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Effects of Freeze Drying and Spray Drying on the Microstructure and Composition of Milk Fat Globules

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Running title: Differences of freeze-dried and spray-dried milk fat globules

Abstract

Freeze drying and spray drying are conventional methods for converting milk into powder in the dairy industry. The purpose of this study was to investigate the effects of these drying methods on the physical, chemical and structural features of milk fat globules (MFGs). The globule sizes increased after freeze drying and spray drying, but the integrity of their microstructures was still maintained. Compared with that in untreated MFGs, the composition of the fatty acids underwent fewer changes under the two different drying conditions. The main phospholipids showed some differences between the freeze-dried milk and spray-dried milk, and the saturation of the fatty acids of phospholipids was increased after the drying treatments. A reduction in the cholesterol content was observed after the freeze-drying and spray-drying processes. The level of unsaturation decreased as the diameter of the globules increased. The results indicated that freeze drying had a significant effect on the liquid-ordered domains in the MFG membrane, which was formed by sphingolipid and cholesterol. However, spray drying influenced the liquid-disordered domains, which were formed by glycerophospholipid. Thus, the drying method appears to affect the physical, chemical and microstructure characteristics of MFGs, which may affect the stability of the globules in milk and the way the milk is digested.

Keywords: Milk fat globules, Freeze drying, Spray drying, Structure, Composition

Introduction

Milk secreted by mammals can supply nutrition and immunological protection to the young. However, the milk preservation period is generally limited due to staling and spoilage caused by microbial growth at high water activity. Microbial spoilage is by far the most common cause of spoilage as indicated by visible growth (slime, colonies), textural changes (polymer degradation) or off-odours and off-flavours.[1] The preservation techniques for liquid milk are especially important and necessary.

Applications of freeze drying or spray drying to convert milk into powder without changing its nutritional and sensory characteristics to extend the shelf-life of milk have been documented.[2-4] Freeze drying, which is time consuming and expensive, is an important drying process for milk to conserve its flavour, bioactivities and other properties.[5] However, the spray-drying process only requires a very short time.[6] Both the freeze-drying and spray-drying methods can enhance the apparent solubility of milk concentrate powders.

The influence of the drying methods on the surface properties and morphological characteristics of milk fat globules (MFGs) has been reported. However, previous studies emphasised the changes in size, shape and surface proteins of milk powders produced by spray drying.[7-11] The choice of drying methods affects some physical and chemical characteristics of milk. Both freeze-dried and spray-dried milk powders have essentially the same flavour characteristics. However, freeze-dried milk powder frequently acquires a fruity flavour that is not apparent in spray-dried milk.[12] Freeze-dried and spray-dried MFG membranes (MFGM) are morphologically different when examined under light microscopy. The freeze-dried MFGM are irregular flaky translucent sheets with sharp edges, whereas the spray-dried MFGM are spherical particles.[13] Drying methods also increase the degree of protein denaturation and surface

fat coverage.[14] It had been proved that adverse effects are reduced when using lower temperature than is conventionally used in spray drying.[15] The drying methods also can alter the thermodynamic state of the phospholipids in MFGM.[13] To our knowledge, there are few studies of the composition and changes in the microstructure of freeze-dried and spray-dried MFGM. Moreover, the chemical fingerprint of individual MFGs of different sizes from freeze drying and spray drying are also poorly described.

In the present study, the microstructure and lipid composition of freeze-dried and spray-dried milk were measured and compared. The differences in composition of the MFGs according to their sizes obtained with these methods were also reported. Our research will provide insight into the influence of the different methods of drying on MFGs.

Materials and Methods

Milk samples

Raw milk (RM): Cow milk samples from Chinese Holstein cows were purchased from a local producer (Tianzi Dairy Industry Co., Ltd., Wuxi, Jiangsu, China). The milk was left to cool at room temperature and was characterised within 12 h after milking. Freeze-dried milk (FDM): Raw milk was frozen at -20°C and then freeze-dried for 24 h at 5×10^{-3} mbar and -45°C in a Freeze Dry System (Labconco, Kansas City, MO, USA). One gram of dried milk was then dissolved in 10 mL of deionised water at room temperature. Spray-dried milk (SDM): Raw milk was spray-dried in a laboratory-scale SD-1500 spray dryer (Voldy Science & Technology Co., Ltd., Shanghai, China). The milk was fed into the main chamber through a peristaltic pump, and the feed flow rate was controlled by the pump rotation speed. The inlet air temperature was 160°C and the outlet air temperature was 80°C. The spray-dried powder (1 g) was then dispersed in 10

mL of deionised water for analysis.

Particle size measurements

The MFG size distributions were determined by laser light scattering using a Mastersizer 2000 (Malvern Instruments, Malvern, UK), equipped with a He/Ne laser (λ=633 nm) and an electroluminescent diode (λ=466 nm). The refractive index of milk fat was taken to be 1.460 at 466 nm and 1.458 at 633 nm. The milk samples were diluted in about 100 mL of water directly in the measurement cell of the apparatus to reach 10% obscuration. The casein micelles were dissociated by adding 1 mL (35 mM EDTA/NaOH, pH 7.0) buffer to the milk in the apparatus. The size distributions of MFGs were characterised by the volume-weighted mean diameter $D_{4,3}$, defined as $\sum n_i d_i^4 / \sum n_i d_i^3$, and the volume/surface mean diameter $D_{3,2}$, defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of fat globules of diameter d_i.

Apparent zeta potential

MFG electrophoretic mobility was measured by electrophoretic light scattering using a Malvern Zetasizer 2000 (Malvern Instruments, Worcestershire, UK). Samples were prepared by suspending 10 μL milk in 10 mL buffer (20 mM imidazole, 50 mM NaCl, 5 mM $CaCl_2$, pH 7.0), and the zeta potential was measured at 25°C. The averages of three measurements were reported as zeta potentials.

Surface tension

Surface tension of the milk was determined by a DCAT21 surface tension meter (DataPhysics, Filderstadt, Germany). The surface temperature of both the raw and dried milk remained constant at 25°C.

