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Original Research Article

Optimisation of Non-Thermal Ultrasonication Process for Retaining Nutritional Quality in Cow Milk: Effects of Fat Percentage and Treatment Parameters

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Abstract: The thermal unit operation used in milk processing causes changes in milk nutritional quality. Hence, optimising the non-thermal ultrasonication process at 25°C can be a potential method to retain milk quality. The objective of this research is to optimise the ultrasonication process of standardised fresh cow's milk at various fat percentages and elucidate its effect on nutritional values using response surface methodology (RSM). A central composite design based on fat (1–4%); ultrasound amplitude (10–70%), and sonication time (1.5–10.5 min) was used. Data analysis shows that the ultrasonication process significantly (p<0.05) affects the percentage of fat, free fatty acid (FFA), protein, solid non-fat; solid non-fat (SNF), lactose, casein, and total solid (TS). The adjusted R^2 for fat, free fatty acid, and TS with goodness-of-fit were 0.940, 0.975, and 0.955, respectively. The generation of FFA in raw cow milk was observed with some ultrasonication conditions. As a conclusion, the processing parameters to get the highest fat and lowest FFA is a combination of 4% milk fat, 10% amplitude, and 1.95 min sonication time with 46.8 J/mL energy density can be applied to control the release of further FFA generation from the milk processing line.

Keywords: Cow milk; high-power ultrasound; non-thermal processing; nutritional quality; central composite design

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1. Introduction

Ultrasonic non-thermal technology in milk processing lines has attracted much interest in recent years. The significance of the process on milk nutritional qualities and functional properties was observed. The benefits such as improved homogenisation, enhanced emulsification and smaller fat globule size were responded by nutritional and chemical changes after sonication (Carrillo-Lopez *et al.*, 2021; Paniwnyk, 2017).

The physical mechanism of ultrasound in liquid systems involves the interaction of the dissolved gas and liquid with acoustic waves, leading to rarefaction and compression that ultimately result in acoustic cavitation (Sulaiman & Silva, 2023). As microbubbles collapse, high energy released causes smaller fat globules to form from the compression and rarefaction cycle (Ashokkumar *et al.*, 2010; Bermudéz-Aguirre *et al.*, 2008). These smaller fat globules formed provide larger surfaces for binding activities.

Apart from that, milk fat globule membrane proteins were retained better with ultrasonication compared to conventional shear-homogenisation. For instance, Astráin-Redín *et al.* (2023) reported that sonicated milk at 20 kHz prior to pasteurisation shown to have better emulsion homogeneity compared to commercial milk. The homogenised milk processed with ultrasonication was resistant to changes after pasteurisation processes; regardless of high hydrostatic pressure technology, pulsed electric field, or microwave pasteurisation

Acoustic cavitation produces intense forces such as micro-jet shearing and turbulence in breaking the fat globules and fat coalesce in milk ultrasonication. Hence, more interaction between fat and whey, protein whey aggregations and/or κ-casein/casein micelle proteins aggregations from the sonication mechanism via hydrophobic bonding, reactive thiol groups or disulfide crosslinking (Hong Bui *et al.*, 2021; Silva *et al.*, 2018; Chandrapala *et al.*, 2012). The milk sonication at 20 kHz showed that smaller protein aggregates were formed and resistant towards further aggregation through post-heating (Chandrapala *et al.*, 2012). The preservation of low viscosity in whey protein post-freezing or spray-drying was also observed after the powder was reconstituted.

These mechanisms are responsible for rendering pathogenic microorganisms and milk spoilage (Lauteri *et al.*, 2023; Van Hekken *et al.*, 2018; Cameron *et al.*, 2009) and changes in biochemical interaction in liquid food such as milk (Hong Bui *et al.*, 2021; Liu *et al.*, 2021; Obeid *et al.*, 2019). Thus, ultrasonication is a potential technology for improving milk functionalities without any added chemicals and additives with minimal degradation of milk quality.

Numerous high-intensity ultrasonication processes have been studied in the dairy industry, including the enhancement of milk emulsion from the reduction in fat globule size, enhanced rheological qualities, increase in its bioactive activity, and improvement of milk's lactose crystallisation (Carrillo-Lopez *et al.*, 2021; Khaire & Gogate, 2018; Monteiro *et al.*, 2018; Patel & Murthy, 2009). These significant quality enhancements were attained using ultrasonication which is not a traditional homogenisation process to stabilise the emulsion and prevent phase separation by lowering the size of its fat globules and simultaneously controlling lipid oxidation (Juliano *et al.*, 2014).

The extent of milk functionalities is derived from its nutritional composition. The chemical changes from protein and fat components were caused by specific nutrition concentration or percentage and ultrasonic processing conditions such as sonication time, amplitude, and frequency (Zhou *et al.*, 2020; Van Hekken *et al.*, 2019; Ertugay *et al.*, 2004). Therefore, ultrasonic homogenisation possibly affects fat content and lipid oxidation processes (Juliano *et al.*, 2014) and protein-fat content in milk (Mudgil *et al.*, 2022; Obeid *et al.*, 2019; Sutariya *et al.*, 2017). Most ultrasound treatments applied are made across either different ultrasonic conditions and/or a combination of raw and processed milk (Mudgil *et al.*, 2022; Liu *et al.*, 2021; Balthazar *et al.*, 2019; Leong *et al.*, 2016; Juliano *et al.*, 2014).

