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Composition of bovine milk fat globules by confocal Raman microscopy

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ABSTRACT

Confocal Raman microscopy was used to determine the chemical fingerprint of individual bovine milk fat globules of different sizes and from milk sourced from different breeds. No detectable level of triglycerides was found in the small milk fat globules of 1 μm diameter or less in size. These small fat globules also delivered information about the lipids of the milk fat globule membrane. In addition, the lipid composition varied according to the size of the globules with an increasing concentration of carotenoids and decreasing level of unsaturation with increasing diameter. The carotenoids content also varied according to the breed origin of the milk, with Jersey milk fat globules containing more carotenoids than the Friesian-Jersey milk fat globules. The results suggest that the milk fat globules have different properties according to their size.

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1. Introduction

Raman spectroscopy is not commonly used as an analytical tool in the dairy industry (McGoverin, Clark, Holroyd, & Gordon, 2009, 2010; Moros, Garrigues, & de la Guardia, 2007); infrared and near-infrared spectroscopies are the most common vibrational spectroscopy techniques used in the food industry (Nafie, 2001) and have been used in the study of milk (Kher, Udabage, McKinnon, McNaughton, & Augustin, 2007; Ozaki, McClure, & Christy, 2007; Patel, Anema, Holroyd, Singh, & Creamer, 2007; Wu, Feng, & He, 2007). Raman spectroscopy is more suitable for the *in vivo* or *in situ* study of aqueous samples, as water has weak Raman scattering properties (Li-Chan, 1996). Raman spectroscopy is based on the inelastic scattering of photons (from a laser) upon interaction with sample molecules (McGoverin, Rades, & Gordon, 2008). Raman spectroscopy allows one to obtain information about the properties and the structure of molecules from their stretching and bending vibrational transitions (Nafie, 2001).

Raman spectroscopy allows sufficiently rapid study of samples *in situ* and does not require any chemical labels (Krafft, Neudert, Simat, & Salzer, 2005). Raman spectral data of milk products and compounds are well documented (Bernuy, Meurens, Mignolet, & Larondelle, 2008; Li-Chan, 2007; McGoverin et al., 2009), and

lipids of various origins have been extensively studied (Krafft et al., 2005; Weng, Weng, Tzeng, & Chen, 2003). Raman spectroscopy has been applied to the examination of milk fat globules (MFGs), one from bovine milk (Forrest, 1978) and the other one from human milk (Argov et al., 2008), and to the comparative study of bovine milk and bovine milk fat, providing information on the milk fat globule membrane (MFGM) (Larsson, 1976).

Variations in the fatty acid composition and lipid content according to the size of the globules were reported over 20 years ago (Timmen & Patton, 1988). This is of great importance as it suggests heterogeneity in the secretion and assembly of the milk fat globules. In addition, it has a technological potential, as globules of different composition possess different properties leading to use in various dairy products for their specific physicochemical characteristics after separation by size subclasses (Michalski, Michel, & Geneste, 2002; Michalski et al., 2006). Timmen and Patton (1988) associated the difference in fatty acid composition and globule size with a different secretion pathway. Small globules originate from the secretion of microlipid droplets in the endoplasmic-reticulum. Intermediate-sized milk fat globules result from cytoplasmic droplets formed by fusions of microdroplets. Larger globules arise from post-secretion fusion of globules, as some exceed the size of the secreting cells.

The difference in composition of bovine MFGs according to their size (Fauquant, Briard, Leconte, & Michalski, 2005; Michalski et al., 2006) has been reported. Gravity separation, centrifugation (Timmen & Patton, 1988) and microfiltration (Michalski et al., 2006) are the most common techniques used to separate the MFGs based upon size. However, the cream phase, made of the larger MFGs,

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usually still contains some small MFGs, and the skim milk phase contains a fraction of small globules but also some larger milk fat globules. Even with the most efficient separation technique, the resulting fractions of globules contain a pool of globules with a broad range of sizes. The overall composition of the globules in the cream phase is “contaminated” by the residual small MFGs, and the large fat globules, “contaminating” the skim milk phase, biasing the chemical composition toward an overestimate of the amount of triglycerides. In the work presented here, the composition of the MFGs was determined for bovine MFGs of particular diameters.

Individual bovine milk fat globules from Jersey and Friesian-Jersey cows were analyzed using confocal Raman spectroscopy as a function of size, and their chemical signature was obtained and compared according to the size and to the breed. The difference in composition of the bovine MFGs according to their size and the breed of cow was confirmed. Differences in the lipid content and fatty acid composition of MFGs of particular diameters were investigated.

2. Materials and methods

Bovine raw milk samples from Jersey cows and Friesian-Jersey cows were kindly provided from a local farm (Port Chalmers, Otago, New Zealand). The Jersey breed milk was chosen for its high concentration of non-aqueous components (Auldist, Johnston, White, Fitzsimons, & Boland, 2004) and large fat globules (Couvreux & Hurtaud, 2007). All the cows were pasture-fed in the same manner. The milk, straight after milking, was left to cool at room temperature and then centrifuged for 5 min at $3000 \times g$. The top cream layer was collected and reconstituted to a 20% fat content in phosphate buffered saline (PBS) buffer, pH 7.2 and then diluted ten-fold.

For confocal Raman microscopic observations and spectra collection, the sample must be quiescent. Diluted cream samples (25 μL) were deposited onto a concave microscopic slide and agarose (50 μL , 0.5%, w/v, in deionized water) was added to fix the samples on the slides. A quartz cover slip was then applied rapidly without excessive pressure. The Raman spectrum of agarose did not present any characteristic peak, besides the broad peak of water Raman bands at $3100\text{--}3400\text{ cm}^{-1}$ due to O–H stretching, therefore, the Raman spectrum of agarose did not interfere with the spectra of MFGs.

The Raman spectral data were collected using a Senterra dispersive Raman microscope (Bruker Optics, Ettlingen, Germany). An Olympus BX confocal microscope (Olympus Europa GmbH, Hamburg, Germany) was used to observe and acquire Nomarski differential interference contrast images of the MFGs. The Raman microscope allows a depth profiling of $2\text{ }\mu\text{m}$ and high spatial resolution down to $1\text{ }\mu\text{m}$.

OPUS version 6.5 (Bruker Optics) was used to control the microscope and collect the spectra. An Olympus $100\times$ objective (numerical aperture 0.9) with a $50\text{ }\mu\text{m}$ confocal pinhole was used to collect the Raman signal from the centre of the globule sphere. The options ‘Sure_Cal’, ‘spectral shape correction’ and ‘cosmic spike removal’ were implemented within OPUS to ensure wavelength repeatability and transferability. An excitation wavelength of 532 nm from a diode laser (Sentinel, Bruker Optics), with a 5 mW power before the objective, and a 1200 groove per millimetre grating were used to record the Raman spectra of milk fat globules of different size and from different breeds of cow. A low laser power was used to avoid overheating the samples and therefore changing the ratio of liquid/crystalline fat. The spectral region recorded was $35\text{--}3707\text{ cm}^{-1}$ for both the Jersey and Friesian-Jersey MFGs. Each spectrum was the co-addition of thirty 10 s exposures and was

recorded at room temperature. Each peak intensity was measured and an average of intensity from three globules of the same size was calculated. The globules were classified as follows: ‘small’ for globules less than $2\text{ }\mu\text{m}$, ‘intermediate-sized’ for globules between $3\text{ and }9\text{ }\mu\text{m}$, and ‘large’ for globules over $10\text{ }\mu\text{m}$.

3. Results

3.1. Identification of the peaks

The main Raman peaks and their assignments are listed in Table 1. The peaks observed in the Raman spectra of milk fat particles mainly arise from the lipid molecules and can be readily assigned to well-known major bands of lipid vibrations by comparison with previously published studies of pure lipids, lipid mixtures or native lipid-containing samples. Due to their overwhelming contribution to the Raman spectra of MFGs, the intensity of the lipid bands would mask any putative peaks of proteins associated with the milk fat globules, as some protein Raman peaks overlap with Raman lipid peaks. The resonance enhancement of the carotenoid peaks at $1160\text{ and }1520\text{ cm}^{-1}$, due to conjugated systems of double and single bonds, masks the C–C stretching vibrations at 1100 cm^{-1} (Larsson, 1976).