120

121 **Confocal laser scanning microscopy (CLSM)**

122 The microstructures of MFGs were analysed with a Zeiss LSM 710 Meta confocal
123 microscope. A 63 × (NA 1.4) oil immersion objective was used for all images. Confocal
124 experiments were performed using an argon laser operating at 488 nm excitation
125 wavelength and a He-Ne laser operating at 543 nm excitation wavelength. The milk
126 sample for observation was prepared as previously reported.[16] Lipid-soluble Nile Red
127 fluorescent dye (9-diethylamino-5H-benzoalpha-phenoxazine-5-one; Sigma-Aldrich,
128 St. Louis, MO, USA) (42 μg/mL in acetone) was used to stain the triacylglycerol core
129 of the MFGs. The fluorescent dye N-(lissamine rhodamine B sulfonyl)
130 dioleoylphosphatidylethanolamine (Rh-DOPE; Avanti Polar Lipids, Inc., Alabaster,
131 AL, USA) (1 mg/mL in chloroform) was used to label the phospholipids.

132

133 **Extraction of total lipids**

134 Total lipids of milk were extracted by homogenising with 2:1 chloroform-methanol
135 (v/v).[17] The homogenate was treated by ultrasonic waves for 10 min and then
136 centrifuged for 10 min at 4500 × *g*. The organic phase, which contained the milk lipids,
137 was shaken and equilibrated with one-fourth volume of a saline solution (NaCl 0.86%,
138 w/w). The extract was moved to a separatory funnel for 20 min, and the liquid at the
139 bottom was filtered and evaporated under vacuum.

140

141 **Gas chromatography (GC) analysis of fatty acids**

142 Twenty milligrams of milk fat in 2 mL hexane and 500 μL of 2 mol/L KOH-CH_3OH
143 were added in a screw-capped tube. The reagents were incubated for 5 min at room
144 temperature, and then 5 mL of deionised water was added. The upper layer was

recovered and analysed by GC.

Fatty acid methyl esters were measured on an Agilent 7820 GC (Agilent Corp., USA) equipped with a flame ionisation detector and a capillary column TRACE TR-FAME (60 m × 0.25 mm × 0.25 μm, Thermo Fisher, USA). The oven temperature was programmed as follows: 60°C for 3 min, then raised to 175°C at 5°C min^{-1} and held for 15 min, and finally raised to 220°C at 2°C min^{-1} and held for 10 min. The injector and detector temperatures were 230°C and 250°C, respectively.

High-performance liquid chromatography analysis of phospholipids

Phospholipids were analysed with a high-performance liquid chromatograph equipped with an evaporative light-scattering detector.[18] A silica column (4.6 mm × 250 mm, 5 μm particle size) conjugated with a precolumn was used in this study. Nitrogen was used as the nebulising gas at a flow rate of 1 L min^{-1}, and the evaporating temperature was 85°C. The elution programme was carried out under isocratic conditions with 87.5:12:0.5 (v/v/v) chloroform/methanol/triethylamine buffer (pH 3, 1 M formic acid) from 0 to 10 min and then a linear gradient with 87.5:12:0.5 (v/v/v) at 11 min to 28:60:12 (v/v/v) at 45 min. The mobile phase was brought back to the initial conditions at 47 min, and the column was allowed to equilibrate until the next injection at 55 min. The flow rate was maintained at 0.5 mL min^{-1}, the injection volume was 10 μL, and the samples and the column were equilibrated at 40°C.

Analysis of the fatty acids of phospholipids

Phospholipids classes were separated by one-dimensional double-development high-performance thin-layer chromatography using hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Bands of absorbent containing the phospholipids fraction were

scraped off the plates into test tubes. Then, the phospholipids were extracted three times with chloroform (1 mL each). Fatty acid methyl esters of the phospholipids were prepared with a method adapted from Lopez et al.[19] The procedure was then continued as described above for the analysis of total fatty acids.

Analysis of sterols

Sterols of milk samples were extracted according to the method of Fraga.[20] Sterols samples were analysed by a Thermo Scientific France DSQ GC-MS equipped with a DB-5 MS capillary column (30 m; 0.25-mm i.d., 0.52-μm film thickness; Agilent Corp.). The oven temperature was held at 150°C for 1 min and then increased to 300°C at a rate of 10°C min^{-1} and held for 15 min at 300°C.

Confocal Raman microscopy analysis

Milk samples (50 μL) were deposited onto a microscopic slide, and 50 μL of 0.5% (w/v) agarose was added to fix the samples, which were then analysed by confocal Raman microscopy (HORIBA Jobin Yvon SAS, Longjumeau, France).[21] The spectral region recorded was 400-3200 cm^{-1} for the MFGs. The Raman spectral data acquisition was performed using Labspec6 software (HORIBA Jobin Yvon SAS). The peak intensity was measured, and the average peak intensity of the MFGs of the same size was calculated. The intensities of the Raman spectral bands were analysed and calculated using Matlab software (The MathWorks, Natick, MA, USA).[22]

Statistical Analysis

All sample results are expressed as mean ± standard deviation (SD). The experiments were run in triplicate. Statistical analysis software (version 9.0, SAS Institute, Inc.,

Cary, NC) was used for data treatment. Results were considered statistically significant at $P<0.05$.

Results and Discussion

Size distribution, zeta potential and surface tension of MFGs

To determine the influence of the drying processes used in the preparation of milk powder on MFGs, their size distribution, zeta potential and surface tension were measured and compared (Table 1). The size distributions of MFGs in RM (control), FDM and SDM are shown in Figure 1.