Yanjun *et al.* (2014) observed significant changes in reduced particle size, reduced viscosity, increased solubility, increased storage modulus, and increased emulsifying activity in reconstituted milk protein concentrate under ultrasound pre-treatment at 20 kHz, 600 W maximum power and 50% amplitude prior spray-drying. The ultrasonication pre-treatment increased surface hydrophobicity with no significant change in protein molecular weight observed in the reconstituted milk protein concentrate (MPC) using the SDS-PAGE profile of the protein size. It was suggested that ultrasound duration treatment was not enough to escalate the secondary and primary structure of the protein’s coiled structure, which was elaborated further by Zhou *et al.* (2020) on whey protein conformation interchanged after different amplitude and exposure time with ultrasonication.

The homogeneity of ultrasonicated milk fat globule size distribution was comparable at 1.0 kJ/mL (Astráin-Redín *et al.*, 2023) with commercial whole milk (Astráin-Redín *et al.*, 2023; Bermudéz-Aguirre *et al.*, 2008). At higher energy densities of 7.0 kJ/mL, fat globules and casein particles may aggregate by re-coalesce occurrences, thereby adsorbing the whey proteins and the casein micelles on the interface to link new bridges among fat globules (Abesinghe *et al.*, 2020; Scudino *et al.*, 2020). Astráin-Redin *et al.* (2023) demonstrated that

ultrasonication was able to homogenise stable milk fat globule size without high temperature before pasteurisation.

Ultrasonication has emerged as a promising technique for controlling free fatty acids (FFAs) release (Van Hekken *et al.*, 2018; Juliano *et al.*, 2014; Riener *et al.*, 2009) and milk fat globule membrane deterioration (Sun *et al.*, 2022; Liu *et al.*, 2021; Leong *et al.*, 2016) in milk processing. Therefore, the technique also offers potential improvements in dairy product quality and nutritional quality.

In addition to favourable physical effects, cavitation can also induce chemical changes in liquid milk including redox reactions. Liu *et al.* (2021) proved that ultrasonication was as efficient as shear-homogenisation to reduce milk fat globule size, at which simultaneous volatile free fatty acid was released. Through the shearing process in the dairy system, induced lipolysis caused altered milk functionality such as foaming, crystallisation and emulsification among others (Ho *et al.*, 2023; Carrillo-Lopez *et al.*, 2021; Paniwnyk, 2017; Yanjun *et al.*, 2014).

The application of ultrasonic waves during milk treatment can effectively disrupt fat globules, leading to enhanced fat emulsification and reducing FFA release. Liu *et al.* (2021) concluded that ultrasonication retained more milk fat globules compared to shear-homogenisation by elaborating on the effect of different power used (40 W and 60 W) following a short time window (30–180 s) compared to a longer time used which can vary between 5-25 min (Hong Bui *et al.*, 2021; Juliano *et al.*, 2014; Riener *et al.*, 2009).

Response surface methodology (RSM) is a statistical method used for modelling and optimising a process. RSM could be used for optimising milk nutritional quality assessment upon ultrasonication such as to investigate the optimal power intensity and treatment needed including the duration of exposure at specified milk fat percentages to control FFA while maximising the percentage of total solid (TS). Previously, Khatkar *et al.* (2018) demonstrated the application of RSM using three independent variables; whey protein concentration (10–15%) from dairy experimental material, sonication amplitude (20–40%), and sonication time (10-20 min), and were optimised to exhibit the best-modified whey powder functionality.

The purpose of this study was to evaluate the optimal standardised milk fat percentage with ultrasonic conditions on milk nutritional values. Therefore, identifying the ideal combination of these parameters may help to ensure the effectiveness and efficiency of the ultrasonication process towards nutritional values. For this purpose, RSM using the central composite design was designed based on three factors and five levels; fat percentage (1–4%),

ultrasound amplitude percentage (10–70%), and sonication time (1.5–10.5 min) was carried out to validate milk nutritional values with specified optimisation.

2. Materials and Methods

2.1 Sample Preparation

Raw milk was obtained from UPM Ladang 16 dairy farm. The milk was warmed until 40°C before being centrifuged and separated into cream and skimmed milk. The cream was added into skimmed milk to prepare milk for respective fat percentage (1.0%, 1.5%, 2.5%, 3.5%, 4.0%) and coded as - α until + α using the standardisation formula (Bird, 1993). As shown in Table 1, the standardised milk was kept at 4°C and used within 24 h prior to ultrasound processing.