The principle pigment composing 95% of the total carotenoids present in milk fat is β -carotene and it is found only in the milk fat core (MacGibbon & Taylor, 2006). Milk of Jersey cows contains more carotenoids than milk of Friesian and Friesian-Jersey cows

Table 1
Main Raman peaks assignments^a.

Origin	Wavenumber (ν/cm^{-1})	Assignment/structural information obtained
Phospholipid headgroup	845–895	846: Inositol residue 860: Phosphatidic acid 875: Choline
Carotenoids	1006	“Breathing” mode of ring compounds
Fatty acid	1063–1083	$\nu(\text{C}=\text{C})$ stretching Symmetric phosphoryl stretching <i>gauche</i> C–C
Saturated fatty acid	1125–1133	$\varphi(\text{C}=\text{C})$ Out-of-phase C–C solid fat
Carotenoids	1158 1213	$\nu(\text{C}=\text{C})$ Antisymmetric phosphoryl stretching
PC, PI and PS	1270	C=C <i>cis</i> unsaturation C–H in-plane bending of ethylene groups
PC, PI and PS	1303	$\delta(\text{CH}_2)$ twisting unsaturation
Cholesterol, PC, PI and PS	1443	Saturated fatty acid $\delta(\text{CH}_2)$ scissoring
Carotenoids	1526	$\nu(\text{C}=\text{C})$
PC, PI and PS	1654	$\nu(\text{C}=\text{C})$ <i>cis</i> unsaturation
Triacylglycerol	1744	Ester $\nu(\text{C}=\text{O})$
Acyl chains	2715–2726	$\nu(\text{C}=\text{H})$
Acyl chains in liquid state	2850	CH_2 symmetric stretching
Cholesterol, cholesterol ester	2874	CH_3 symmetric stretching
Acyl chains in crystalline state	2885 ± 5	Fermi resonance CH_2 stretching CH_2 anti-symmetric stretching
Acyl chains	2900–2910	$\nu(\text{C}=\text{H})$ of acyl chain
Acyl chains	2935 2960	CH_3 symmetric stretching out-of-plane and CH_3 anti-symmetric stretching
	3007	<i>cis</i> -unsaturated =CH stretching

^a Adapted from Faiman & Larsson, 1976, Forrest, 1978, Bresson, Marssi & Khelifa, 2005, Krafft et al., 2005, Argov et al., 2008, and McGoverin et al., 2009.

(Newstead, 1976). Friesian cows have a lower level of β -carotene and a higher level of vitamin A than Jersey and Guernsey cows (MacGibbon & Taylor, 2006). These two components are present at very low concentration of about 0.1% in milk fat globules (Forrest, 1978), and show absorption maximum below 500 nm. Carotenoid bands appear as intense peaks even in 1064 nm excited Raman spectra at concentrations as low as 0.02% (Ozaki, 1999). At lower concentration, the peaks are weaker but still detectable (Li-Chan, 1996). Pre-resonance Raman effects cause the strong enhancements of the Raman bands at 1010, 1160 and 1525 cm^{-1} due to carotenoids (Ozaki, 1999).

More peaks were observed in the Raman spectra of larger Jersey and Friesian-Jersey milk fat globules than for small and intermediate-sized globules, and they presented higher intensities. However in the region from $2700\text{ to }3100\text{ cm}^{-1}$, the Raman spectra of small Jersey and Friesian-Jersey milk fat globules presented more detailed peaks in the region of CH stretching, as the peak at 2885 cm^{-1} was weaker than in the spectra of larger globules. In Fig. 1, the fatty acid components looked like non-uniform, saturated fatty acids in the region $2700\text{--}3050\text{ cm}^{-1}$ (Krafft et al., 2005); the lipids of MFGs contain a large range of fatty acid residues (Fauquant et al., 2005).

In the Raman spectra of pure lipids and samples of high-lipid content, the region from $400\text{ to }1800\text{ cm}^{-1}$ is more informative for the identification of lipids than the region from $2700\text{ to }3500\text{ cm}^{-1}$, which contains strongly overlapping broad Raman bands from stretching vibrations of CH, NH and OH groups (Krafft et al., 2005). The latter also provides information about the hydrocarbon chain structure in lipids (Faiman & Larsson, 1976). In complex samples, the detection limits of each individual lipid species will depend on the concentration and the signal-to-noise ratio of the Raman spectra.

A distinct vibrational mode from phospholipids other than the carbon bond vibrations is typically observed around 860 cm^{-1} (Argov et al., 2008; Krafft et al., 2005). Identification of the individual phospholipids present in the milk fat globules would be difficult as the milk fat globule membrane contains a complex mixture of phospholipids. As seen in Fig. 1, the peak at around 860 cm^{-1} is quite broad, from $845\text{ to }895\text{ cm}^{-1}$, due to overlapping

contributions from the different polar head groups from the complex mixture of phospholipids.

3.2. Raman spectra of Jersey milk fat globules

The Raman spectra of Jersey milk fat globules (Fig. 2) showed differences in the lipid composition according to the size of the globules. The Raman spectra of all subclasses of globules, small, intermediate-sized and large, contained the following peaks: $607, 846, 1064, 1081, 1158, 1303, 1443, 1526$ and 1654 cm^{-1} . The intensity of the Raman bands at 1158 and 1526 cm^{-1} due to carotenoids increased in intensity with increasing globule diameter (Fig. 2).

The intensities of the Raman bands from the $1\text{ }\mu\text{m}$ MFGs are weak (Fig. 2). The stronger ones are at $1303, 1443$, both due to vibrations of the CH_2 groups, and 1654 cm^{-1} , due to $\nu(\text{C}=\text{C})$ of the hydrocarbon chains (Fig. 2). The bands at 607 and around 846 cm^{-1} were also present, thus we can suggest that these small globules contain mainly phospholipids and cholesterol. The triglyceride peak at 1744 cm^{-1} was undetectable in the MFGs smaller than $1\text{ }\mu\text{m}$ (Fig. 2). The triglyceride peak only appeared in the Raman spectra of MFGs of size $2\text{ }\mu\text{m}$ and larger (Figs. 1 and 2). The peaks at 1303 and 1443 cm^{-1} increased in intensity due to the additional hydrocarbon chains of the triglyceride molecules. The Raman spectra of MFGs larger than $9\text{ }\mu\text{m}$ presented more defined and more intense Raman bands and additional peaks not detected in the Raman spectra of MFGs smaller than $9\text{ }\mu\text{m}$ (Figs. 1 and 2). The extra band at 1006 cm^{-1} corresponded to the “breathing” of the aromatic compounds of carotenoids, and the extra bands at 966 ($\delta(\text{C}=\text{H})$), 1193 (C–C backbone), 1213 (anti-symmetric phosphoryl stretching) and 1269 cm^{-1} (C=C *cis* unsaturation) corresponded to the vibrations of phospholipids and fatty acid chains. The strong intensities of the carotenoids bands ($1006, 1158$ and 1526 cm^{-1}) showed an increase in concentration in carotenoids with increasing size of the milk fat globules (Fig. 2). The concentration of carotenoids in small globules may be too low as the characteristic bands are hardly detected in the Raman spectrum of a $1\text{ }\mu\text{m}$ MFG (Fig. 2).

A linear correlation has been reported between the iodine value and the unsaturation level of lipid-containing foods (Ozaki, Cho, Ikegaya, Muraishi, & Kawauchi, 1992). The unsaturation level was determined by calculating the ratio of the two band intensities (I) at 1654 (C=C stretching modes of *cis* unsaturated fatty acids) and 1744 cm^{-1} (ester $\nu(\text{C}=\text{O})$) and the ratio of the two band intensities at 1654 and 1443 cm^{-1} corresponding to the CH_2 scissoring mode of saturated fatty acids (Fig. 3). The ratio I 1654 :I 1744 of all the MFGs (Fig. 3) is close to 2.27 as reported by Forrest (1978) for milk fat globules at $20\text{ }^\circ\text{C}$, revealing that the Jersey milk fat globules have a low degree of unsaturation. Furthermore, the two ratios (Fig. 3) show that the percentage of unsaturation decreases with decreasing size of globules. Butter has a low iodine value of 31 and gives a weak C=C stretching band at 1654 cm^{-1} (Ozaki et al., 1992). The ratio I 1654 :I 1443 (Fig. 3) indicates that milk fat globules have a low degree of unsaturation, and thus a low iodine value.