The size distribution in RM was characterised by a main peak at 3.89 μm, which was similar to previous results.[23] This corresponded to $D_{3,2}$ and $D_{4,3}$ values of 3.31 ± 0.11 and 3.70 ± 0.31 μm, respectively. The shapes of the size distributions changed markedly with the treatments of freeze drying and spray drying, with significant decreases in the main peak at 3.89-5.04 μm. Particle size distribution curves were polydispersed and multimodal in FDM samples, with 3 peaks at 3.89, 11.99 and 16.96 μm, whereas the samples of SDM had bimodal size distributions at 5.04 and 13.08 μm. High values of $D_{3,2}$ and $D_{4,3}$ were characteristic of FDM and SDM that exhibited peaks that corresponded to high size values in the particle size distribution curves. These changes were commonly attributed to the formation of a small amount of MFGs bigger than those of the control. The size increases after heat treatment were presumably due to coalescence of the globules and increasing protein denaturation.[24] The coalescence might be related to altered protein-lipid interactions in the membrane. Freezing was found to be very important in affecting the particle size. Two explanations for the growth of globule size during the freezing process have been proposed: (1) the ice crystal formation repelling foreign material away from the interstitials causes the

aggregation of globules; and (2) the osmotic de-swelling of the globules causes them to recombine into larger globules.[25] The drying processes caused a range of structural and physicochemical modifications, which in turn influenced the reconstitution and absorption of milk proteins and caused the increase in MFG sizes.

The zeta potential was thought to be particularly suitable to estimate the degree of MFG surface coverage by plasma proteins as an indicator of the degree of damage.[26] The apparent zeta potential calculated for RM of -9.44 ± 0.66 mV was not significantly different from that of -9.72 ± 0.43 mV for FDM. However, the zeta potential of SDM (-10.35 ± 0.72 mV) was significantly higher than that of RM and FDM. The increase of about 9.6% in the absolute value of the apparent zeta potential may be related to the adsorption of casein micelles and whey proteins to the MFGM.[27] This means that possible damage to MFGs during the spray-drying process may result in variation of the interface of the fat globules. Particularly, the heat treatment process can alter the composition of the membrane that may be accompanied by changes in the surface charge of the fat globules.

Surface tension can also be used to evaluate the degree of damage of MFGM. The values of surface tension of RM, FDM and SDM were measured and can be compared in Table 1. The values obtained in RM and FDM were not significantly different, whereas the value in SDM was lower than that in RM, which indicated that some surface-active materials were lost from the MFGM. Phospholipids, which have a polar head group and two hydrophobic hydrocarbon tails, are the most abundant lipids of MFGM. During the spray-drying process, the high temperature and high shear stress may cause the phospholipids to become a kind of surfactant that reduces the surface tension.

Microstructure of MFGs

CLSM was used to visually assess the structural changes of the MFGs during the different drying processes, and the structural details of the MFGs and MFGM could be observed. Figure 2 shows the CLSM images of RM, FDM and SDM, which were stained using Nile red fluorescent probe for the triacylglycerol core of MFG and Rh-DOPE for MFGM, respectively. The MFGs in RM and the two drying samples were present in the form of spherical droplets with polydispersed size distributions. This was in agreement with the results of laser light-scattering measurements, which showed that parts of the MFGs in FDM and SDM were bigger than those in RM (Figure $2A_1$, B_1, C_1).

The emission fluorescence of Rh-DOPE in the form of red rings at the periphery of the MFGs was still detected after both the cold- and heat-drying methods (Figure $2B_2$, C_2), indicating that the MFGM still maintained their structural integrity after processing. Despite the fact that no significant changes or disruption was found in the MFGM of the FDM and SDM samples, some small damage was still present in the globules. As observed in Figure $2B_2$, the surfaces of some MFGs after freeze drying became thicker than those of RM. A possible explanation may be that the amphiphilic phospholipids tend to accumulate on the surface and then the fat globules coalesce with each other during the freeze-drying process.

Figure $2C_2$ shows that some MFGs aggregated rather than evenly dispersed throughout an aqueous phase. At the same time, the results also revealed that freeze-dried milk powder dissolved in water showed better stability than spray-dried milk powder. One reason may be that with the changes that occur in the physical and chemical properties of MFGM with rapid water removal during spray drying, the powder particles are likely to join together due to changes in their surface properties.

Lipid Composition of MFGs

Cow milk contains about 3-5% total lipids. Milk fat is a complex mixture of compounds with a wide range of polarities and structures. Over 98% of the lipids in all milk types are in the form of triglycerides, with the phospholipids and sterols accounting for less than 1% and 0.5%, respectively, of the total lipids. Given the fact that they are the main lipids of MFGs, the assessment of their stabilities after different drying methods is of interest. Because the results from physical and microstructural studies still do not adequately explain the differences in the MFGs between RM, FDM and SDM, the chemical compositions were also compared in our study to examine the effects of spray drying and freeze drying on the composition of these lipid compounds.

Fatty acids

The compositions of fatty acids detected in the control and drying-processed samples are shown in Table 2. The predominant fatty acids of cow milk were myristic (C14:0), palmitic (C16:0), stearic (C18:0) and oleic (C18:1) acids, and the values were consistent with those previously reported.[28] Saturated fatty acids (SFA) constituted 71.75% of the total fatty acids, and monounsaturated fatty acids (MUFA) accounted for 27.31%. The difference between the SFA and unsaturated fatty acids (UFA) observed in this study could be due to the different treatments. Low and high temperatures at the later stages of the drying processes may inactivate milk lipases, which means that triglycerides in spray-dried and freeze-dried powder will not be hydrolysed by the action of these enzymes and therefore will be less susceptible to further oxidation. As expected, the fatty acid proportions of both the spray-dried and freeze-dried samples had no statistically significant differences compared with the control samples.

Phospholipids

Phospholipids interacting with membrane proteins and sterols determine the structure, stability and fluidity of MFGM. They are the backbones of the membrane due to their amphiphilic structure and emulsifying properties. The main phospholipids located in the MFGM are phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS).

The compositional data reported in Table 3 provided evidence that the drying methods affected the phospholipids. The proportion of phospholipids in FDM and SDM were significantly changed. We observed decreases in SM (from 34% to 29%) and PS (from 12% to 7%) in FDM (P<0.01). However, the changes in FDM were different from those in SDM. Decreases were observed in the proportion of PC (from 29% to 26%) and PS (from 12% to 9%) in SDM (P<0.01). SM in SDM pointed to high stability, whereas the relative distribution of PE and PI were increased.