Table 1. The levels for the Central Composite Design experiment (α = 1.5)

Independent variables	Coded levels				
	- α	-1	0	-1	+ α
Fat, %	1.0	1.5	2.5	3.5	4.0
Time, min	1.5	3	6	9	10.5
Amplitude, %	10	20	40	60	70

2.2 Sonication

For a better understanding of raw milk nutritional changes at different milk fat percentages, it is necessary to standardise ultrasonic settings as independent factors. Ultrasonication treatments were carried out using an ultrasonic processor (450 W Branson Digital Sonifier, Branson Ultrasonic Corporation, Danbury, Connecticut, USA) with maximum operating power at 400 W with a constant frequency of 20 kHz. The processor was fitted with an ultrasonic disruptor horn of a 25.4 mm diameter tip. The sonicator was utilised in the pulse-pause mode (59 s sonication followed by 10 s pause). The ultrasound probe was positioned at the centre of the beaker at a depth of 20 mm from the beaker base. Milk samples (100 mL) were transferred to a 120 mL beaker and immersed into the ice bath to ensure the temperature of the sample was below 40°C to avoid milk fat separation. The output power was used by Raso *et al.* (1999) to determine the energy density range using Equation (1).

$$Energy\ density\ \left(\frac{1}{ml}\right) = \frac{Output\ power\ (W) \times Processing\ time\ (s)}{Volume\ (mL)} \quad (1)$$

2.3 Determination of Protein, Solid Non-Fat, Total Solid, Lactose, Casein, Fat, and Free Fatty Acid Content

The protein, solid non-fat (SNF), TS, lactose, casein, fat and FFA contents of milk were determined using Fourier transfer infrared (FTIR) spectroscopy using a Foss FT1 Milkoscan (Foss Electric, Hillerod, Denmark) (Van Hekken, 2018). Specifications of instruments complied with the AOAC International (2012). All measurements were conducted in triplicate after 50 mL of each sample was warmed at 40°C and shaken prior to analysis.

2.4 Experimental Design

The optimum condition for determining the nutritional value of sonicated cow milk was determined by central composite design with RSM using 5 levels of 3 factors in Minitab (version 17.1.0, Lead Technologies, Inc., State College, PA, USA. The quadratic models used three independent variables i.e. treatment times (X1:1.5-10.5 min), power amplitude (X2:10%-70%), and raw milk fat (X3:1.0%-4.0%) combinations with 6 replicates at the centre of the design at a continuous application of ultrasound at a power output of 400 W in Table 1. Respectively, 100 mL of sonicated raw milk was frozen in a -20°C deep freezer and used within 3 days before further analysis. The responses from the independent variables (%) were total fat, protein, solid non-fat, total solid, lactose, casein, and FFA (Table 2).

Table 2. Experimental design of independent variables for milk ultrasonication

Experimental run	Fat, %	Time, min	Amplitude, %
1	- α	0	0
2	+1	-1	+1
3	0	0	0
4	0	0	0
5	0	0	0
6	0	- α	0
7	0	0	+ α
8	+1	+1	-1
9	-1	-1	+1
10	-1	+1	+1
11	0	0	0
12	-1	-1	-1
13	0	0	0
14	+ α	0	0
15	0	0	0
16	-1	+1	-1
17	0	+ α	0
18	+1	-1	-1
19	+1	+1	+1
20	0	0	- α

The predicted response value at optimised conditions for fat, TS and FFA percentages were compared with the experimental values and the percentage of error was calculated using Equation (2).

$$Percent\ error = \frac{|predicted\ value - experimental\ value|}{predicted\ value} \times 100\% \quad (2)$$

2.5 Numerical Optimisation of Process

The data obtained from these experiments were analysed using Minitab software, to assess the model's significance, lack-of-fit from F-value, regression values from the coefficient of determination (R^2 and R^2_{adj}), and second-order quadratic polynomial equation. The final prediction was then generated from responses of all experimental data using Equation (3) (Montgomery, 2008).

$$Y = ß_0 + ß_1X_1 + ß_2X_2 + ß_3X_3 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{23}X_2X_3 + ß_{11}X_1X_1 + ß_{22}X_2X_2 + ß_{33}X_3X_3 \quad (3)$$

The regression polynomial equation is where $ß_0$ is a constant coefficient, $ß_1$, $ß_2$, $ß_3$ are the linear coefficients, $ß_{12}$, $ß_{13}$, $ß_{23}$ are the interaction, and $ß_{11}$, $ß_{22}$, $ß_{33}$ are the quadratic coefficients. Y was the dependent factor from X_1, X_2, and X_3 independent variables. All 20 experimental runs were randomised to reduce the bias of unexpected variability in the observed response (Montgomery, 2008).

For every dependent factor, a polynomial equation was obtained and the variables significant at $p<0.05$ levels were considered for the construction of the final model. Analysis of variance (ANOVA) was used to examine the statistical significance of the terms in the regression equation for each response. Responses to examine correlation coefficients between independent variables were obtained from RSM optimisation.

3. Results and Discussions

3.1 Mathematical Model Fitting and Validation

Response surfaces were used to fit the best mathematical model to establish the influence of independent variables on dependent variables. Table 3 shows the significant effect of independent variables towards responses from Table 2. Experimental design for the ultrasonic process parameters was optimised to achieve the highest fat and lowest FFA and from fat percentage, amplitude percentage, and duration time. From the experimental data, coefficients of the polynomial equation were calculated to predict responses.