Another ratio from Raman spectra is indicative of the configuration around the C=C bond in unsaturated fatty acids (Ozaki et al., 1992). *Cis* and *trans* unsaturated fatty acids give C=C stretching bands in the regions $1650\text{--}1665$ and $1670\text{--}1680\text{ cm}^{-1}$ respectively. In Fig. 2, only the band at 1654 cm^{-1} was observed, thus the *cis* configuration around the C=C bonds was dominant in the lipids of the Jersey milk fat globules.

A last indicator of the unsaturation level is the band at 1269 cm^{-1} (Ozaki et al., 1992). Butter shows a weak band related to the CH in-plane deformation modes of the ethylene groups due to a low degree of unsaturation. In Fig. 2, the band at 1269 cm^{-1} was weak and not even detected in the Raman spectrum of the $1\text{ }\mu\text{m}$ MFG. This band almost appeared as a shoulder of the more intense

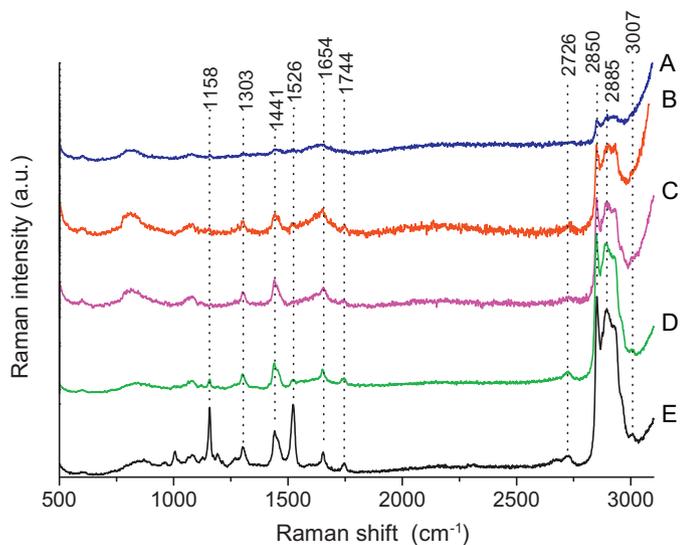


Fig. 1. Raman spectra of Jersey milk fat globules of size $1\text{ }\mu\text{m}$: spectrum A; $2\text{ }\mu\text{m}$: spectrum B; $5\text{ }\mu\text{m}$: spectrum C; $9\text{ }\mu\text{m}$: spectrum D; $15\text{ }\mu\text{m}$: spectrum E. (a.u.: arbitrary unit).

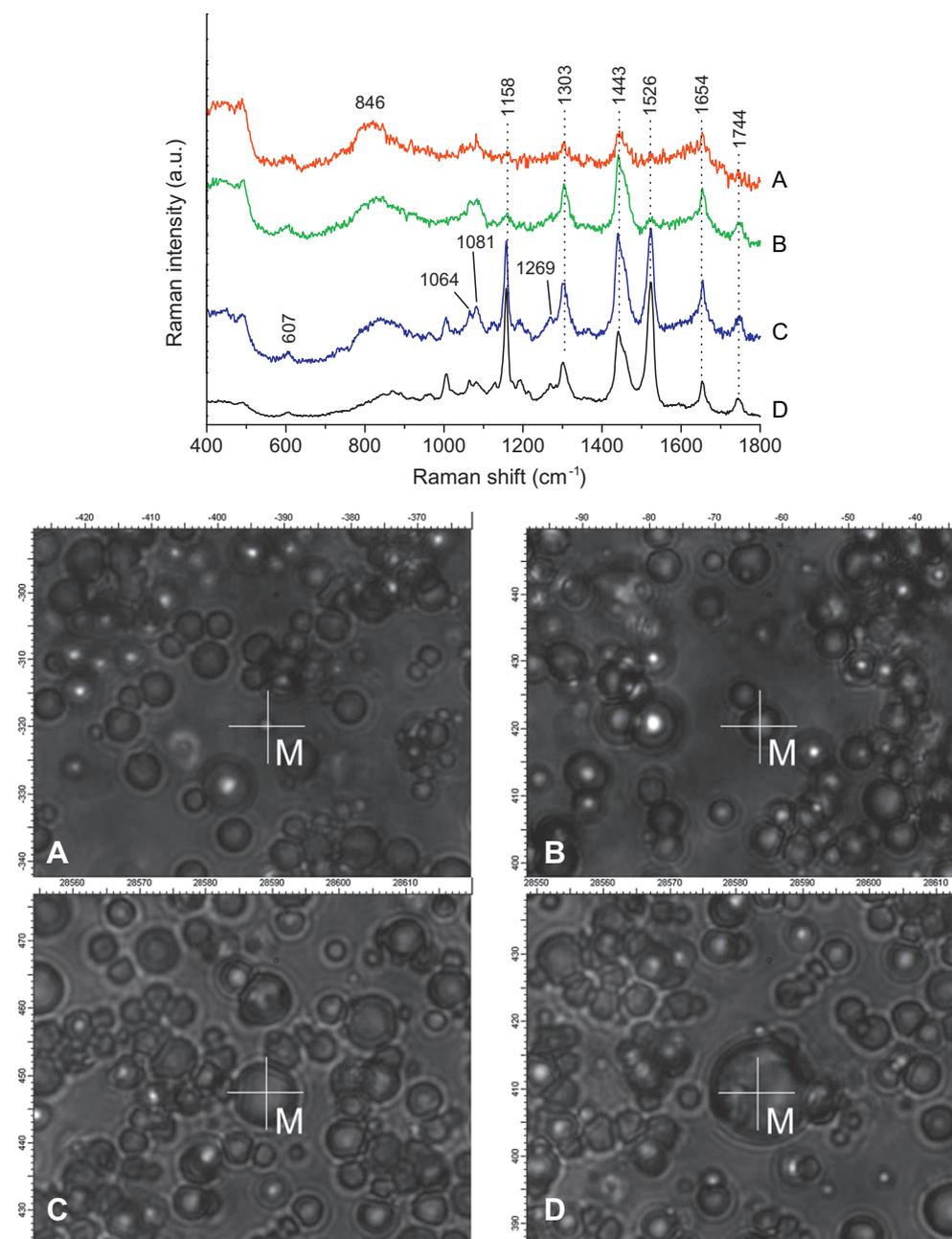


Fig. 2. Raman spectra of Jersey milk fat globules and associated differential interference contrast images of size 1 μm : spectrum and image A; 5 μm : spectrum and image B; 9 μm : spectrum and image C; 15 μm : spectrum and image D.

band at 1303 cm^{-1} . This, again, was characteristic of a low degree of unsaturation.

The intensities of the bands at 1063 , 1083 and 1130 cm^{-1} (Figs. 1 and 2), and at 2850 and 2885 cm^{-1} (Fig. 1) are representative of milk fat globules in a fluid state, respectively the C–C mobility and the C–H mobility (Forrest, 1978). However, the 1063 , 1083 , 1130 , 2850 and 2885 cm^{-1} peaks of few globules exhibited a less fluid state (Fig. 4), similar to the peaks of the Raman spectrum of milk fat globules at $8\text{ }^\circ\text{C}$ (Forrest, 1978).

3.3. Raman spectra of Friesian-Jersey milk fat globules

In the region from 2700 to 3100 cm^{-1} (Fig. 5), related to vibrations of the hydrocarbon chains (Table 1), differences between

Friesian-Jersey milk fat globules of varying size were observed. The Raman spectrum of $1\text{ }\mu\text{m}$ MFG was missing the band at $2720 \pm 20\text{ cm}^{-1}$. This band could be related to a barely detectable level of triglycerides in the small MFGs as the triglyceride band at 1739 cm^{-1} was also missing (Fig. 6). In the region 2800 – 3100 cm^{-1} , the same bands were detected in all spectra but with an increasing intensity with increasing size of the globules (Fig. 5). This was particularly true for the bands at 2850 (acyl chain in a liquid state) and 2885 cm^{-1} (acyl chain in a crystalline state). Most globule lipids were in a fluid state (Fig. 5), however the 1063 , 1083 , 1130 , 2850 and 2885 cm^{-1} peaks of the $16\text{ }\mu\text{m}$ MFG exhibited a less fluid state (Figs. 5 and 6).