The changes in the compositions of phospholipids seemed to be attributable to the locations of the phospholipids in the membrane. Native MFGs are enveloped by tri-layer membranes, with the inner layer originating from the endoplasmic reticulum and the outer bilayer originating from regions of the apical plasma membrane of mammary epithelial cells. PC and SM are present predominantly in the outer leaflet. PE and PI reside mainly in the inner leaflet, whereas PS is located almost exclusively in the inner leaflet of the plasma membrane.[29] Therefore, PC and SM located in the outer leaflet of the membrane are more susceptible to treatment. According to the recent structural model of MFGM, the liquid-disordered phase is composed of the glycerophospholipids (PE, PC, PI and PS), whereas the liquid-ordered phase domain is composed of SM.[30] In light of our results, we suspect that the liquid-disordered phase is

more influenced by high temperature, but the liquid-ordered phase is more sensitive to low temperature. Whether the reported decreases in the respective contents of SM and PC in FDM and SDM are valid will require further investigation.

Fatty acids of phospholipids

The total fatty acids composition of phospholipids in the RM, FDM and SDM is presented in Table 4. The main fatty acids present in the phospholipids were C14:0, C16:0, C18:0, C18:1 and C18:2. These results were consistent with the report by Fong.[31] Some significant differences were also found between the dried samples and control samples. The SFA of phospholipids in FDM and SDM were 66.03% and 61.36%, respectively, versus 57.18% in RM. Phospholipids of RM were less saturated than those in the FDM and SDM. The MUFA of phospholipids were 19.98% in FDM, 23.89% in SDM and 30.51% in RM. Finally, the contents of polyunsaturated fatty acids of the phospholipids were 13.99% and 14.75% in FDM and SDM, respectively, but only 12.31% in RM.

Regarding the SFA composition of the phospholipids, C16:0 and C18:0 were significantly increased, whereas the UFA of C16:1 and C18:1 were significantly decreased in FDM and SDM. According to the data reported in the literature, SM was found to contain high amounts of long-chain fatty acids, with C16:0 being the major fatty acid (≥25%). C16:0 and C18:1 were the main fatty acids in PC, and a high amount of UFA existed as PE (C18:1≥50%).[31, 32] Therefore, the significant differences of fatty acids of phospholipids were related to the changes in the phospholipids profile during the drying processing. The increased SFA in FDM and SDM might be due to the respective decreases of PC and SM. Drying treatments may lead the PC, SM and PE to convert to phosphatidic acids, which are easy to extract.

345

346 ***Minor sterols***

347 Cholesterol is mainly distributed on the entire plasma membrane, and it also
348 concentrates in specialised sphingolipid-rich domains called liquid-ordered phase
349 domains of MFGM. The content of cholesterol in RM was 292 mg/100 g, and it
350 decreased to 233 mg/100 g in FDM and to 240 mg/100 g in SDM (Figure 3). The
351 greatest reduction in cholesterol content was caused by the drying treatment.
352 Cholesterol is a monounsaturated lipid with a double bone on carbon-5 and is
353 susceptible to oxidation by a free radical mechanism.[33] The decrease in cholesterol in
354 FDM probably results from oxidation during the heat treatment. However, the change
355 in cholesterol in SDM may be caused by the alteration of the membrane structure
356 during the low-temperature process because freeze drying results in osmotic shock
357 and loss of membrane integrity from intracellular ice formation and recrystallisation.[34]
358 The changes in the cholesterol content were in accordance with the changes in the SM
359 content during freeze drying. Thus, we demonstrated the damage that occurs on the
360 sphingolipid-cholesterol membrane domains by freeze drying. Lanosterol and
361 lathosterol, the other sterols found in milk, accounted for 4.6 mg/100 g and 5.6 mg/100
362 g of the milk fat in RM, respectively, but only small changes were observed in these
363 sterols during the drying processes.

364 Squalene is a precursor of cholesterol, and it may be viewed as a more flexible
365 structure in the membrane than in the rigid cholesterol molecule. The function of
366 squalene in the membrane is not yet clear, but it appears to have some roles in
367 stabilising the membrane structure. The content of squalene increased from 9.1 mg/100
368 g in RM to 28.3 mg/100 g in FDM and to 12.3 mg/100 g in SDM under the effects of
369 drying. The reason for the increase of squalene in FDM and SDM was unclear. This

observed increase illustrates that modification of the structure of the MFGM during the drying processes resulted in increasing solubility in the extraction solvents.

Chemical fingerprints of RM, FDM and SDM by confocal Raman microscopy

Raman spectroscopy is ideally suited to the characterisation of different sized MFGs as it allows the study of aqueous samples in situ.[35] Therefore, Raman spectroscopy combined with optical microscopy was applied here with the aim of comparing lipid profiles of MFGs in the two drying methods and among different size classes. Raman spectra were generated and interpreted for comparison of the chemical composition of three different MFG size classes (small for globules less than 3 μm, medium for globules between 3-7 μm and large for globules over 7 μm in diameter) in RM, FDM and SDM.

The Raman spectra of MFGs in RM, FDM and SDM were different according to the size of the globules. As shown in Figures 4 and 5, where visual differences in the peak heights of the spectra were noted, the relevant literature was consulted for information about the nature of these peaks. The prominent peaks in the Raman spectra were found at 1654 cm^{-1} (ν(C=C) cis double bond stretching of RHC=CHR), 1443 cm^{-1} (δ(C–H) scissoring of $–CH_2$), 1269 cm^{-1} (δ(C–H) bending at the cis double bond in R–HC=CH–R), 1303 cm^{-1} (C–H twisting of the $–CH_2$ group) and 1742 cm^{-1} (ν(C=O) stretching of RC=OOR).[36]

The peak at 860 cm^{-1}, which is quite broad (from 810 to 897 cm^{-1}) (Figure 4), was due to the contributions from the complex mixture of the polar head groups from the phospholipids[37]. The intensity of the band at 860 cm^{-1} was decreased after the freeze-drying and spray-drying processes. According to the model proposed by Gallier et al.[35], the two ratios I_{1654}/I_{1742} and I_{1654}/I_{1443} are indicative of the degree of

unsaturation of the samples. The band at 1269 cm^{-1} is a last indicator of the unsaturation level. In Figure 4, the relative intensity of the band at 1269 cm^{-1} was weak compared with that of the band at 1303 cm^{-1}, which was characteristic of the low degree of unsaturation. The Raman spectra of MFGs in RM, FDM and SDM showed decreased levels of unsaturation as the globule size increased (Figure 5). This could be explained by the content of the triglyceride core increasing with the increasing size of the MFGs. The changing trends in the degree of unsaturation were similar under the freeze-drying and spray-drying processes for the small and medium MFGs, whereas the degree of unsaturation demonstrated a decreasing trend for MFGs with larger diameters.