Table 3. Dependent responses from independent variables

Experimental run	Responses (%)						
	Fat	Protein	SNF	TS	Lactose	Casein	FFA
1	0.948	3.065	8.135	8.888	4.263	2.430	10.007
2	3.005	3.113	8.215	11.033	4.333	2.405	10.396
3	1.955	3.085	8.173	9.913	4.295	2.403	10.202
4	2.098	3.048	8.093	9.965	4.273	2.375	10.238
5	2.015	3.078	8.163	9.970	4.295	2.398	10.209
6	2.245	3.030	8.080	10.125	4.275	2.363	9.971
7	2.400	3.088	8.178	10.353	4.305	2.390	10.534
8	2.833	3.100	8.203	10.885	4.340	2.403	10.312
9	1.230	3.033	8.073	9.075	4.253	2.378	10.002
10	1.485	3.050	8.118	9.345	4.258	2.390	10.303
11	1.888	3.070	8.140	9.815	4.280	2.410	10.219
12	1.418	2.995	8.015	9.213	4.245	2.356	9.850
13	1.925	3.075	8.173	9.895	4.308	2.405	10.226
14	2.950	3.155	8.290	11.078	4.368	2.430	10.458
15	2.155	3.065	8.138	10.080	4.2875	2.383	10.278
16	1.418	3.010	8.045	9.238	4.2375	2.375	9.901
17	2.100	3.090	8.145	10.063	4.305	2.408	10.402
18	3.028	3.078	8.150	11.010	4.3225	2.378	10.091
19	3.385	3.143	8.263	11.425	4.340	2.400	10.875
20	2.355	3.025	8.088	10.248	4.283	2.355	10.004
p-value	<.0001	0.0130	0.0134	<.0001	0.0099	0.0132	<.0001

From the experimental data, coefficients of the polynomial equation were calculated to predict responses. The nutritional results of 20 sets of experiments were analysed and the interpretation was provided. Significant values at (p<0.05), lack-of-fit of (p>0.05) for each variable and coefficient of determination (I^2) on ultrasonic and fat percentage treatment on dependent parameters are shown in Table 4.

Table 4. Estimated regression equations for dependent variables from A: Fat (%), B: Amplitude (%) and C: Time (min)

Particulars	**Regression parameter coefficients**						
	Fat %	Protein %	SNF %	TS %	Lactose %	Casein %	FFA%
Intercept	2.036	3.0609	8.1417	9.9858	4.2911	2.3922	10.2238
Linear							
A: Fat, %	0.7763**	0.0384**	0.0650**	0.8614**	0.0400**	0.0066^{ns}	0.1835**
B: Amplitude %	0.0382^{ns}	0.0199**	0.0312**	0.0552^{ns}	0.0057^{ns}	0.0088**	0.1774**
C: Time, min	0.0178^{ns}	0.014**	0.0218**	0.0375^{ns}	0.0054^{ns}	0.0092**	0.1359**
Interaction							
AB	-	-	-	-	-0.0022^{ns}	-	0.0393*
BC	-	-	-	-	0.0003^{ns}	-	0.0633**
AC	-	-	-	-	0.0034^{ns}	-	0.0435**
Quadratic							
A^2	-	0.014**	0.0237*	-	0.0080^{ns}	0.0122**	-
B^2	0.1676**	-	-	0.1517**	-0.0015^{ns}	-0.0133**	-
C^2	-	-	-0.0207**	-	-0.0032^{ns}	-	-
R^2	0.9529	0.8855	0.9069	0.9644	0.9351	0.6846	0.9826
R^2 (adj)	0.9403	0.8549	0.8737	0.9549	0.8766	0.5720	0.9745
F-values	0.112^{ns}	0.302^{ns}	0.867^{ns}	0.071^{ns}	0.517^{ns}	0.511^{ns}	0.143^{ns}

** $p<0.01$ significant at 1% level of probability

* $p<0.05$ significant at 5% level of probability

ns not significant

According to the statistical analysis (ANOVA) results, the experimental data described by a linear and quadratic model, with coefficient of determination (R^2) values on nutritional composition (%) for total fat, protein, solid non-fat, TS, lactose, casein, and FFA was 0.940, 0.855, 0.874, 0.955, 0.876, 0.572, and 0.975, respectively.

The coefficient of the equation and the results of ANOVA were performed on the models to evaluate the significance at linear, interaction, and quadratic levels of the independent variables on dependent responses as shown in Table 4. The lack-of-fit F-values from dependent variables implies that the lack-of-fit is not significant relative to the pure error and, therefore is desirable. The predictive regression models designed for independent (X) and dependent (Y) variables in terms of nutritional composition were presented in Equations 4–10. The R^2 in this study shows that the influence of raw milk, ultrasound amplitude, and exposure time towards nutritional composition was adequately described with linear, interaction, and quadratic polynomial models.

$$\text{Fat \%} = 0.655 + 0.7763A - 0.0316B + 0.0059C + 0.000419B^2 \quad (4)$$

$$\text{Protein \%} = 2.9847 - 0.0318A + 0.000995B + 0.00467C + 0.01403A^2 \quad (5)$$

$$\text{SNF \%} = 7.9388 - 0.0537A + 0.001560B + 0.0349C + 0.02374A^2 - 0.002300C^2 \quad (6)$$

$$\text{TS \%} = 8.254 + 0.8614A - 0.02759B + 0.0125C + 0.000379B^2 \quad (7)$$

$$\text{Lactose \%} = 4.2072 - 0.0022A + 0.00083B + 0.00296C + 0.00793A^2 - 0.000004B^2 - 0.000353C^2 - 0.000109AB + 0.00115AC + 0.000005BC \quad (8)$$

$$\text{Casein \%} = 2.3629 - 0.0546A + 0.003103B + 0.00307C + 0.01224A^2 - 0.000033B^2 \quad (9)$$

$$\text{FFA \%} = 9.806 + 0.0179A - 0.00238B - 0.0331C + 0.001967AB + 0.01449AC + 0.001056BC \quad (10)$$