The intensity of the band at 1443 cm^{-1} was much stronger than the intensity of the band at 1654 cm^{-1} (Figs. 6 and 7) revealing that

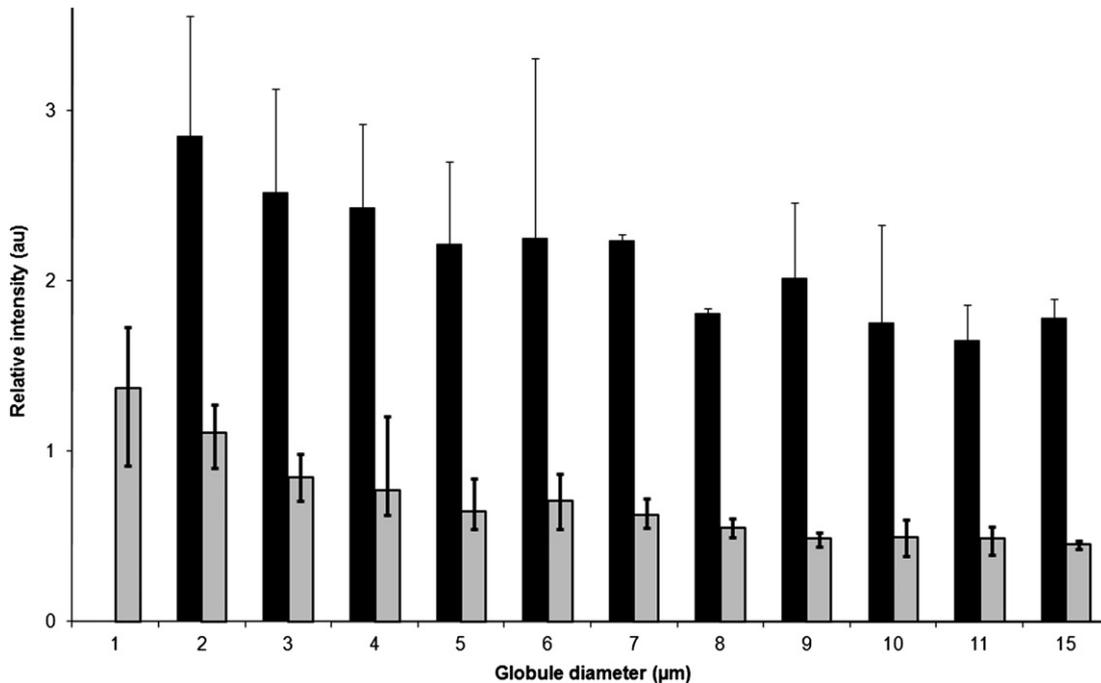


Fig. 3. Qualitative evaluation of the unsaturation level of Jersey milk fat globules (MFGs) with varying size. The columns indicate the double stretching band (1654 cm^{-1}) intensities (I) relative to either the triglyceride band (\blacksquare , 1744 cm^{-1}) or the CH_2 scissoring band (\blacksquare , 1443 cm^{-1}) intensities. There is no detectable level of triglycerides in MFGs of size $1\text{ }\mu\text{m}$.

the Friesian-Jersey MFGs had a low degree of unsaturation, and thus a low iodine value. No band at $1670\text{--}1680\text{ cm}^{-1}$ was detected (Fig. 6); only the band at 1654 cm^{-1} was observed, thus the *cis* configuration around the $\text{C}=\text{C}$ bonds was dominant in the lipids of the Friesian-Jersey MFGs. Both Jersey and Friesian-Jersey MFGs had a decreasing level of unsaturation with increasing size of the globules (Fig. 7). The band at 1269 cm^{-1} is very weak (Fig. 6), appearing as a shoulder of the stronger band at 1303 cm^{-1} . This, again, is characteristic of a low degree of unsaturation.

As stated above for Jersey MFGs, the lipids of Friesian-Jersey MFGs were in a fluid state. A few globules, such as the $16\text{ }\mu\text{m}$ MFG (Figs. 5 and 6), exhibited a less fluid state, as the 1063 , 1083 , 1130 , 2850 and 2885 cm^{-1} band intensities looked similar to the peaks for MFGs at $8\text{ }^\circ\text{C}$ reported by Forrest (1978).

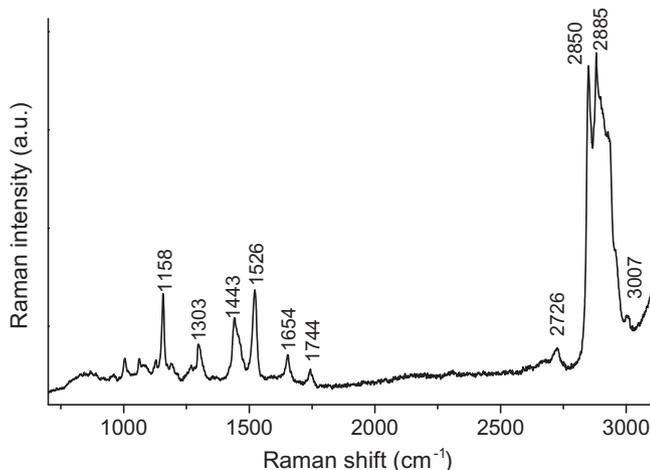


Fig. 4. Raman spectrum of an $11\text{ }\mu\text{m}$ Jersey milk fat globule in the region $800\text{--}3100\text{ cm}^{-1}$.

In the region from 400 to 1800 cm^{-1} (Fig. 6), the Raman spectra of small globules of less than $2\text{ }\mu\text{m}$ showed very weak bands at 605 , approximately 850 , 1064 , 1078 , 1298 , 1443 and 1654 cm^{-1} . These bands were due to the vibrations from phospholipids and cholesterol. The intensities of the bands in the Raman spectrum of the $3\text{ }\mu\text{m}$ MFG were slightly stronger than in the Raman spectrum of the $1\text{ }\mu\text{m}$ MFG (Fig. 6), and additional bands were present at 1125 and 1158 cm^{-1} , both weak, and at 1744 cm^{-1} due to the presence of triglycerides. The Raman spectra of MFGs larger than $8\text{ }\mu\text{m}$ presented similar bands to the Raman spectrum of intermediate-sized MFGs (Fig. 6), but with increased intensity and an extra band at 1526 cm^{-1} due to carotenoids, however the spectra of the $8\text{ }\mu\text{m}$ and $16\text{ }\mu\text{m}$ MFGs lacked the carotenoid band around 1006 cm^{-1} (Fig. 6). This band was detected in the Raman spectra of $12\text{ }\mu\text{m}$ and $17\text{ }\mu\text{m}$ MFGs (Fig. 8), therefore heterogeneities in composition exist between larger MFGs of varying diameters. The $8\text{ }\mu\text{m}$ and $16\text{ }\mu\text{m}$ MFGs observed in Fig. 6 contained a lower amount of carotenoids than the $12\text{ }\mu\text{m}$ and $17\text{ }\mu\text{m}$ MFGs observed in Fig. 8.

3.4. Comparison of Jersey and Friesian-Jersey milk fat globules

3.4.1. Raman spectra of milk fat globules of less than $2\text{ }\mu\text{m}$

The Raman spectra of small milk fat globules (less than $2\text{ }\mu\text{m}$) presented very weak bands (Fig. 9). One striking characteristic was the absence of a detectable triglyceride band near 1744 cm^{-1} (Fig. 10). Both Jersey and Jersey-Friesian small MFGs have a similar composition. Their Raman spectra revealed the presence of mainly phospholipids and cholesterol, probably from the MFGM. The band at 1443 cm^{-1} appeared clearly (Fig. 9), denoting the presence of saturated fatty acids, probably from MFGM sphingomyelin molecules, which are highly saturated and tightly packed (Gallier, Gragson, Jimenez-Flores, & Everett, 2010).