Three intense bands near 1010, 1160 and 1530 cm^{-1} corresponded to the aromatic compounds of carotenoids.[38] This carotenoid band appeared very strong for the small, medium and large MFGs in RM. However, the FDM and SDM presented very weak bands. This suggests the concentration of carotenoids was decreased by both the high- and low-temperature processing.

The Raman spectra in the region around 2600-3200 cm-^{1} contain information about the mobility and structure of the hydrocarbon chains of lipids. The C-C mobility of the hydrocarbon chains indicated by the intensities of the bands at 1065, 1080 and 1130 cm^{-1} and the increased intensity of the band at 2850 cm^{-1} relative to the band at 2885 cm^{-1} are indicative of higher mobility of the hydrocarbon chains. The change in the intensities of these bands after the freeze-drying and spray-drying processes indicated that the fatty acid composition of globules of different sizes varied during the drying processes. Therefore, MFGs of different sizes may have different melting points and could be at different states at the same temperature.

Conclusions

This study revealed the changes in physical, chemical and structural properties of MFGs caused by the freeze-drying and spray-drying methods. The sizes of the fat globules increased as a result of coalescence and gathering of the MFGs during the drying processes, and CLSM clearly showed evidence of microstructural changes of the MFGs undergoing freeze drying and spray drying. Our results indicated that neither freeze drying nor spray drying significantly affected the contents of the fatty acids. However, the changes in the phospholipids, the fatty acids of the phospholipids and the sterol profiles were obvious. The peak intensities of the MFGs of different sizes also showed some changes between RM, FDM and SDM by Raman spectroscopy. The present study provided evidence that both the freeze-drying and spray-drying processes can affect the MFGs. The effect of the two drying methods needs to be taken into consideration when researching dairy powder properties because they may affect the stability of the globules in milk and the way the reconstituted milk is digested.

Conflict of interest

The authors declare that there are no conflicts of interest.

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512

513 **Figure Captions**

514 **Figure 1.** Particle size distribution of the milk fat globules at different drying methods
515 determined by laser light scattering.

516

517 **Figure 2.** CLSM images of milk fat globules from raw milk (A1, A2), freeze-dried milk
518 (B1, B2) and spray-dried milk (C1, C2). A1, B1 and C1 stained with Nile Red. A2, B2
519 and C2 stained with Rh-DOPE. Scale bar = 10 μm.

520

521 **Figure 3.** Comparison of sterols in raw milk, freeze-dried milk and spray-dried milk.

522

523 **Figure 4.** Raman spectra of milk fat globules in raw milk, freeze-dried milk and
524 spray-dried milk of small size (≤3 μm), medium size (>3 μm and <7 μm) and large size
525 (≥7 μm) in the region 400-1800 cm^{-1} (left) and in the CH region 2600-3200 cm^{-1} (right).
526 (a.u.: arbitrary units).

527

528 **Figure 5.** Qualitative evaluation of the unsaturation level and the liquid/crystal fat
529 ratio of RM, FDM and SDM fat globules with different sizes.

530

531 **Table 1** Size distribution, zeta-potentials and surface tension of milk fat globules after

532 different drying methods.

Size parameters	Raw milk	Freeze-dried milk	Spray-dried milk
$D_{3,2}$ (μm)	3.31±0.11	8.67±0.23	6.95±0.41
$D_{4,3}$ (μm)	3.70±0.31	5.02±0.34	4.50±0.33
zeta-potentials (mV)	-9.44±0.66	-9.72±0.43	-10.35±0.72
surface tension (mN/m)	41.207±0.023	41.993±0.030	35.414±0.029

533 $D_{3,2}$, average size of surface area; $D_{4,3}$, average size of volume.

534

535 **Table 2** Fatty acid composition (expressed as percentage of total fatty acids) of milk fat
536 globules in raw milk, spray-dried milk and freeze-dried milk [a].

Fatty acid (%)	Raw milk	Freeze-dried milk	Spray-dried milk
C4:0	1.00±0.04	1.49±0.03 **	1.44±0.06 **
C8:0	1.00±0.03	1.16±0.04 **	1.09±0.03 *
C10:0	2.86±0.09	3.10±0.08 *	3.00±0.07
C11:0	0.08±0.01	0.08±0.01	0.10±0.02
C12:0	3.88±0.09	4.00±0.08	3.99±0.07
C13:0	0.14±0.02	0.15±0.02	0.15±0.04
C14:0	12.89±0.23	12.77±0.14	12.78±0.15
C14:1	1.44±0.08	1.45±0.05	1.46±0.05
C15:0	1.45±0.13	1.43±0.03	1.44±0.06
C15:1	0.02±0.00	0.02±0.00	0.02±0.00
C16:0	35.65±0.24	35.09±0.16	35.11±0.11
C16:1	1.95±0.10	1.82±0.04	1.81±0.05
C17:0	0.79±0.07	0.78±0.04	0.79±0.04
C17:1	0.30±0.02	0.29±0.03	0.30±0.04
C18:0	11.71±0.11	11.67±0.09	11.60±0.13
C18:1t	0.42±0.05	0.43±0.03	0.46±0.04
C18:1	23.17±0.15	23.06±0.19	23.24±0.13
C18:2t	0.27±0.02	0.36±0.02 **	0.28±0.03
C18:3n-6	0.06±0.01	0.05±0.01	0.07±0.01
C18:3n-3	0.30±0.03	0.30±0.03	0.33±0.02
C20:0	0.22±0.02	0.20±0.02	0.21±0.01
C21:0	0.04±0.01	0.04±0.01	0.05±0.01
C20:3n-6	0.15±0.01	0.14±0.03	0.15±0.02
C22:1	0.01±0.00	0.01±0.00	0.02±0.00
C20:5n-3(EPA)	0.14±0.02	0.05±0.01 **	0.06±0.01 **
C23:0	0.05±0.01	0.04±0.01	0.05±0.01
C22:2	0.01±0.00	0.01±0.00	0.00±0.00
SFA	71.75±0.87	72.00±0.76	71.80±0.81
MUFA	27.31±0.40	27.08±0.34	27.31±0.31
PUFA	0.94±0.09	0.91±0.10	0.89±0.09