Fat, FFA and TS are strongly related (R^2 >0.960) based on the determined fat percentage, sonication time, and sonication amplitude percentage. Significant changes (p<0.01) in TS after sonication indicated that sonicated raw milk may contribute to viscosity changes (Yanjun *et al.*, 2014). Van Hekken *et al.* (2019) concluded that ultrasonication can modify protein-protein and protein-lipid interactions, hence demonstrating the slight changes in fat, protein, and TS percentage differences in this study.

The fat percentage of ultrasonicated cow milk at different amplitude and the exposure time varied between 0.95 to 3.39% (Table 3). From the linear model, the fat factor was linear and had a significant (p<0.01) positive correlation on response, showing that different fat percentage as a factor also increases the fat level of sonicated milk. At the quadratic level, amplitude was positively correlated with high significance (p<0.01) explaining whether the low or high level of amplitude affected the sonicated milk fat. Previously, Ertugay *et al.* (2004) reported that the smallest fat globule diameter was 0.725 μm at a power level of 450 W for 10 min at the highest homogenisation efficiency. The same study reported that fat globule diameters at 180 W for 10 min showed no differences in the smallest fat globule diameter as compared to conventional homogenisation. The emulsion was stabilised as cavitation assisted in dissolved-oxygen removal. Milk fat globules consist of nonpolar triglycerides and cholesterol esters within a size between 0.1 to 10 μm. The main constitutions of milk triglycerides are the three fatty acid molecules esterified to a glycerol molecule and stabilised a milk fat globule membrane (MFGM) layer. The MFGM is a protective coat on the milk fat spherical globule against lipolysis degradation (Kelly & Larsen, 2010). The results of the lowest amplitude of 40% were observed to be in agreement

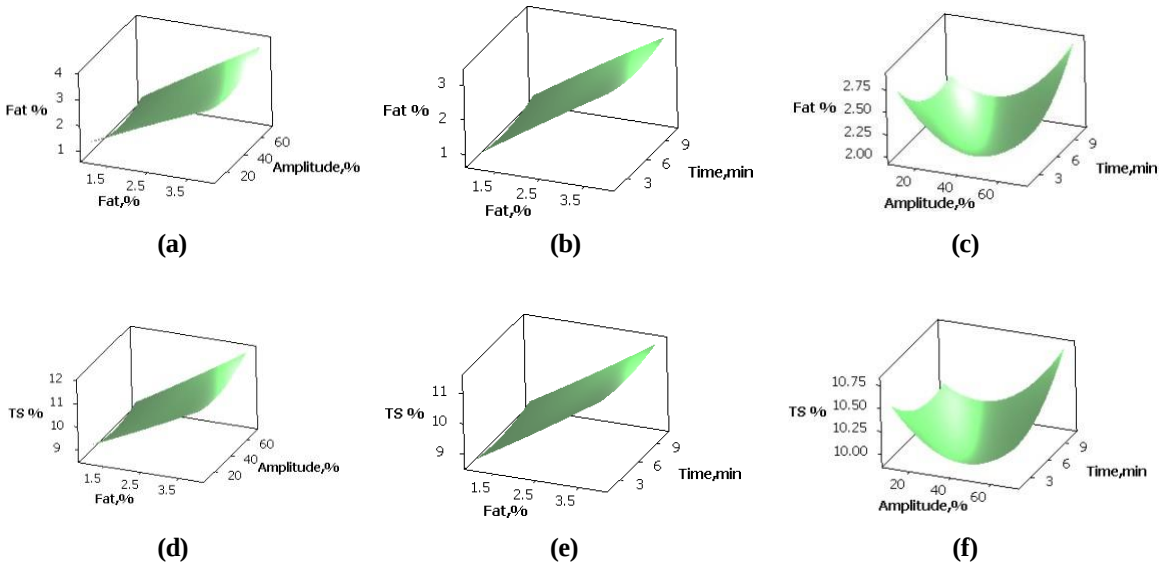
with a previous study by Hong Bui *et al.* (2021) which stated that the concentration of fat (2, 4 and 6% w/w) in reconstituted cow milk suspension at different times (0.5 to 10 min) and different energy density influenced the final intensities of modified fats, triglycerides, and lipid-protein complexes due to the shear pressures from sonication.

