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Harmonic Microchip Acoustophoresis: A Route to Online Raw Milk Sample Precondition in Protein and Lipid Content Quality Control

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A microfluidic approach for raw milk sample preconditioning prior to protein and lipid content analysis has been developed. The system utilizes microchip acoustophoresis and is a further extension of our previously reported multiple node ultrasonic standing wave focusing platform (Grenvall, C.; Augustsson, P.; Matsuoka, H.; Laurell, T. *Proc. Micro Total Anal. Syst.* 2008, 1, 161–163). The microfluidic approach offers a method for rapid raw milk quality control using Fourier transform infrared spectroscopy (FT-IR). Two acoustophoresis modes are explored, $2\lambda/2$ and $3\lambda/2$, offering lipid content enrichment or depletion, respectively. Lipid content depletion above 90% was accomplished. FT-IR data on microchip-processed raw milk samples, enabling direct lipid and protein content analysis, are reported. Most importantly, the harmonic operational modes bypass the problem of lipid aggregation and subsequent clogging, inherent in $\lambda/2$ acoustophoresis systems.

Raw milk quality control is becoming increasingly important worldwide, partly due to the fast growing industrial dairy sector in large countries like China and India, and partly due to the continuous improvements in dairy herd control and production of dairy products.

Rapid and well-established methods already exist for measuring the concentration of the most abundant solids in milk; in particular lipid, protein, and lactose. These solids determine the value of the milk as a raw material for a dairy product, and typically, the price of the traded milk is directly calculated from the lipid and protein concentrations.

In this respect, one of the main nuisances in raw milk is the presence of lipid globules, which leads to pronounced light scattering and prevents the use of conventional microscopy or spatially resolved spectroscopy for analysis. A simple method of removing the lipid particles in milk may thus be a very useful pretreatment step for microbiological analysis. Alternatively, the selective removal and concentration of the lipid globules may facilitate new methods for analyzing the detailed milk fat properties, e.g., determining the fatty acid composition, the amount of unsaturated fat, or trans fat.

Although being slow and requiring manual handling centrifugation is still a major work horse as a sample preprocessing step. Microchip-based acoustophoresis, on the other hand, has enabled new noncontact means of manipulating particles and cells in a continuous flow microfluidic format.^{1–6} Acoustophoresis offers a rapid and continuous flow based mode of operation, using the acoustic standing wave forces as a gentle sample pretreatment method to reduce biocomplexity. Early work in the ultrasonic standing wave technology (USW) field of research aimed to use the acoustic radiation force for spatial handling of microparticles within the acoustic field, in different media.^{7,8} Later work has reported USW as a means to discriminate between different types of bioparticles or particle size.^{9,10} Previous experiences gained in acoustophoretic separation of lipid microemboli in shed blood^{11,12} serve as a knowledge basis for the work reported in this paper, addressing separation requirements in other complex biofluids.

A key process step to enable milk quality control is the discrimination of the lipid emulsion in raw milk. Microchip integrated acoustophoresis has therefore in this paper been evaluated as a fundamental platform to either provide raw milk samples with depleted lipid emulsion content for further bioanalytical milk quality control or to generate concentrated lipid emulsions enabling direct FT-IR spectroscopic analysis of the lipid content.

MATERIALS AND METHODS

Channel Design. The acoustic resonator channels were fabricated according to earlier reported microfabrication processes

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Figure 1. Schematic of the acoustic separator chip with the silicon channel visible through the glass lid, the aluminum block on the actuation side underneath the channel with the piezo crystal (dark gray) attached at the bottom. Fluidic docking ports and external tubing are also visible.

ing.² Two different chip designs were used, channels 150 μm deep and 23 mm long, with widths of 750 and 1125 μm , respectively. Each channel has a center inlet with a separate fluidic connection and two side inlets, which share the same fluidic connection. The same design was used for the outlets, Figure 1. The fluidic network was managed by syringe pumps (WPI sp210iwz, World Precision Instruments Inc., Sarasota, FL).