3.4.2. Raman spectra of milk fat globules in the size range $2\text{--}8\text{ }\mu\text{m}$

The Raman spectra of $5\text{ }\mu\text{m}$ Jersey MFGs presented additional bands compared to $5\text{ }\mu\text{m}$ Friesian-Jersey MFGs. These new features

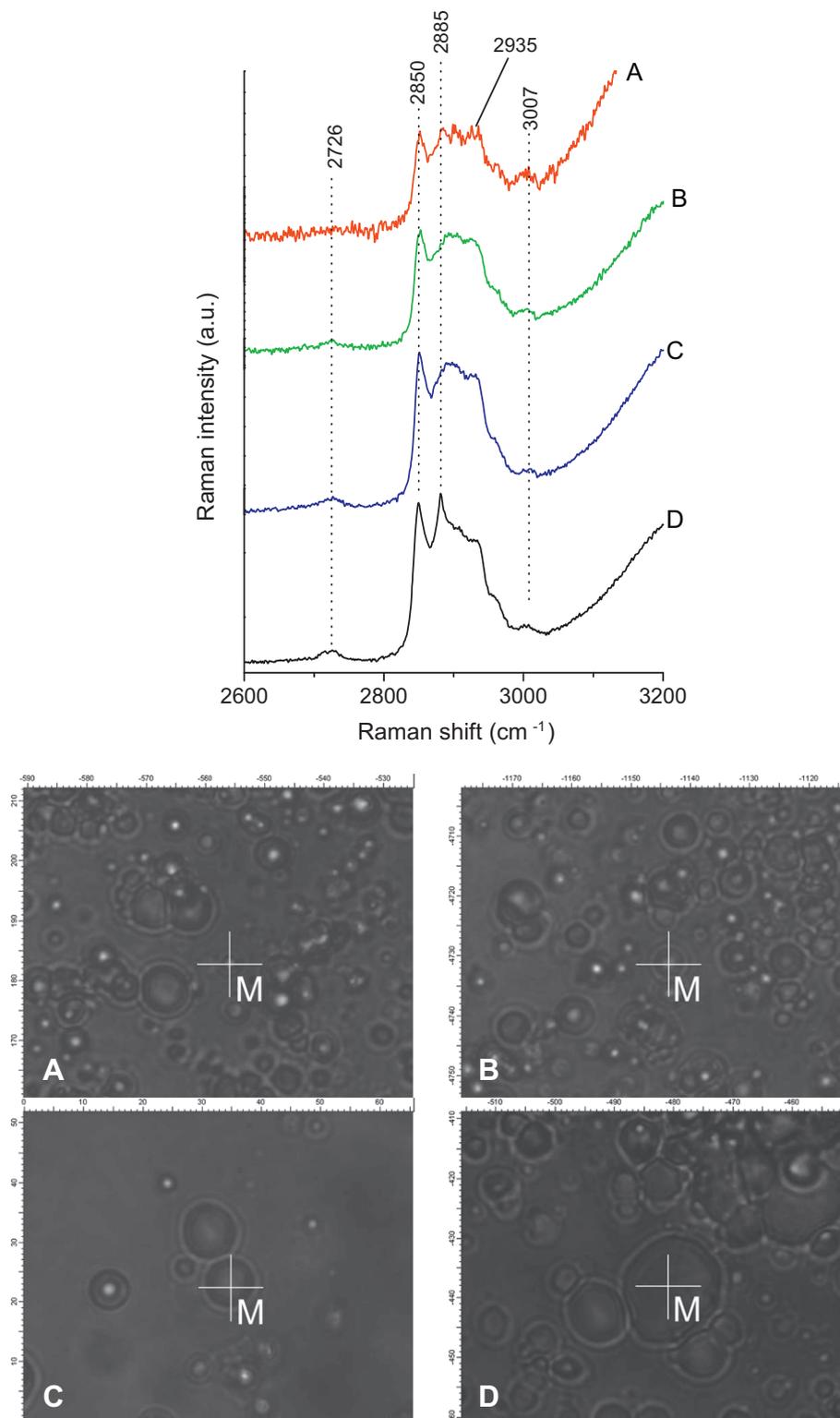


Fig. 5. Raman spectra of Friesian-Jersey milk fat globules and associated differential interference contrast images of size 1 μm : spectrum and image A; 3 μm : spectrum and image B; 8 μm : spectrum and image C; 16 μm : spectrum and image D.

lie at 1161 and 1526 cm^{-1} (Fig. 11) and are due to the presence of carotenoids. The bands around 1065–1083 cm^{-1} were broader in the spectra of the Jersey MFGs than in the spectra of the Friesian-Jersey MFGs (Fig. 11). This suggests the increased presence of *gauche* rotamers and phospholipids, also observed by the more defined peaks around 820–846 cm^{-1} .

The carotenoid bands (1006, 1158 and 1526 cm^{-1}) appeared clearly in the Raman spectra of 9 and 10 μm Jersey MFGs (Fig. 12). The band at 1158 cm^{-1} appeared very weak in the Raman spectrum of the 8 μm Jersey MFG, whereas the two other carotenoid bands were not detected (Fig. 12). Thus, the concentration in carotenoids in the Jersey MFGs increased with increasing size of the globules

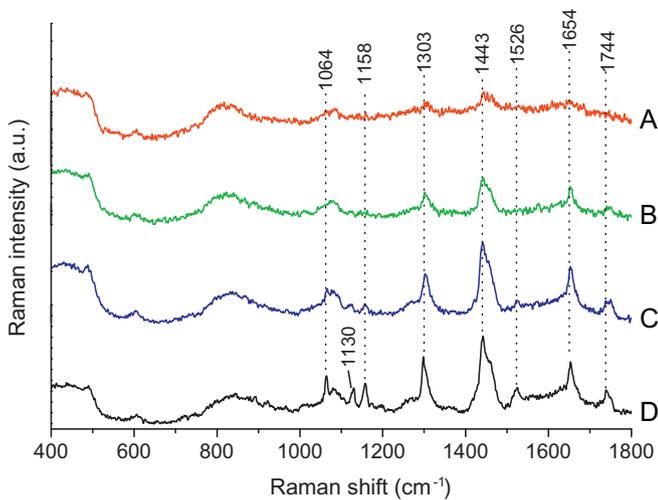


Fig. 6. Raman spectra of Friesian-Jersey milk fat globules focused on the more informative region from 400 to 1800 cm^{-1} (1 μm MFG: A; 3 μm MFG: B; 8 μm MFG: C; 16 μm MFG: D).

and reached a detectable level in the MFGs larger than 9 μm . The Raman spectra of the 9 μm Jersey MFG and the 8 μm Friesian-Jersey MFG (Fig. 12) lacked the band at 1368 cm^{-1} assigned to the presence of saccharides, possibly originating from the presence of molecules such as galactocerebroside and gangliosides (Krafft et al., 2005). Both molecules are found in the MFGM at small concentrations (Rombaut & Dewettinck, 2006). The 9 μm Friesian-Jersey MFG did not contain a detectable level of carotenoids and the Raman spectrum of the 8 μm Friesian-Jersey MFG only displayed a weak band at 1524 cm^{-1} (Fig. 12). The presence of carotenoids was only detected in the Friesian-Jersey MFGs of size 10 μm (Fig. 12). The intensities of the carotenoid bands were stronger in the Raman spectra of the Jersey MFGs than in the spectra of the

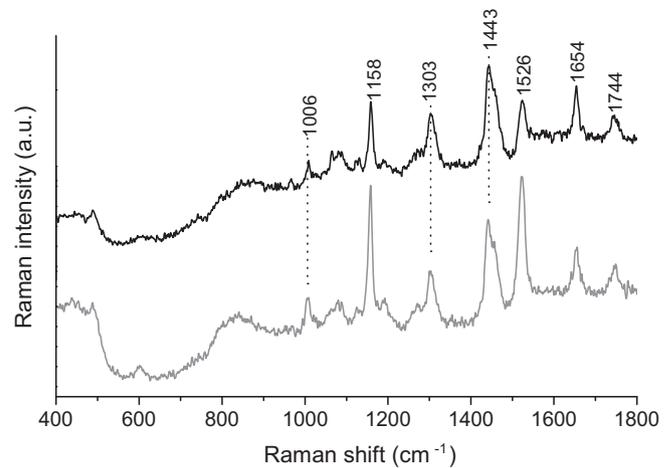


Fig. 8. Raman spectra of 12 μm (black) and 17 μm (gray) Friesian-Jersey milk fat globules in the region 400–1800 cm^{-1} .