The linear effect of all three variables (milk fat percentage, amplitude percentage, and time) in Table 4 was all positively correlated and significant (p<0.01) for protein changes as response variables. There was no interaction between the factors with no effect on the protein. The quadratic effect of milk fat percentage revealed that sonication on different fat percentages from milk significantly affects protein percentages, and others are not significant. In milk protein, ultrasonication destabilises casein (Chandrapala *et al.*, 2012) and delays serum separation in the final product (Paniwnyk, 2017). The changes enhance protein solubilisation as cohesiveness and increase water-holding capacity (Zhao *et al.*, 2014). Current results (Table 4) indicated that protein content was significantly modified (p<0.05) after ultrasonication at linear and quadratic levels with R_{adj} at 0.8549. A previous study on ultrasonication treatment combination of power level (78W and 104 W) at 20 kHz and duration of the treatment (4, 6 and 8 min) on semi-skimmed sheep milk protein fraction proved to remain unchanged (Balthazar *et al.*, 2019). A small range of protein percentage for all sonicated milk was observed and suggested that the protein conformation interchanged in β-lactoglobulin term of decreased β-sheets and increased α-helix and random coils after ultrasonication (Zhou *et al.*, 2020).

SNF percentage of ultrasonicated cow milk at different amplitude and exposure time ranged between 8.015 to 8.290% (Table 3). All linear factors were significantly positive (p<0.01) on SNF, showing that factors also increase the SNF percentage level of sonicated milk. No interaction among factors toward SNF percentage as the response. At the quadratic level, the fat percentage factor was positively correlated with high significance (p<0.05) explaining that changes to the fat percentage will increase the percentage of SNF, while ultrasonication time was negatively correlated with high significance (p<0.01) explaining that the longer ultrasonication will decrease SNF percentage as shown in Figure 1. Ultrasonication can have various effects on milk SNF percentages, which include changing its percentages. SNF is a general term for milk with the exclusion of water and fat. Raw milk's primary components, such as protein, lactose, salts, acids, vitamins, and so on, are crucial markers for assessing the characteristics of dairy products (Lafarge *et al.*, 2004). In milk with higher fat percentages, ultrasonication can cause more significant disruption of the fat globule membrane, leading to increased coalescence and clustering of fat globules. This clustering of

fat globules can result in the separation of fat from the rest of the milk into smaller droplets (Liu *et al.*, 2021), leading to a decrease in the SNF percentage. Consequently, the percentage of SNF would appear lower in ultrasonicated raw cow milk with higher fat content.

Figure 1 shows the surface plot for independent variables on the percentage of TS of ultrasonicated cow milk. At different amplitude and exposure time, the values varied between 8.888 to 11.425% (Table 3). From the linear model, the fat percentage factor was strongly significant (p<0.01) with a positive correlation on response, showing that different fat percentages as a factor increase the percentage of TS level of sonicated milk. At the quadratic level, amplitude was positively correlated with high significance (p<0.01) justifying that the low or high level of amplitude increases the percentages of TS. The longer time (1–10 min) with increasing amplitude (20–40%) induced the unfolding of ß-lactoglobulin and disordered the stable compact fold of native ß-lactoglobulin in goat milk. It was observed that 40% amplitude had given the lowest percentage of TS which may be attributed to the reduction of percentages of ß-sheet and random coils from the interior unfolding process of ß-lactoglobulin molecules (Ciuciu *et al.*, 2016). Zhou *et al.* (2020) supported the increase of TS as amplitude and exposure time of high-intensity ultrasonication improves the thermal stability of the protein. The cavitation phenomenon at the amplitude of more than 40% could have resulted from hydrophobic region exposure to the surface.



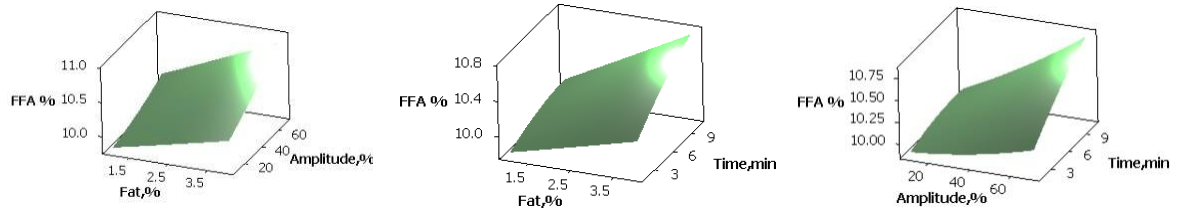


Figure 1. The 3D response surface curve relating to the effects of fat %, amplitude %, and exposure treatment time.

Acoustic cavitation occurs in milk from ultrasonication to produce longitudinal sound waves. It creates rapid compression and rarefaction cycles that interact with the liquid and dissolved gas, leading to the growth of microbubbles available in the milk. Total solids encompass all the solid components present in milk, including fats, proteins, carbohydrates, minerals, and others. Once the microbubbles reach their maximum sizes, they will violently collapse (Ashokkumar *et al.*, 2010) and may create higher total solid concentrations. Turbulence associated with acoustic cavitation promotes the partial mobility of particles by enhancing the aggregations among MFGM-protein complexes. Subsequently, new MFGM-protein complexes from sonication promote gelation and solubility (Sun *et al.*, 2022). TS is one of the important determinants in milk yield of different ruminant/non-ruminant breeds. It was reported that milk composition and milk yield were to be negatively correlated due to water content (Zhou *et al.*, 2018). Therefore, it was suggested that ultrasonication might be useful to improve the TS of the milk yield from the same breed of interest.