Actuation Setup. Acoustic actuation was performed with a piezo crystal (12 mm \times 12 mm \times 1 mm, PZ26, Ferroperm Piezoceramics AS, Kvistgaard, Denmark) resonant at 2 MHz, placed underneath the microchip. An aluminum block (10 mm \times 10 mm \times 16 mm), placed between the piezo crystal and the microchip, act as a heat-sink when doing experiments at high driving voltages. For improved acoustic coupling ultrasound gel (Aquasonic Clear, Parker Laboratories Inc., Fairfield, NJ) was applied between the microchip and the aluminum distance and between the aluminum distance and the piezo crystal. Electronic actuation of the piezo crystal has previously been reported.² The mounted chip can be seen in Figure 1.

Analysis Instrumentation. To evaluate the composition of the separated fractions, two different analytical methods were used: multiangle light scattering (MALS) and (FT-IR). The FT-IR spectrometer was a prototype of OenoFoss (FOSS Analytical A/S, Hillerød, Denmark), with a spectral range of 1000–3500 cm^{-1} and a resolution of 14 cm^{-1} , equipped with a deuterated L-alanine-doped triglycerine sulfate (DLATGS) pyroelectric detector. Each spectrum was obtained with an integration time of 5 s. MALS was performed with a MasterSizer X (Malvern Instruments Ltd., Malvern, U.K.) and used to determine the size distribution of particles in the liquids by measuring the angle-resolved scattering of monochromatic light incident on the sample. Since the analysis is based on the theory of Mie scattering, it is possible to quantify the amount of particles with diameters both larger and smaller than the wavelength of the light source (632.8 nm).

FT-IR provides a full absorption spectrum of the samples in the spectral range of 1000–3000 cm^{-1} , where organic molecules have unique absorption peaks due to vibrational resonances, which allow for identification and quantification. For milk, the main absorption peaks are due to CH_2 and CH_3 bonds in lipid

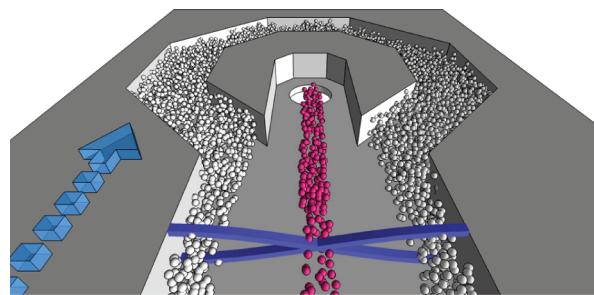


Figure 2. Schematic of original configuration for lipid microemboli separation in blood using a $\lambda/2$ separator. Lipid particles subject to an acoustic force field will focus into either the node (ϕ -positive red blood cells, red) or antinode (ϕ -negative lipid microemboli, white).

and lactose molecules, the ester bond in lipid molecules, the amide bond in proteins, and a number of so-called “fingerprint” absorptions in the low-energy range, 1000–1500 cm^{-1} . In addition to this, very strong water absorption is observed particularly in the range of 1600–1700 cm^{-1} , which typically masks all other information in this region.

THEORY

Acoustic Force. The main driving force in acoustophoresis is the primary acoustic radiation force (PRF) that is exerted upon suspended particles in the presence of a resonant acoustic field (eq 1).^{13–15} The PRF will move particles toward either the pressure nodes of the standing wave, or toward the pressure antinodes, depending on the acoustic contrast factor (ϕ -factor, eq 2) of the particles. The ϕ -factor is determined by the density and compressibility (eq 3) of the particles relative to that of the surrounding medium. Consequently, a particle with a higher density than that of the suspending medium has a tendency to move toward the pressure nodes, whereas particles of densities lower than that of the suspending medium move toward the pressure antinodes (Figure 2). Similarly, the compressibility of a particle with respect to the surrounding medium will impact the acoustic contrast factor.

$$F_r = -\left(\frac{p_0^2 V_p \beta_m \pi}{2\lambda}\right) \phi(\beta, \rho) \sin\left(\frac{4\pi x}{\lambda}\right) \quad (1)$$

where

$$\phi = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m} \quad (2)$$

and

$$\beta = \frac{1}{c^2 \rho} \quad (3)$$

The parameters of eqs 1 and 2 have the following notation:

p_0 , pressure amplitude;

λ , ultrasonic wavelength;

x , distance from a pressure node in the direction of the wave;

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V_p , particle volume;
 β_m , compressibility of the suspending medium;
 β_p , compressibility of the particle;
 ρ_m , density of the suspending medium;
 ρ_p , density of the particle.