537 SFA: saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total
538 polyunsaturated fatty acids. Values are means ± standard. Calculations were based on 5
539 samples with three replicate measurement per milk sample.
540 [a] The contents of fatty acids in freeze-dried milk and spray-dried milk were compared
541 with the control (raw milk) separately. Means with different superscript letters are
542 significantly different (* $P < 0.05$, ** $P < 0.01$).
543

544 **Table 3** Phospholipids composition of cow milk fat globules after spray-drying and
545 freeze-drying.

Phospholipids (%)	Raw milk	Freeze-dried milk	Spray-dried milk
PE	18.09±1.65	26.63±2.21 **	22.92±1.55 **
PI	5.76±0.46	6.04±0.37 *	6.73±0.42 **
PS	12.29±0.79	7.23±0.51 **	9.77±0.73 **
PC	29.72±1.42	30.62±1.27 **	26.59±1.72 **
SM	34.15±1.38	29.48±1.66 **	33.99±1.84

546 PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC,
547 phosphatidylcholine; SM, sphingomyelin. Values are means ± standard. Calculations
548 were based on 5 samples with three replicate measurement per milk sample. The
549 contents of phospholipids in freeze-dried milk and spray-dried milk were compared
550 with the control (raw milk) separately. Means with different superscript letters are
551 significantly different (* $P < 0.05$, ** $P < 0.01$).
552

553

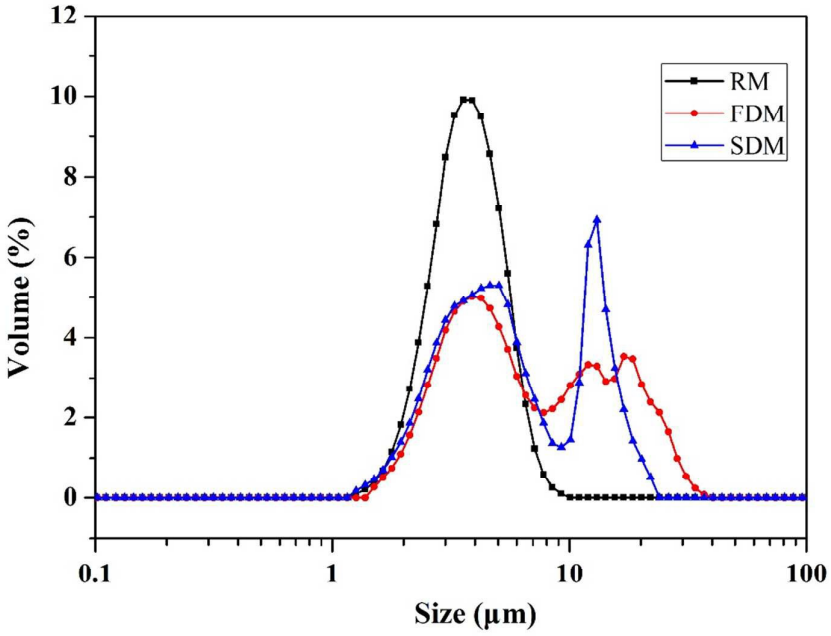
554 **Table 4** Fatty acid composition of phospholipids of milk fat globules in raw milk,
555 spray-dried milk and freeze-dried milk [a].

Fatty acid	Raw milk (%)	Freeze-dried milk (%)	Spray-dried milk (%)
C4:0	0.14±0.02	0.47±0.04 **	0.54±0.04 **
C6:0	0.16±0.01	0.22±0.02 **	0.57±0.05 **
C8:0	0.19±0.04	0.18±0.02	0.66±0.04 **
C10:0	0.59±0.06	1.84±0.07 **	1.22±0.04 **
C11:0	0.11±0.02	0.37±0.03 **	0.26±0.02 **
C12:0	1.19±0.04	1.60±0.03 **	1.76±0.11 **
C13:0	0.16±0.02	0.46±0.06	0.39±0.04
C14:0	6.87±0.08	3.51±0.10 **	5.66±0.02 **
C14:1	0.47±0.02	0.38±0.02 **	0.59±0.11 **
C15:0	1.12±0.04	0.53±0.03 **	1.14±0.04
C15:1	0.20±0.01	0.00±0.00 **	0.21±0.01
C16:0	27.71±0.12	31.78±0.16 **	28.73±0.13 **
C16:1	1.80±0.04	1.10±0.20 **	1.43±0.05 **
C17:0	0.82±0.03	0.51±0.04 **	0.91±0.06
C17:1	0.30±0.03	0.19±0.02 **	0.50±0.06 **
C18:0	16.40±0.19	21.02±0.22 **	18.45±0.12 **
C18:1	27.48±0.23	16.52±0.19 **	20.91±0.59 **
C18:2t	0.38±0.04	0.54±0.04 **	4.62±0.44 **
C18:2	9.81±0.12	11.26±0.24 **	8.84±0.14 **
C18:3n-6	0.10±0.02	0.02±0.00 **	0.11±0.01
C18:3n-3	0.64±0.03	0.86±0.09 **	0.62±0.08
C20:0	0.61±0.03	0.77±0.07 **	0.48±0.06 **
C21:0	0.15±0.02	0.26±0.03 **	0.13±0.02
C20:3n-6	0.65±0.04	1.32±0.02 **	0.56±0.04
C22:1	0.27±0.03	1.80±0.11 **	0.26±0.03
C20:5n-3EPA	0.64±0.03	0.00±0.00 **	0.00±0.00 **
C23:0	0.98±0.10	2.54±0.06 **	0.48±0.04 **
C22:2	0.09±0.01	0.00±0.00 **	0.00±0.00 **
SFA	57.18±0.82	66.03±0.98	61.36±0.87
MUFA	30.51±0.36	19.98±0.54	23.89±0.78
PUFA	12.31±0.29	13.99±0.39	14.75±0.71

556 SFA: saturated fatty acids; MUFA: total monounsaturated fatty acids; PUFA: total
557 polyunsaturated fatty acids. Values are means ± standard. Calculations were based on 5
558 samples with three replicate measurement per milk sample.
559 [a] The contents of fatty acids of phospholipids in freeze-dried milk and spray-dried milk
560 were compared with the control (raw milk) separately. Means with different superscript
561 letters are significantly different (* $P < 0.05$, ** $P < 0.01$).