The percentage of lactose in ultrasonicated cow milk was shown to have a non-significant (p>0.05) effect on factors interactions and quadratic term except positively correlated with different levels of fat percentages as factors. The values varied between 4.24 to 4.37% (Table 3). From the linear model, the fat percentage factor was linear and had a significant (p<0.01) and positive correlation on response, showing that different fat percentages as a factor also increase the percentage of lactose level of sonicated milk. Lactose crystal nucleation improvement came from the heterogeneous surface of the acoustic cavitation bubbles. The shear forces associated with ultrasound are suggested to affect the changes in crystal size and morphology from the dissolution of developing agglomerates in the milk (Nalajala & Moholkar, 2011) after treatment.

Meanwhile, the percentage of casein had the weakest relationship between the ultrasonication treatment and fat percentage of cow milk (R^2_{adj}=0.5720). The percentage of casein values varied between a small range of 2.36 to 2.43% (Table 3). The percentage of fat factor was linear and had a significant (p<0.01) positive correlation on response, showing

that different fat percentages as a factor also increase the fat percentage level of sonicated milk, and no interaction was seen among all factors. At the quadratic level, the percentage of casein was significant (p<0.05) and positively correlated with determined milk fat percentages, but negatively (p>0.05) affected by amplitude as a factor, further explaining whether the low or high level of amplitude did affect the sonicated milk. Casein micelles are regarded as colloidal milk proteins due to their composition of aggregated caseins with a stabilising surface layer of colloidal calcium phosphate (Dalgleish, 1998). However, Abesinghe *et al.* (2020) and Chandrapala *et al.* (2012) reported the average size of the remaining fat globules in milk fat was reduced and did not significantly change the size of the casein micelles. Zhang *et al.* (2018) related that high-intensity ultrasonicated microfiltered casein protein retentates treated for 0.5–5 min undergo changes in their functional properties. It was explained that casein protein reassembles through the exposure of hydrophobic regions of partially unfolding protein from the interior to the surface of the molecules, the reduction in particle size, and changes in protein secondary structures. Chandrapala *et al*. (2012) and Villamiel and Jong (2000) agreed that non-thermal ultrasonication at 50% amplitude neither reduced particle size nor affected the native casein structure, as determined by the Bradford assay. In contrast, conventional heating and ultra-pasteurisation processes may precipitate soluble calcium, solubilise colloidal calcium phosphate, and disassociate κ-casein and some α-caseins from micelles, which ultimately leads to aggregation and complexation with serum proteins and other individual caseins (Abesinghe *et al.*, 2020; Cameron *et al.*, 2009).

Finally, Table 3 showed that the percentage of FFA varied between 9.85 to 10.88% after cow milk was ultrasonicated at different amplitude and exposure times with the strongest correlation. All linear factors had a significant (p<0.01) positive correlation on the percentage of FFA, showing that different fat percentages as a factor also increase the FFA percentage level of sonicated milk. All factors were shown to be highly significant (p<0.01), dependent, and interacted with each other towards FFA percentage. There was no quadratic level from all factors towards FFA, explaining that the low or high level of all individual factors did not affect the fat percentage of sonicated milk. The adjusted R^2 from the percentage of FFA second-order polynomial equation was 0.9745, showing that the equation exhibits the strongest relationship between factors and responses. During this process, Leong *et al.* (2016) described that milk fat was separated into fractions. The stabilised emulsion in sonicated milk leads to smaller fat globule size as compared to commercial milk (Astráin-Redín *et al.*, 2023). Milk fat globules membrane proteins may be lost from the milk fat globules surface and allow caseins and whey proteins to simultaneously migrate to the newly

formed milk fat globule surface, forming a casein/whey-based surface layer (Lee & Sherbon, 2002) and release lipid content including FFA from triglycerols (Liu *et al.*, 2021). Fat % and FFA % changes affected by physical forces from ultrasound, at low-frequency ultrasound led to the disruption of milk fat globule membranes (Bermúdez-Aguirre *et al.*, 2008). From the process optimisation study, FFA in milk was controlled by a short sonication time of 1.5–10.0 min. The result was in agreement with a study performed by Juliano *et al.* (2014), which stated that lipid oxidation content can be reduced by decreasing the sonication time as well as temperature.

3.2 Numerical Process Optimisation

The selected responses from optimisation were based on their goodness-of-fit of more than 0.900; namely percentages of milk fat, TS and FFA (0.940, 0.955, and 0.975, respectively). For simultaneous numerical optimisation of factors and responses, the independent factors were determined with realistic optimum levels (Figure 2).

The minimum value of milk fat percentage was determined to be more than 3.25% as a reference requirement for processed milk, raw milk, and fresh milk in the Malaysian Food Act (1983), although it may vary in other countries. The range of the TS percentage was set at maximum while FFA was set at minimum.

The optimum factors were found to be using 4% fat, 10% amplitude, and 1.95min exposure time with a prediction to produce 10% FFA, 11.49% total solid %, and 3.50% fat after ultrasonication, at a composite desirability of 0.996 (Figure 2). The signified optimum process with a density power of 46.8 J/mL energy density, could be seen at the 4.0% fat raw cow milk ultrasonication to obtain the highest TS and lowest FFA content as the outcome from RSM.