The particle diameter (d_p) is an important factor as the velocity of a particle in the pressure field is mainly determined by the PRF ($\propto d_p^3$) and the counteracting Stokes' drag force ($\propto d_p$).¹⁶ Small particles thus move slower in the suspending medium compared to larger particles. Molecular material or debris will consequently not be subjected to any considerable movement in a sound field that has been properly adjusted to separate cells or lipid particles.

Lipid Emulsion Manipulation in Milk. In view of acoustophoresis as the means of depleting milk from the lipid emulsion a most straightforward approach is to employ the strategy originally pursued in lipid microemboli reduction in shed blood during cardiac surgery, Figure 2.¹⁷ A major difference in the system composition for milk separation is the fact that the lipid content in raw milk is substantially much higher (typically 3.5–4.5%) than in shed blood processing (0.1–0.5%).¹⁸ This fact calls for attention to increased problems of lipid emulsion trapping along the side walls of the separation channel, a phenomenon beneficial to some applications.¹⁹ Lipid trapping along the side walls, although initially not noted, has later also been observed in blood processing after extended period of operation. Once the lipid trapping is initiated along the side walls, it rapidly accumulates at localized areas along the acoustophoresis channel, disturbing the flow profile and thus the separation efficiency. With the high lipid content in raw milk the accumulation of lipid emulsion on the side walls of the acoustophoresis channel occurs within seconds of operation at sample flow rates of 10 $\mu\text{L}/\text{min}$. Figure 3a–c shows a sequence of images taken over 30 s illustrating the initiation of lipid trapping and the further growth of the lipid aggregate. In this perspective the strategy of removing lipids by translation to the side walls of the separation channel is not a viable route for lipid emulsion separation or enrichment in raw milk quality control.

The core of the problem is that the $\lambda/2$ excitation mode of operation drives the lipids to the side walls where the flow rate is at its minimum in the channel, which in turn means that the arrival rate of lipid particles to the side walls easily becomes higher than the removal rate. As the lipid particle density builds up along the side wall some lipid vesicles also collapse and stick to the wall surface, initiating a nucleation site for the growth of a lipid constriction in the channel. Although earlier work on lipid separation in shed whole blood, at substantially lower lipid concentration than raw milk, initially did not reveal this sticking problem, lipid concentrations of several percent as the case is for raw milk becomes an immediate problem. Operation of the sample preparation chip for sufficient time, typically 10 min to collect a fraction of 100–200 μL , for subsequent FT-IR analysis was not possible.

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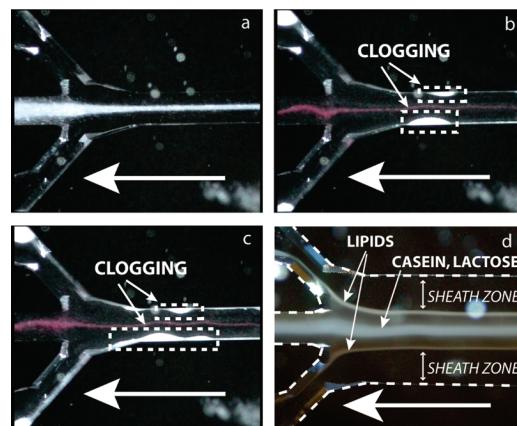


Figure 3. (a) Laminated flow of 5% lipid emulsion spiked with 1 μm polystyrene particles as model bacteria (red) leaves through the center outlet in a $\lambda/2$ wide channel; ultrasound is inactive. (b) When the ultrasound is activated, lipids start to accumulate along the side walls (white aggregates formed along the side walls) and red particles are focused to the channel center. (c) Thirty seconds after ultrasound activation the flow is erratic, and lipid clogging is severe. (d) As comparison, a 3 $\lambda/2$ wide channel with raw milk and sheath flow between the sample and the channel walls (dashed lines) shows no sign of lipid aggregation 30 min after ultrasound activation. Milk lipids go to the side outlets while casein and lactose go to the center outlet.