562

563 **Figure 1**



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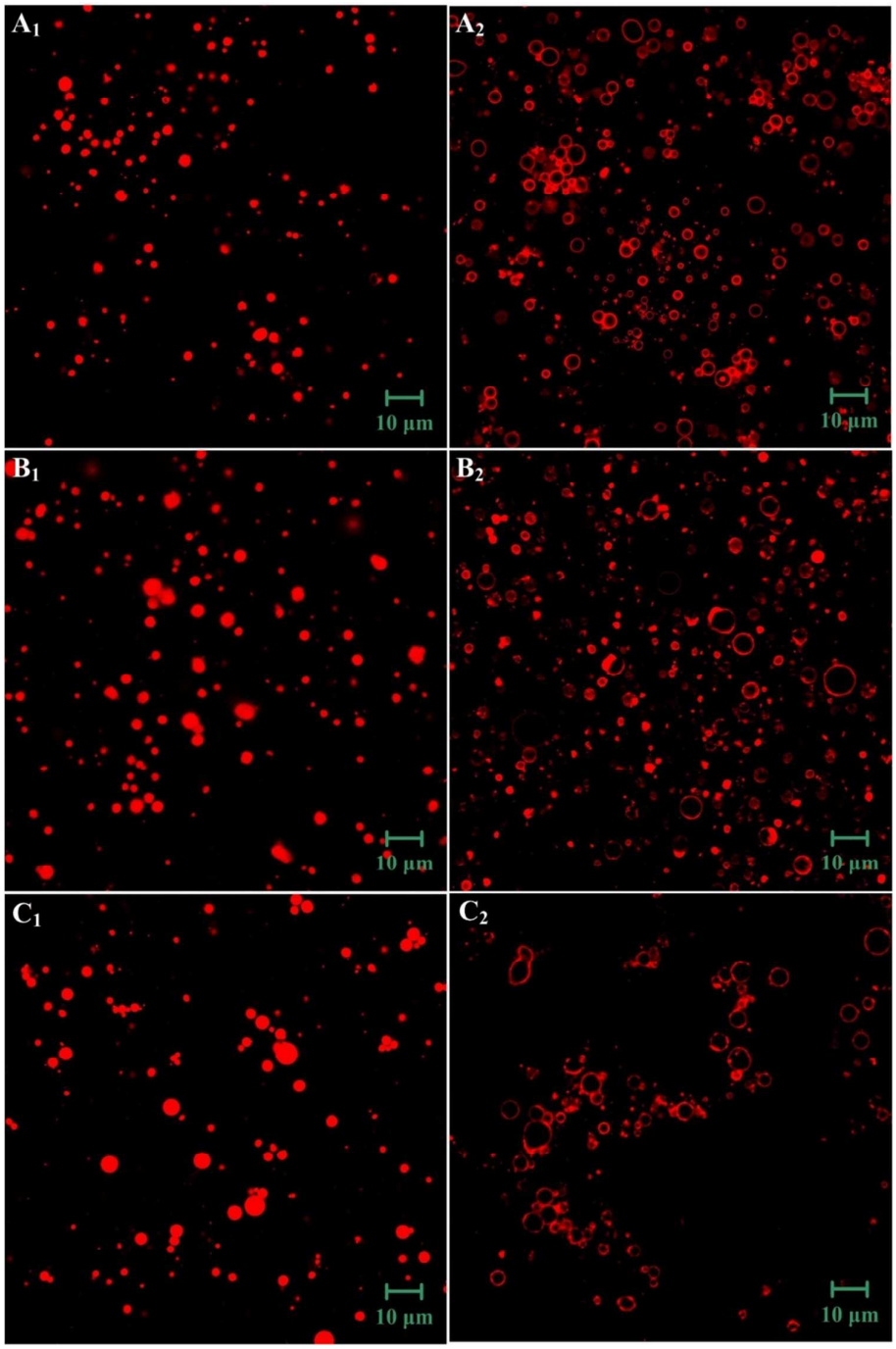
566 **Figure 2**



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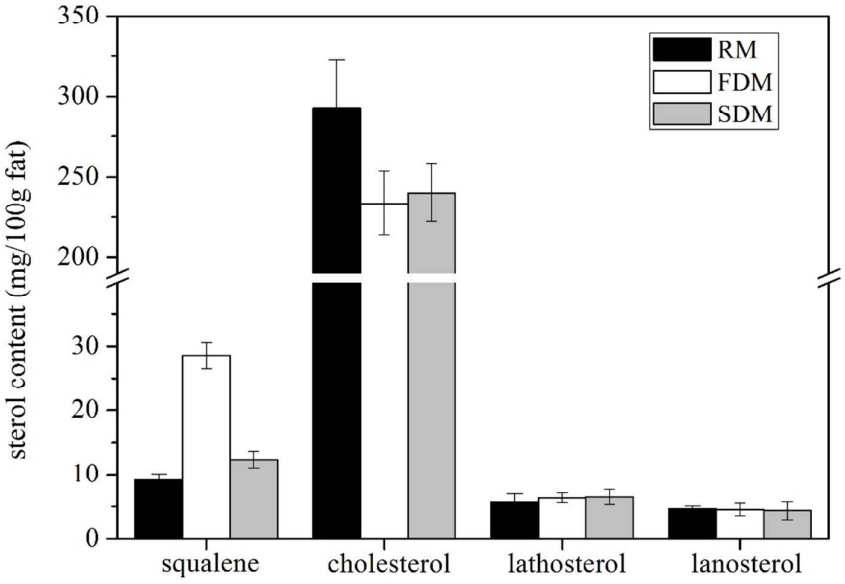
570 **Figure 3**