The predicted results were verified with experimental tests with its % error shown in Table 5. The experimental response values at optimised conditions for the percentages of fat, TS and FFA were 3.65%, 11.43%, and 10.12%, respectively.

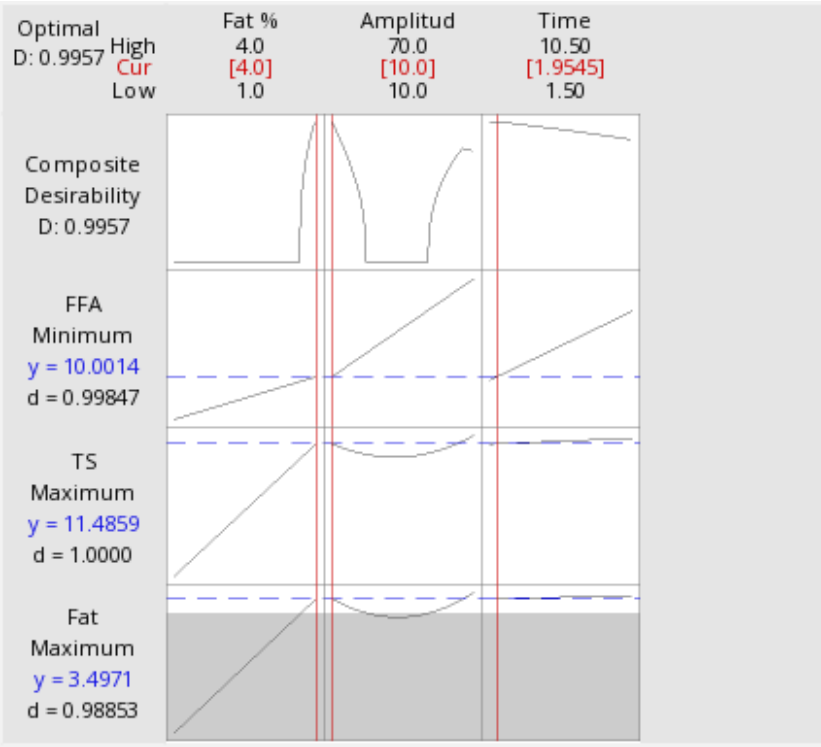


Figure 2. Optimised conditions to produce ultrasonicated cow milk from RSM

Table 5. Predicted and experimental values of response variables

Response variables	Optimised values (%)		% Error
	Predicted	Experimental	
Fat %	3.50	3.65±0.14	4.10
TS %	11.49	11.43±0.88	0.52
FFA %	10.00	10.12±0.09	1.19

3.3 Correlation Between the Responses

Pearson's correlation revealed that the milk at different fat percentages exhibited a highly significant ($p< 0.01$) positive correlation with all milk (r=0.942 for fat and r=0.952 for TS) components except casein. Meanwhile, the percentage of amplitude % exhibited a weak (r<0.600) positive correlation with protein, SNF, casein and FFA. Furthermore, there was no significance seen in the changes of amplitude percentages towards fat and lactose, which indicated that ultrasonication amplitude did not affect the respective nutritional composition. Ultrasonication exposure time was significant, positive, and weakly correlated with protein, casein and FFA. In addition, there was also no significant correlation between exposure time among fat, SNF, TS, and lactose of ultrasonicated milk. Notably, the protein was highly significantly correlated with SNF (r=0.957). Among all factors, FFA was

shown to be significantly (p<0.01) associated with independent variables and positively highly significance (p<0.01) correlated with other nutritional components.

Table 6. Pearson's correlation between factors

Pearson	Fat %	Amplitude %	Time	Fat	Protein	SNF	TS	Lactose	Casein	FFA
Fat %	1.000	0.000 ns	0.000ns	0.942**	0.664**	0.650**	0.952**	0.755**	0.173 ns	0.601**
Amplitude %		1.000	0.000 ns	0.046 ns	0.344**	0.312**	0.061 ns	0.108 ns	0.231*	0.582**
Time			1.000	0.022 ns	0.242*	0.218 ns	0.041 ns	0.102 ns	0.242*	0.446**
Fat				1.000	0.595**	0.593**	0.994**	0.723**	0.034ns	0.650**
Protein					1.000	0.957**	0.667**	0.819**	0.735**	0.762**
SNF						1.000	0.665**	0.839**	0.702**	0.729**
TS							1.000	0.780**	0.121 ns	0.674**
Lactose								1.000	0.421**	0.604**
Casein									1.000	0.374**
FFA										1.000

** p<0.01 significant at 1% level of probability
* p<0.05 significant at 5% level of probability
ns not significant

5. Conclusions

In this study, the effects of ultrasonication on milk nutritional components were compared by the Milkoscan infrared milk analyser. The results showed that the percentages of fat, TS and FFA have the strongest relationship with ultrasonication amplitude, time, and raw milk fat percentage. The highest milk fat percentage with the lowest percentages of TS and FFA using factors in sonication was optimised with a combination of 4% milk fat, 10% amplitude, and 1.95 min of sonication time. The recommended conditions for ultrasonication and raw milk fat percentage can be employed as non-destructive processing to control FFA production induced within the milk processing line at 46.8 J/mL energy density. However, further studies are necessary to evaluate the volatile compounds released from the sonication process.

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