To avoid lipid accumulation along the side walls a system that focused the lipid emulsion in a high flow rate region was therefore designed. This can be accomplished either by operating the acoustic resonator channel at a higher harmonic of the fundamental resonance or by increasing the channel width in $\lambda/2$ steps to accommodate additional nodes and antinodes. In this paper we have investigated either 2 $\lambda/2$ or 3 $\lambda/2$ wide channels as operating modes.

Operating at the Second Harmonic (2 $\lambda/2$): Lipid Enrichment. The reason for running a system at a higher harmonic is to generate multiple nodes and antinodes in the microchannel. Exciting the resonator at a given harmonic n will produce n nodes and $n + 1$ antinodes. By utilizing hydrodynamic (sheet flow) focusing it is possible to choose a subset of these nodes and antinodes toward which particles are directed. Precise sample collection is then facilitated by adjustments to the outlet flow ratios. The possibility to choose different sheath flow media to match the current operation mode is also a benefit with this system. In lipid enrichment mode of operation the exciting frequency is set to match one acoustic wavelength across the acoustic resonance channel. In this paper a 750 μm channel was produced to allow 2 $\lambda/2$ mode of operation at the same excitation frequency, 2 MHz, and input power as used in the narrower 375 μm channel, and thus similar acoustic forces can be anticipated to drive the system. The particle suspension (raw milk) enters the channel in the center inlet with flow conditions set to sheath flow media (skimmed milk or water) laminate the particle suspension into the central one-third of the channel, Figure 4a. When particles enter the acoustic field they focus into nodes and antinodes within the one-third central channel section, in this case the center antinode with its two adjacent nodes. Lipid particles focus in the center antinode since they are prevented from reaching the side wall antinodes by the two nodes on both sides of the center antinode, which act as acoustic barriers in addition to the fluidic

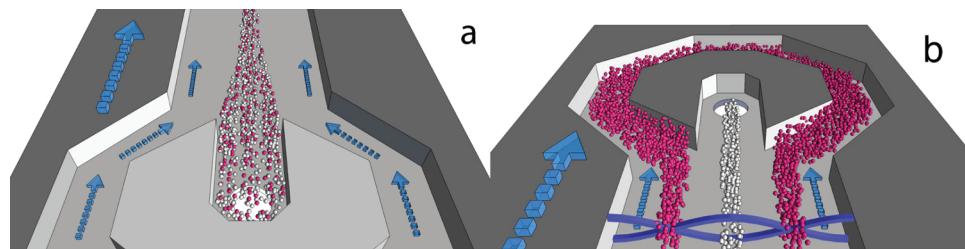


Figure 4. Schematic of lipid enrichment in raw milk using a $2 \lambda/2$ separator. (a) Sample sheet flow configuration at the chip inlet. (b) By utilizing the $2 \lambda/2$ channel it is possible to collect a narrow and enriched fraction of lipid particles (white) through the center outlet and a residual fraction (red) through the side outlets, thus preventing lipid particles from reaching the side walls and inducing clogging.

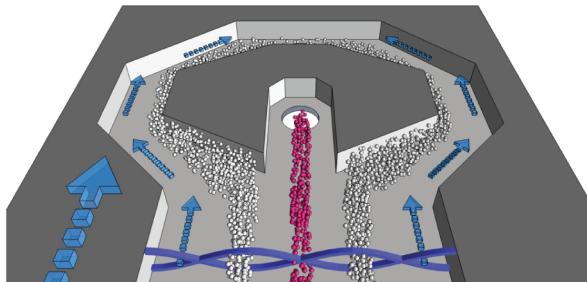


Figure 5. Schematic of lipid depletion in raw milk using a $3 \lambda/2$ separator. By utilizing the $3 \lambda/2$ channel it is possible to collect a narrow fraction containing dissolved species, e.g., lactose and proteins, as well as small casein particles (red) through the center outlet and a residual fraction of lipid particles (white) through the side outlets without risk of clogging. The increased distance between the virtual acoustic barriers and the side walls offers better sheath flow ratio in the $3 \lambda/2$ channel as compared to the $2 \lambda/2$ version.

