The use of Rayleigh-Raman scattering to investigate milk fat globules

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Abstract

Human Milk is a complex mixture of nutritional components.[1, 2] The composition of human milk is dynamic and varies between mother, populations, stage of lactation and within feedings, etc.[2, 3] Understanding human milk composition in important for varies purposes, including the management of infant feeding.[2]
The aim of this study is to firstly, find a correlation between the size of the CH-band and the Rayleigh signal of a trapped particle. Secondly, determine the different types of particles present in the human milk. Lastly, comparing the sizes of fat globules per mother. All using Rayleigh-Raman scattering. It is hypothesised that with Rayleigh-Raman scattering both the size and the composition of individual milk fat globules can be analysed. It is expected to mostly trap Triacylglycerols and extracellular vesicles. It is also expected to trap Casein Micelles in the milk. It is expected that the CH-band intensity and the Rayleigh signal have a positive correlation.
There were 5 healthy volunteers between 24 and 34 years old, who donated milk samples between March and November of 2021. All volunteers lived in the Netherlands with a lactation stage between 2-15 months. There were two batches of samples, pure milk and skim milk. The skim milk was centrifuged in two steps, first 100G for 2 minutes and the second 1000G for 10 minutes at 20°C. The pure milk was 75x diluted and the skim milk was 20x diluted with Phosphate-buffered saline (PBS). The samples were heated to 37°C in a warmth bath for at least one hour. At the setup, 50 μL of the sample was pipette into a microscope glass and covered with a cover glass. The samples were measured at 80μm in the well and 120μm below the cover glass. To investigate the fat globules, a Raman setup-up 640 nm lase beam was used with an power of 35 mW. The calibration of the pre-processing was done with the Quantitative Calibration Routine (QCR) program. For the further analysis of the data two MATLAB files were used. One file was used to read the raw data and to determine the trapping event with the Rayleigh signal. The second file was used to display the calibrated Raman spectrum of the trapping event.
The results show that there is a increase visible between the CH-band intensity and the Rayleigh intensity. The identified particles are triolein, tripalmitolein, Elaidic acid and fluorescent particles. By measuring the CH-band intensity it is possible to determine bigger and smaller particles. However, it is not possible to determine the exact measurements of the particle using this method. The CH-band intensity resonates with the Rayleigh intensity.
It is advised to measure at 40 μm with an power of 70 mW at the sample. It is also advised to glue the cover glass to the microscope glass.

DUTCH - Samenvatting

Mensenmelk is een complexe samenstelling van voedingstoffen.[1, 2] De samenstelling is dynamisch en verschilt per moeder, bevolking, lactatieperiode en binnen een voeding, etc.[2, 3] Het is van belang om de samenstelling van de melk beter te begrijpen o.a. voor het voeden van de baby.[2]
Het doel van dit onderzoek is om een correlatie tussen de grootte van de CH-band intensiteit en Rayleigh intensiteit van een deeltje te vinden. Ook worden de verschillende deeltjes in de melk bepaald. Als laatste, wordt er gekeken naar de grootte van het grootste en kleinste getrapte deeltje in de melk van de verschillende moeders. Dit wordt allemaal gedaan met Rayleigh-Raman scattering. Er wordt verondersteld dat met de Rayleigh-Raman verstrooiing de grootte van de deeltjes in de melk en de samenstelling van de melk geanalyseerd kan worden. Ook wordt er verwacht dat de getrapte deeltjes voornamelijk Triacylglycerols en extracellulair blaasjes zijn. Ook wordt er verwacht Casein Micellen aan te treffen in de melk. Tenslotte wordt er verwacht dat er een positieve correlatie is tussen de CH-band intensiteit en de Rayleigh intensiteit.
Voor het onderzoek hebben 5 gezonde vrouwen tussen de 24 en 34 jaar melk gedoneerd. De donaties vonden plaats tussen maart en november 2021. Alle vrijwilligers wonen in Nederland en hebben een lactatieperiode tussen de 2- 15 maanden. Voor de monsters werden twee subgroepen gemaakt, pure melk en magere melk. De magere melk was gecentrifugeerd in twee stappen, eerst 100G voor 2 minuten, daarna 1000G voor 10 minuten op 20°C. De pure melk is 75x verdund en de magere melk 20x verdund met Phosphate-buffered saline (PBS). De monsters zijn verwarmd op 37°C voor tenminste één uur. Bij de opstelling is 50 μL van de monsters in een microscoopglaasje gepipetteerd en bedekt met een dekglaasje. De meting werden uitgevoerd op 80μm in het welletje en 120μm onder het dekglaasje. Om de vetdeeltjes te meten is een Raman opstelling gebruikt met een 640 nm laser, met een vermogen van 35 mW op het monster. De kalibratie is verwerkt met het Quantitative Calibration Routine (QCR) programma. De verdere analyses zijn uitgevoerd met MATLAB. Met één van de documenten is de ruwe data ingelezen en het trapping moment bepaald met het Rayleigh signaal. Het andere document is gebruikt het gekalibreerde Raman spectrum van het trapping moment.
Uit de resultaten bleek dat er een toename zichtbaar is tussen de CH-band intensiteit en de Rayleigh intensiteit. Triolein, tripalmitolein, Elaidic acid en fluorescente deeltjes zijn geïdentificeerd. Ook is het mogelijk om grote en kleine deeltjes te onderscheiden met de CH-band intensiteit. Echter is het met deze methode niet mogelijk om de exacte afmetingen van het deeltje te bepalen. Verder is het gebleken dat de CH-band intensiteit resoneert met de Rayleigh intensiteit.
Het wordt geadviseerd om het vermogen van de laser naar 70 mW bij het monster te verhogen. Ook wordt het geadviseerd om minder diep in het welletje te meten, bijvoorbeeld 40 μm. Verder wordt het geadviseerd om het dekglaasje te verlijmen met het microscoopglaasje.