Friesian-Jersey MFGs (Fig. 12), revealing a higher concentration in carotenoids in the Jersey MFGs.

3.4.3. Raman spectra of milk fat globules larger than 10 μm

The intensities of the Raman bands in the spectra of the large Jersey MFGs were stronger than in the spectra of the Friesian-Jersey MFGs (Fig. 13). The spectra of the large Jersey MFGs presented four additional bands at 866 (phospholipids), 890 (phospholipids), 960 (saturated fatty acid) and 1269 cm^{-1} (*cis* unsaturation) (Fig. 13). The Raman spectrum of the 16 μm Friesian-Jersey MFG (Fig. 13) was missing the carotenoid band at 1006 cm^{-1} , whereas the other carotenoid bands were quite weak compared to those from the Jersey MFGs. The Raman spectrum of the 17 μm Friesian-Jersey MFG (Fig. 13) did not contain a band at 1064 cm^{-1} .

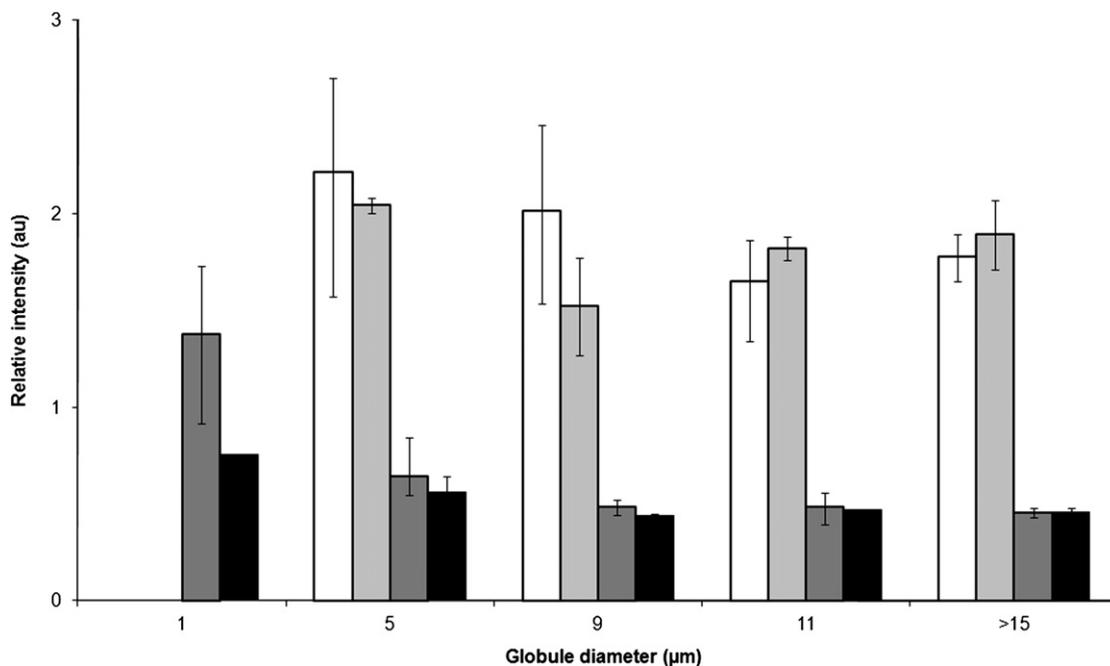


Fig. 7. Qualitative evaluation of the unsaturation level of Jersey (□, ■) and Friesian-Jersey (□, ■) milk fat globules (MFGs) with varying size. The columns indicate the double stretching band (1654 cm^{-1}) intensities (I) relative to either the triglyceride band (□, ■; 1744 cm^{-1}) intensities or the CH₂ scissoring band (□, ■; 1443 cm^{-1}) intensities. There is no detectable level of triglycerides in MFGs of 1 μm .

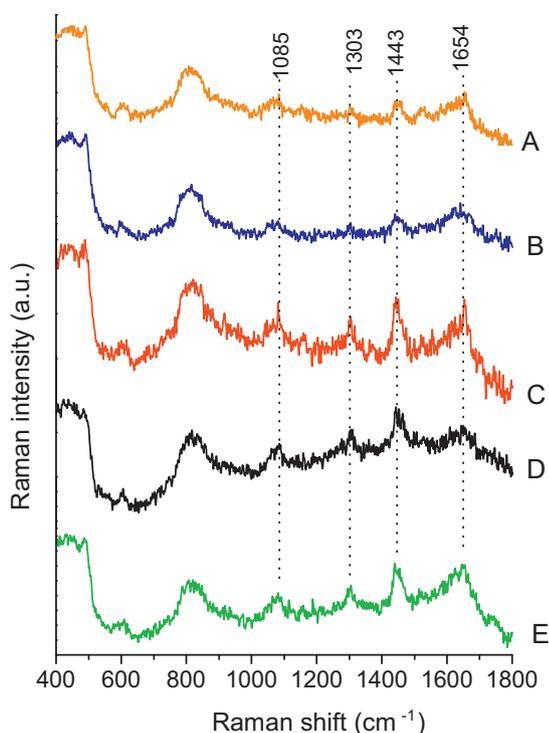


Fig. 9. Raman spectra of Jersey and Friesian-Jersey milk fat globules and associated differential interference contrast images (less than 1 μm Jersey MFG: spectrum A; 2 μm Jersey MFG: spectrum B; 1 μm Jersey MFG: spectrum C; 1 μm Friesian-Jersey MFG: spectrum D; 2 μm Friesian-Jersey MFG: spectrum E).

Butter also does not contain this band at 1064 cm^{-1} (Ozaki et al., 1992). The greater intensity of the band at 1081 cm^{-1} relative to the bands at 1064 and 1126 cm^{-1} was associated with the presence of *gauche* rotamers in the acyl chains, and thus the presence of mobile hydrocarbon chains. Upon cooling, the intensity of the former decreased whereas the intensities of the latter two increased (Forrest, 1978). It was also suggested that the ratio of *trans* to *gauche* chains, and thus the lipid chain mobility, could be determined by calculating the C–C intensity ratio $(I_{1130} + I_{1060})/I_{1080}$ and the C–H intensity ratio I_{2885}/I_{2850} (Forrest, 1978). The

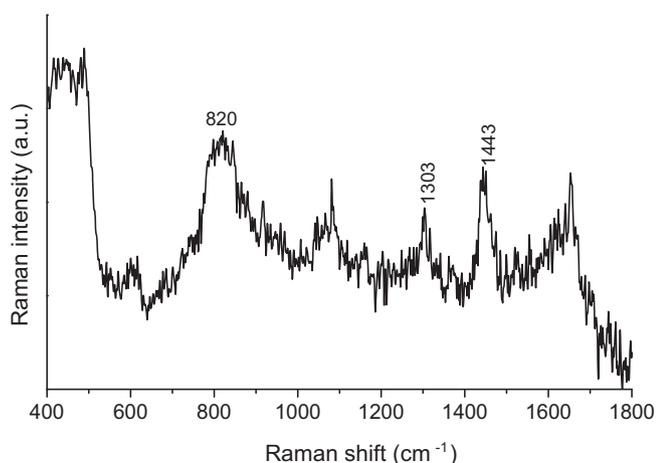


Fig. 10. Raman spectrum of a 1 μm Jersey milk fat globule in the region 400–1800 cm^{-1} .

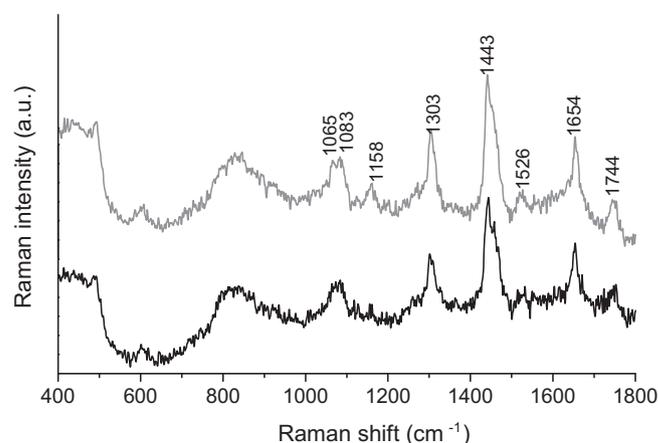


Fig. 11. Raman spectra of Jersey (gray) and Friesian-Jersey (black) 5 μm milk fat globules focused on the more informative region from 400 to 1800 cm^{-1} .

intensities of the bands at 1064 , 1081 and 1125 cm^{-1} for both Jersey and Friesian-Jersey MFG spectra in Figs. 12 and 13 looked similar to the intensities of the same bands of milk fat globules at $25\text{ }^\circ\text{C}$ (Forrest, 1978), with the exceptions stated above, thus the lipid acyl chains of both types of MFGs are in a fluid state.