barrier created by the sheath flows, Figure 4. Ideally this mode allows for extraction of all the lipids through the center outlet in a volume fraction (typically $1/10$) of the initial one-third of the channel volume, offering a substantial lipid enrichment and improved analytical conditions. Particles that focus into the nodes will simultaneously be collected in the side outlets.

Operating at the Third Harmonic ($3 \lambda/2$): Lipid Depletion. When operating the chip for lipid depletion it is more beneficial to move the larger lipid particles into the sheath flow while keeping the protein and lactose content in the center portion of the channel, thereby eliminating any dilution of this fraction. This translates into placing a pressure node in the center of the channel, and the lipid content will thus be focused into the antinodes adjacent to the channel center. In order to maintain the actuation frequency as reasoned above, a new chip was produced, having an $1125 \mu\text{m}$ wide channel which matched three half-wavelengths ($3 \lambda/2$) at 2 MHz. As in lipid enrichment mode, raw milk entered the chip through the center inlet. Sheath flows in combination with additional nodes and antinodes constrained the solid particle content of the raw milk to the channel center, Figure 5. Since lipids are now focused into the adjacent antinodes it is possible to extract a lipid depleted sample in the center outlet and collect a lipid fraction in the side outlets. Figure 3d shows a photograph of raw milk separation after 30 min of operation using the $3 \lambda/2$ excitation mode. Lipid trapping is effectively avoided.

RESULTS AND DISCUSSION

Data $2 \lambda/2$ Mode of Operation: Lipid Enrichment. In Figure 6, the FT-IR spectra of the emulsions taken from the center outlet are shown. Three different samples have been measured

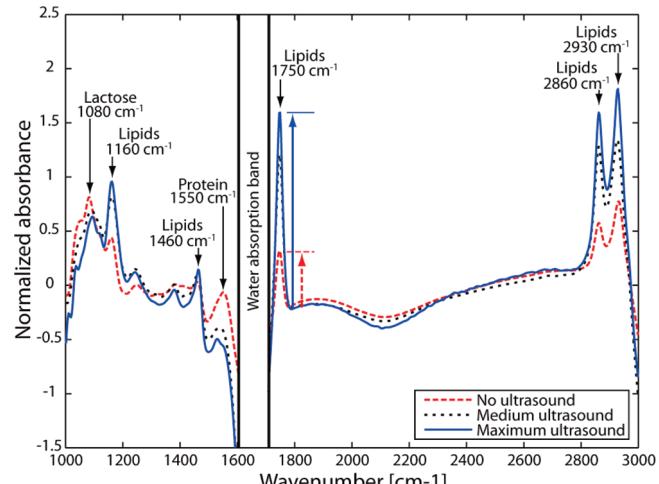


Figure 6. FT-IR spectra of the samples taken from the center outlet in lipid enrichment mode of operation ($2 \lambda/2$). The peak at 1750 cm^{-1} indicates factor 3.5 lipid enrichment with maximum ultrasound applied to the system. Increased lipid concentration prevents correct analysis of the 1550 cm^{-1} protein amide absorption peak.

corresponding to the highest possible ultrasound amplitude (10.5 V), a lower amplitude (8.5 V), and no applied ultrasound. All spectra are normalized using an SNV function (standard normal variate) and referenced against the spectrum of pure water; thus, the ordinate values are not the physical absorbance and may, e.g., be negative. The data in the region of 1600 – 1700 cm^{-1} have been omitted due to the high water absorption here that causes spectral saturation.

