were able to quantify the concentration of the 16:0 and 18:0 fatty acids individually, including their regioisomeric distribution. This has not been achieved before.[2] Use in other materials must be verified on a case-to-case basis, and must be evaluated on basis on the validity of our assumptions.

There is a potential for further refinement of the methodology in conjunction with other methods, but will point out that the presence of short-chain SFA increases the complexity of the C-6′ peaks of long-chain saturated fatty acids dramatically. In a recently reported and closely related effort (Merchak et al., 2017), multivariate linear regression was used for the quantification of 10 different fatty acids from olive oils, mainly using signals in the crowded 29–30 ppm region of the ^{13}C NMR spectra. They have successfully quantified several SFA found in their mixtures. We note that they were unable to obtain regioisomeric distribution on *sn*-1,3 and *sn*-2 for the SFA included in the study. However, olive oils are not the easiest material, as there is only very little SFA in *sn*-2 (Aranda et al., 2004; Yorulmaz et al., 2014). Further refinement of their method could potentially extend its scope, in particular with modified signal processing to bring out the finer structures of the C-6′ peak cluster, and maybe supported by even higher magnetic field strengths.

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[2]Which prompt us to comment that some carbonyl assignments presented elsewhere (Sacchi et al., 1993) are probably in error, as signals assigned to 18:0 and 16:0 in the carbonyl region of *sn*-1,3 probably are related to unsaturated *vs*. saturated neighbors instead.

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Conflict of interest The authors declare that they have no conflict of interest.

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