Argov et al. (2008) observed the separation of the three peaks, 1063 , 1083 and 1130 cm^{-1} only for submicron human milk fat globules. In Figs. 9–13, the three peaks are separated for all sizes of bovine milk fat globules. In addition, Argov et al. (2008) did not detect the bands at about 1066 and 1129 cm^{-1} for human milk fat globules larger than 1 μm in size; this suggests that human milk fat

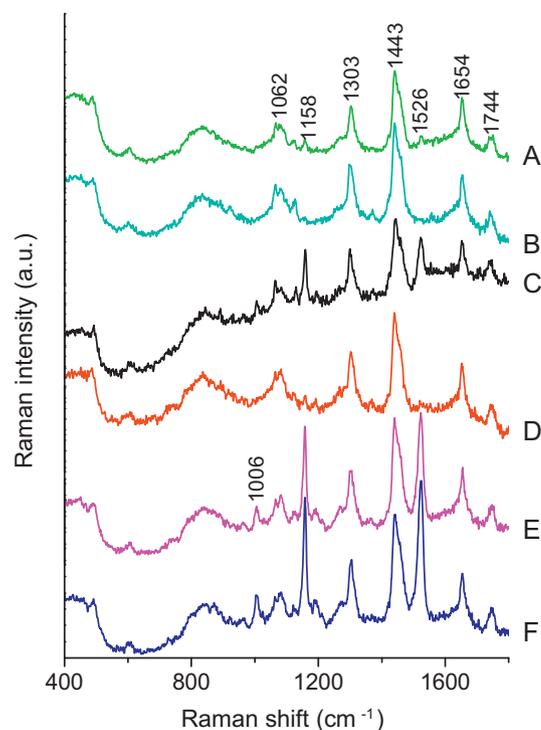


Fig. 12. Raman spectra of Jersey and Friesian-Jersey milk fat globules (8 μm Friesian-Jersey MFG: A; 9 μm Friesian-Jersey MFG: B; 10 μm Friesian-Jersey MFG: C; 8 μm Jersey MFG: D; 9 μm Jersey MFG: E; 10 μm Jersey MFG: F).

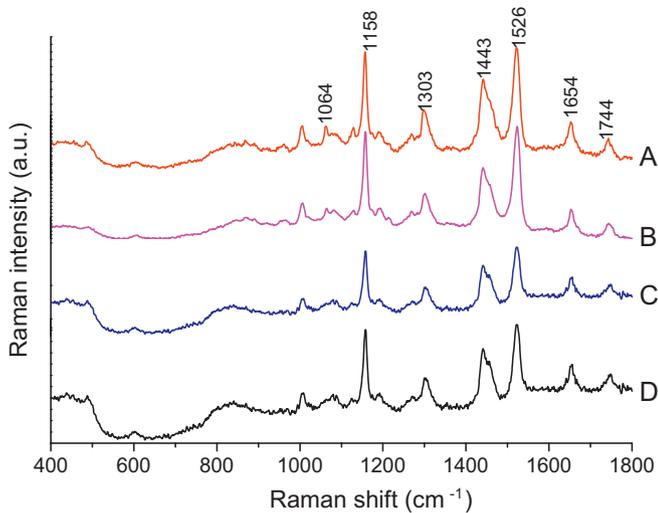


Fig. 13. Raman spectra of Jersey and Friesian-Jersey milk fat globules (11 μm Jersey MFG: A; 15 μm Jersey MFG: B; 16 μm Friesian-Jersey MFG: C; 17 μm Friesian-Jersey MFG: D).

globule lipids are more fluid, less saturated and contain more *gauche* rotamers than bovine milk fat globule lipids.

3.5. Presence of lipids in a crystalline state in the milk fat globules at 20 °C

The increased intensity of the band at 2850 cm^{-1} relative to the band at 2885 cm^{-1} is indicative of higher mobility of the hydrocarbon chains (Faiman & Larsson, 1976; Forrest, 1978). This was confirmed by the increased intensity of the band at 2885 cm^{-1} upon cooling, and by the MFGs of Jersey (Fig. 1) and Friesian-Jersey MFGs (Fig. 14) being typical of MFGs at 25 °C with highly mobile acyl chains (Forrest, 1978). The presence of the bands at 2885 and 2850 cm^{-1} indicates the presence of crystals in the α -form (Forrest, 1978). However the intensities of these bands can vary, even between milk fat globules of the same size (Fig. 15). This indicates that the fatty acid composition varies between globules of different size classes and globules of the same size. Moreover, the fatty acid chains of milk triglycerides and phospholipids contain different fatty acids, varying in chain length, degree of unsaturation and conformation, attached to the glycerol backbone (MacGibbon & Taylor, 2006). Therefore each fatty acid has a different melting point and will be in a different state at a given temperature.

Cholesterol has been found in the lipid core and the MFGM (Mulder & Walstra, 1974). The peak at 2870 cm^{-1} , assigned to CH_3 symmetric stretching of cholesterol molecules, is weak and appeared as a shoulder of the more intense peak at around 2885 cm^{-1} (Fig. 14). The differentiation between the two peaks is clearly seen in Fig. 14 in the pink (3 μm) spectrum.

4. Discussion

4.1. Absence of a detectable triglyceride component in small milk fat globules

Milk fat globules have always been considered as a delivery vehicle of energy in the form of triglycerides. In the current study, a new functional form can be attributed to the fraction of small MFGs, as there was no detectable level of triglycerides in the MFGs of size less than 2 μm using this technique. Milk globules consist of

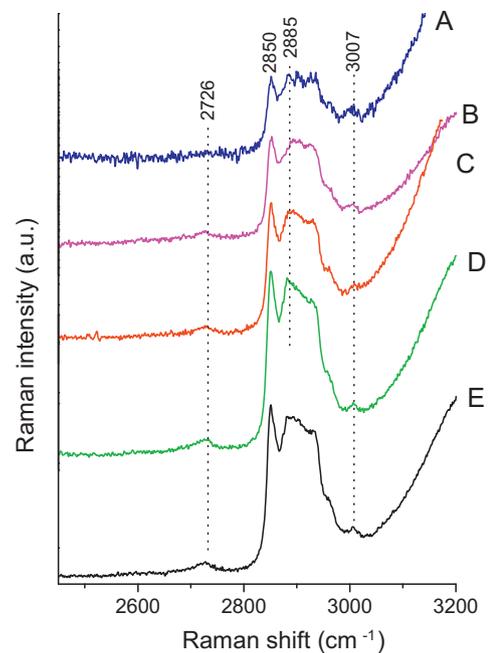


Fig. 14. Raman spectra of Friesian-Jersey milk fat globules in the region 2700–3100 cm^{-1} (1 μm MFG: A; 3 μm MFG: B; 5 μm MFG: C; 9 μm MFG: D; 16 μm MFG: E).

a triglyceride core and a phospholipid trilayer membrane (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999), thus the spectra should reflect contributions from both the core and the membrane. The ratio of MFGM to triglycerides is higher in the small MFGs, and the wavelength of the laser is the same order of size as the small MFGs, therefore the relative contribution by the MFGM to the total lipid spectra will be higher for the smaller globules, as will the contribution from the phospholipids.