The three spectra show a clear increase of all the spectral absorption peaks originating from the lipid molecules when the ultrasound is applied—most prominently the CH_2/CH_3 absorptions at 2860 and 2930 cm^{-1} , the $-\text{CO}-\text{O}-$ ester linkage absorption at 1750 cm^{-1} , and the fingerprint absorptions at 1160 and 1460 cm^{-1} . The lactose concentration is estimated as the difference between the lactose peak at 1080 cm^{-1} and the background level at 1000 cm^{-1} and is practically unaffected by the application of the ultrasound.

The analysis of the protein content based on the amide absorption at 1550 cm^{-1} is complicated by the increasing absorption on the left side (lower wave numbers) due to lipids and the decreasing absorption on the right side (higher wave numbers) due to water displacement. This causes a peak shift toward lower wave numbers and a reduction of the apparent peak height. Thus, we cannot rule out a reduction of the protein content in the center outlet when the ultrasound is applied,

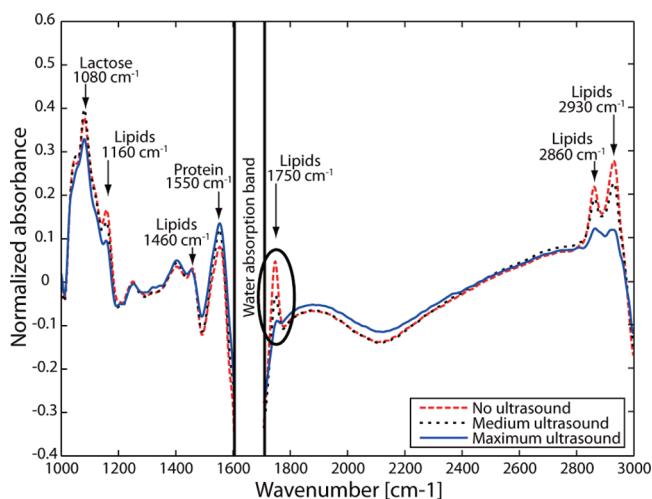


Figure 7. FT-IR spectra of the samples taken from the center outlet in lipid depletion mode of operation ($3\lambda/2$). The lipid peak at 1750 cm^{-1} (circled) indicates significant lipid depletion when running the system with maximum ultrasound applied. The unchanged lactose and protein peaks at 1080 and 1550 cm^{-1} , respectively, suggest that the concentrations of these milk solids are virtually unaffected by the ultrasound.

but the effect is smaller than what is deduced directly from the amide peak height in the FT-IR spectra.

From the FT-IR spectra, it is concluded that the $2\lambda/2$ mode of operation allows a significant concentration of milk lipids in the center outlet, whereas the lactose content is practically unaffected. The increase of the peak at 1750 cm^{-1} , which is unique for lipids in milk, indicates that the lipid is concentrated by a factor of 3.5.

Data 3 $\lambda/2$ Mode of Operation: Lipid Depletion Mode. In Figure 7, the FT-IR spectra of the emulsions taken from the center outlet are shown for increasing ultrasound amplitude. Following the same analysis as for the spectra in Figure 6, it is observed that the lipid content, 1750 cm^{-1} , in the center outlet is significantly reduced as the ultrasound is increased, whereas the lactose and protein contents are practically constant, why the $3\lambda/2$ mode of operation is well-suited for milk sample preconditioning in protein analysis.