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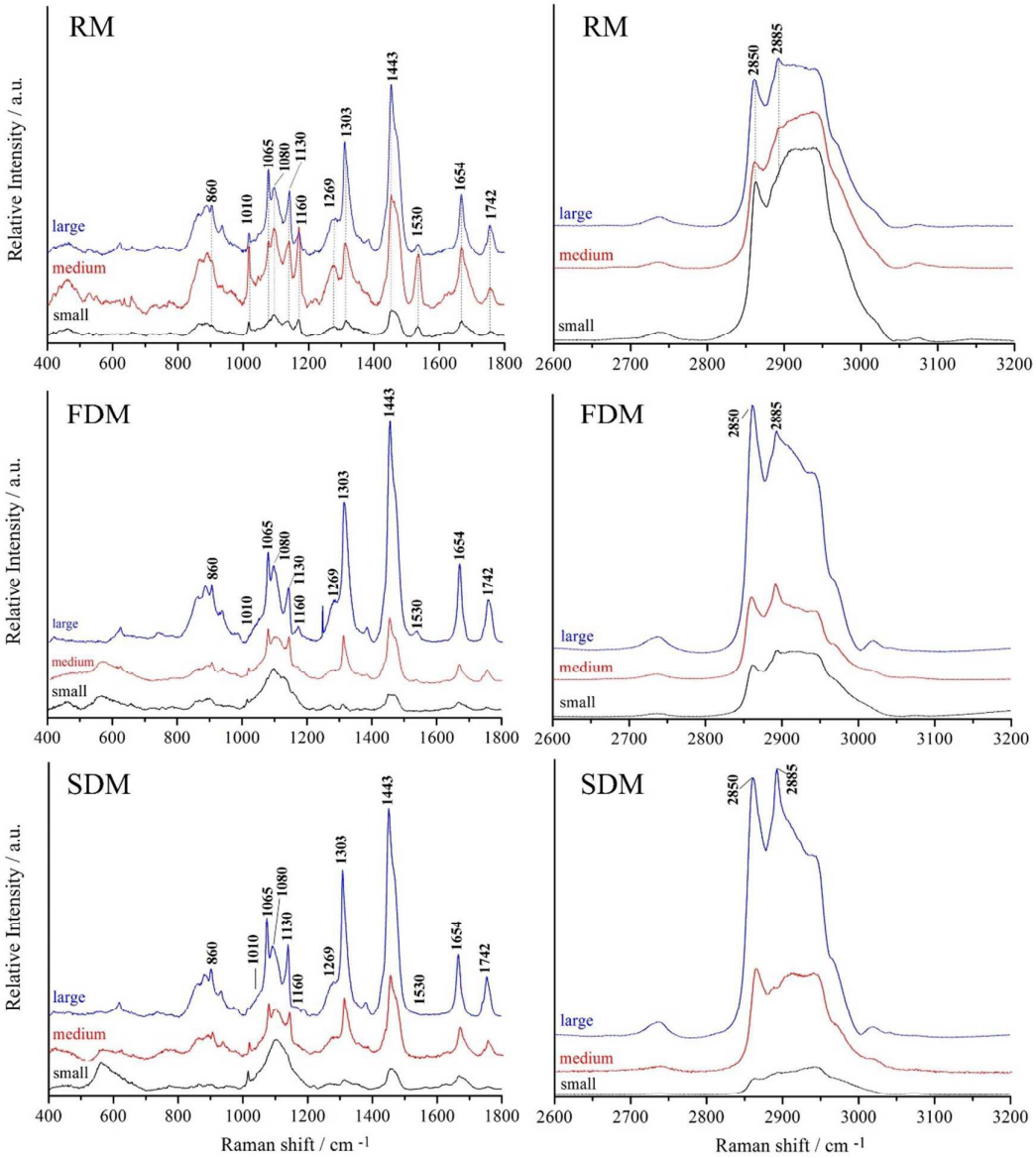
574 **Figure 4**



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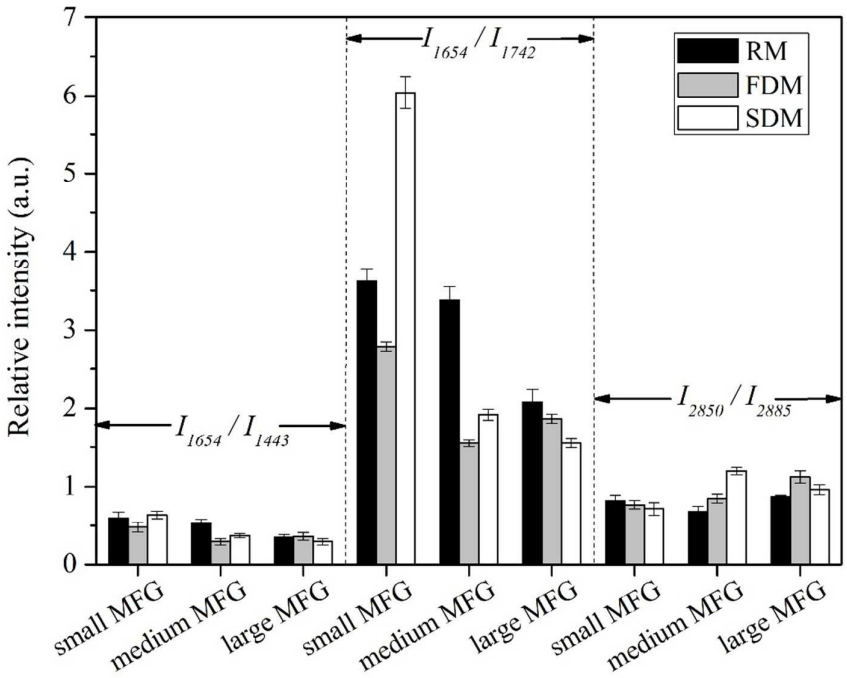
577 **Figure 5**



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