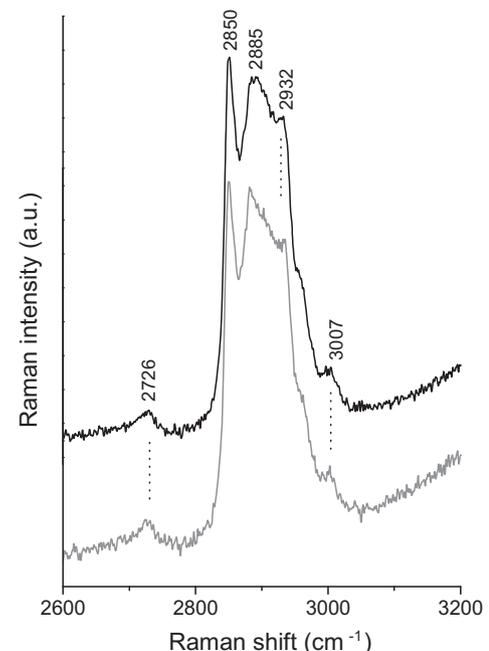


Fig. 15. Raman spectra of 12 μm Friesian-Jersey milk fat globules in the region 2700–3100 cm^{-1} .

Waning, Kalda, Paulsson, Nylander, and Bergenstahl (2003) introduced the concept of isolated milk fat globule membrane vesicles in dairy products. These vesicles might originate from phospholipids released when the milk is cooled and centrifuged or from membrane material lost through processing, such as homogenization and churning. Mulder and Walstra (1974) reported the presence of lipoproteins particles or 'milk microsomes' of the MFGM in milk and buttermilk, however their size range from 10 to 200 nm. The lipoprotein particles contain phospholipids, other glycerides, cholesterol, protein and several enzymes. Michalski et al. (2002) found that very small fat droplets (<500 nm) are present in milk at early processing stages (pumping and transporting) but not in milk analyzed right after milking, whereas Walstra (1969) reported the presence of very small fat globules in milk after milking. Thus, the small MFGs, analyzed after milking without any further processing treatment, and lacking a detectable triglyceride component, could consist mainly of phospholipid or residual MFGM vesicles and be analogous to phospholipid liposomes. They may arise from a different mechanism of formation and have a different biological role such as a higher rate of digestibility (Ward, German, & Corredig, 2006).

The same observation has been made for human milk fat globules studied using laser trapping confocal Raman spectroscopy (Argov et al., 2008). These authors reported the absence of triglycerides in the milk fat globules of submicron and 1 μm size, and named the globules 'lactosomes'. They also observed a difference in both the lipid composition and relative lipid content between classes of particles with different diameters. The submicron particles contained a large amount of unsaturated fatty acids. *Cis* and *trans* isomers of unsaturated fatty acids were unequally distributed between the small and large globules. In addition, Argov et al. (2008) did not detect the presence of carotenoids in human MFGs.

4.2. Differences in milk fat globule composition according to their size

The anti-symmetric stretching vibrations dominate in the C–H stretching region in crystalline phases in Raman spectra (Faiman & Larsson, 1976). The symmetric stretching vibrations dominate in the liquid crystalline phases. The ratio 2885/2850 in milk is higher than the ratio 2885/2850 in milk fat, denoting that the oil/water interface (the MFGM trilayer) at the surface of the globules is mostly in a crystalline state and closely packed (Larsson, 1976) whereas the lipid core is in a more liquid state. Indeed Raman spectrum of milk, and similarly the Raman spectrum of intact milk fat globules, provides information about the MFGM due to total reflection enhancement of the light scattering from the surface layer (Larsson, 1976).

Milk fat is liquid above 40 °C and completely solidified below –40 °C (Mulder & Walstra, 1974). Therefore, at the experimental temperature of 20 °C, milk fat is in a mixed liquid/crystalline state. The Raman spectra of phospholipids in the C–H stretching region present typical bands at 2850 (symmetric CH₂ stretching), 2885 (anti-symmetric CH₂ stretching) and 2935 cm⁻¹ (symmetric CH₃ stretching). The intensity ratios of these three bands provide information about the fluidity of the membrane, interactions of the CH₂ groups and the packing order near the terminal CH₃ group in the hydrophobic centre of the bilayer (Li-Chan, 2007). The ratio 2885/2850 corresponds to the order related to lateral chain-chain interactions, and the ratio 2885/2935 gives information on the inter-chain order and *gauche* conformations along the acyl chains.

Forrest (1978) was the first to report the use of Raman spectroscopy to obtain the chemical fingerprint of the milk fat globules and its membrane. This author determined that, at 20 °C, the MFG

lipids were 30–40% more ordered than the lipids of the intact MFGs, and the milk triglycerides were at 39 ± 5% in the *cis* unsaturated configuration. However, the sample preparation in this study (Forrest, 1978) can be criticized. Indeed, the pretreatment to obtain the milk fat was quite harsh and may have ruptured or involved loss of MFGM, in particular after a long ultracentrifugation (Anderson & Brooker, 1975). The MFGM is more rigid due to the presence of long-chain fatty acids in phospholipids (Dewettinck et al., 2008). The MFGM was isolated prior to the Raman study of Forrest (1978), therefore it is not in its native state but rather in an amorphous state, and the native structure may be completely lost. With his technique, Forrest (1978) detected only very weak carotenoids peaks and did not detect the contributions from the phospholipids around 860 cm⁻¹ in the Raman spectra of MFGs at –97 °C and 20 °C.

The physicochemical properties of MFGs have a major impact on the properties of dairy products, particularly butter, cream and cheese. Milk fat has a wide melting range and presents mostly β' crystals (Walstra, 2002). The smaller the globules, the more crystallization with small and unstable crystals (Couvreur & Hurtaud, 2007). Thus a higher pressure and a longer time are required during churning of the cream in the butter-making process for smaller globules. Butter, made from milk containing small fat globules, contains more intact MFGs and is wetter and, therefore, more spreadable than butter rich in large fat globules. Milk with large fat globules is more suitable for creaming. A cream with small MFGs will have reducing ability to be whipped into Chantilly, or to be churned (Walstra et al., 1999). In cheese, small fat globules easily insert into the protein network, leading to a higher water-retention capacity and wetter cheese (Gougedranche, Fauquant, & Maubois, 2000). The large fat globules tend to be destructive of the protein network as they are often larger than the protein pores. Thus, a cheese with small fat globules of less than 2 μm is less firm than a cheese with globules larger than 2 μm (Gougedranche et al., 2000). The size of native milk fat globules, separated by means of microfiltration, affects functional and physicochemical properties of Camembert cheese (Michalski et al., 2003) and Emmental cheese (Michalski et al., 2004) due to an increased surface area of the native MFGM in small globules compared with larger MFGs. Small fat globule Camembert and Emmental cheeses are less firm, and undergo greater proteolysis, but with reduced lipolysis compared with large fat globule cheeses (Michalski et al., 2003, 2004).

4.3. Differences in milk fat globule composition according to the breed of cow

Scarce data about the characterization of the milk fat globules by breed of cow are available (Couvreur & Hurtaud, 2007). High-butterfat breeds of cow, such as Jersey, produce milk with MFGs larger on average than high-yielding breeds, such as Holstein (also called Friesian). Milk fat from Friesian cows is more concentrated in sphingomyelin than milk fat from Jersey cows, however, the sphingomyelin content in whole milk does not differ between the two breeds due to a higher fat content in milk from Jersey cows (Graves, Beaulieu, & Drackley, 2007). The major difference observed in the current work was the variation in carotenoid content and presence of *gauche* rotamers between the two breeds. Carotenoids are responsible for the yellowish colour of milk (MacGibbon & Taylor, 2006), and thus the colour of Jersey cows' milk may be more yellow than Friesian-Jersey cows' milk, as the former has a higher content of carotenoids.

5. Conclusions

Small bovine milk fat globules may not be true fat globules as no detectable triglyceride peak was found by the Raman spectroscopic

method. They may offer specific physiological and nutritional functions, as well as different technological properties than the larger milk fat globules. The presence of liquid mobile and crystalline acyl chains in milk fat globules confirmed the variation in the fatty acid composition and lipid content within a same globule, but also between globules of different size and from two different breeds of cow. It was also confirmed that Jersey cows have more carotenoids than Friesian cows. The concentration in carotenoids increased with increasing size of globules. Milk fat globule lipids preferably adopt a *cis* configuration and exhibit a low level of unsaturation. It was also shown that Raman spectroscopy may be useful to obtain information on the composition and structure of the milk fat globule membrane, deduced from the Raman spectra of milk fat globules.

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