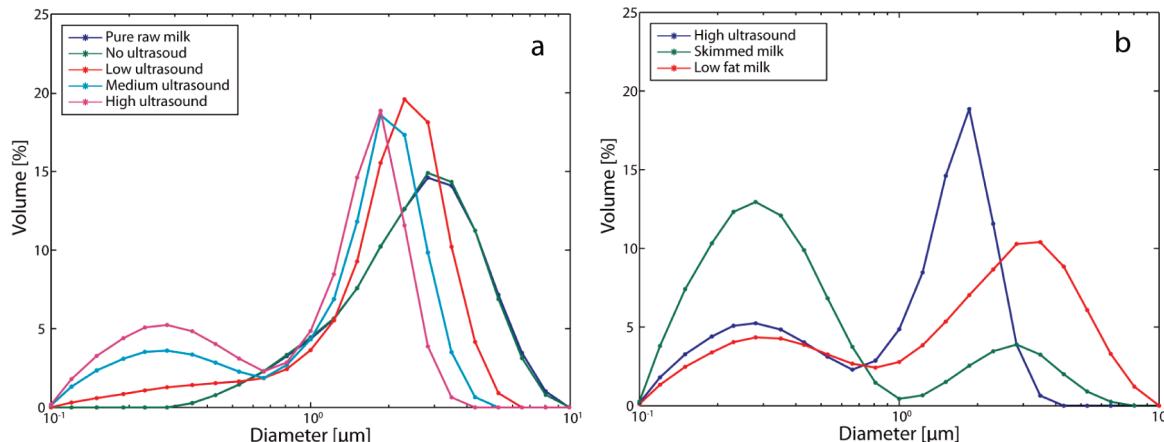


Figure 8. (a) MALS measurements of samples from the center outlet when operating the system in lipid depletion mode with varying ultrasound effects applied. All distributions are normalized; thus, only the curve shapes should be compared. A shift toward smaller fat globules can be seen as ultrasound effect increases. The casein micelle peak at $\sim 0.3\text{ }\mu\text{m}$ is consistent with FT-IR measurements where lipids were depleted, whereas milk proteins are virtually unaffected by the ultrasound. (b) Comparisons between commercially available milk and a high ultrasound center outlet sample also suggest protein content to be unaffected by the ultrasound.

Figure 8a shows the MALS measurements of the particle size distribution in the center outlet for increasing ultrasound amplitude. It is noted that all distributions are normalized to a total area of 100%, and thus only the shapes can be compared, but not the absolute magnitudes. As expected the pure raw milk has the same distribution as a sample from the center outlet when no ultrasound is applied. As the ultrasound amplitude is increased two effects are noted—the peak of the distribution shifts toward smaller diameters, and a second peak centered at approximately $0.3\text{ }\mu\text{m}$ become more prominent.

The particles in the range of $1\text{--}10\text{ }\mu\text{m}$ are the lipid globules in the milk, and thus the shift of the peak toward smaller diameters show that the large lipid globules are separated more efficiently in the ultrasound field than the smaller globules. This is consistent with the larger ultrasound forces that act on large particles compared to small particles.

The particles with diameters below $1\text{ }\mu\text{m}$ are mainly casein micelles (milk protein). Light scattering due to lipid emulsions is much stronger than for casein due to the larger dielectric contrast, and thus the casein peak is not resolved in the MALS measurements until the lipid concentration is sufficiently low. The increasing casein peak is therefore consistent with the decreasing lipid contents observed in the FT-IR measurements.

In Figure 8b the particle size distribution at the highest ultrasound amplitude is compared to that of commercial skimmed milk (lipid content less than 0.1%) and low-fat milk (0.5% lipid content). The commercial milks have the same casein (protein) content and show a similar casein peak shape as the milk exposed to the ultrasound field. Thus, there is no indication that the ultrasound forces alter the size distribution of the casein micelles.

In summary, the $3\lambda/2$ configuration facilitates an efficient removal of lipid emulsion from milk with practically no effect on the lactose and protein composition. The lipid depletion in terms of the total concentration may be better than 90%, and for the highest ultrasound amplitude lipid particles larger than $4\text{ }\mu\text{m}$ disappear below the detection limit of MALS.

CONCLUSIONS

This paper demonstrates that multinodal acoustophoretic operation in continuous flow separation of milk constituents avoids the problem of side wall lipid particle adhesion and subsequent clogging. Two modes of acoustophoretic operation have been investigated offering either lipid enrichment or depletion, where the $2\lambda/2$ acoustophoresis mode opens the route to improved analytical conditions for lipid content analysis in milk sample quality control and the $3\lambda/2$ mode of operation displays excellent raw milk sample preconditioning for protein analysis. Further work will focus on integration of the proposed

raw milk sample preparation with analytical instrumentation for milk quality control.

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