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1 Introduction

Human Milk is a complex mixture of nutritional components.[1, 2] The composition of human milk is dynamic and varies between mother, populations, stage of lactation and within feedings, etc.[2, 3] Understanding human milk composition is important for varies purposes, including the management of infant feeding.[2] Often human milk is researched by using biomarkers. Some of the used methods to measure fats are Micro-Rose-Gottlieb (gravimetric) and Creamatocrit. [4] There have been researches [5] in which they looked at the degree of saturation of lipids. They observed the lipids using Raman spectroscopy. In the synchronized Rayleigh-Raman research[6], it was possible to use a new method, based on synchronized Rayleigh and Raman light scattering from a single laser beam, to optically trap single Extracellular Vesicles. This method uses a purge of an optical trap at regular time intervals. Each time it starts with and empty trap. This technique is nondestructive. The technique is applicable on diluted samples. For this technique to be successful a sample should have a concentration of 3,6 E8 -5 E10 particles/mL[6] of milk.
There is still little known about human milk. In order to learn more, more research is needed. There is still little known about Casein Micelles, more is known about bovine milk than human milk.
The aim of this research is to firstly, find a correlation between the size of the CH-band and the Rayleigh signal of a trapped particle. Secondly, determine the different types of particles present in the human milk. Lastly, comparing the sizes of fat globules per mother. All using Rayleigh-Raman scattering. This leads to the following research questions. Is there a correlation between the size of the CH-band intensity and the Rayleigh signal of a trapped particle? Can the different types of particles in the human milk be determined? Can the sizes of the particles be compared by the CH-band intensity?
Based on the information found in previously performed experiments, it is hypothesised that with Rayleigh-Raman scattering both the size and the composition of individual milk fat globules can be analysed. It is expected to mostly trap Triacylglycerols and extracellular vesicles. It is also expected to find Casein Micelles in the milk. It is expected that the CH-band intensity and the Rayleigh signal have a positive correlation. The ratio give an indication of the saturation of the fat globules, higher ratio means more unsaturated fat particles.

2 Theory

In this paragraph the human milk, extracellular vesicles, Casein micelles, Raman scattering and Rayleigh scattering will be discussed.

2.1 Human milk

Human milk is a complex mixture of nutritional components.[1, 2] These nutritional components are macronutrients (carbohydrates, fat and proteins), micronutrients (minerals and vitamins) and bioactive components (growfactors and immunologic factors).[2] The composition of human milk is dynamic and varies between mothers, populations, stage of lactating and within feedings, etc [2, 3]. Understanding human milk composition is important for multiple purposes including the management of infant feeding.[2]. Approximately 50% of the infant's energy requirements comes from fatty acids (FA)[7].

2.1.1 Milk fat globules

The fat in human milk is variable. It changes in content over the course of a feed, during a day and over the course of lactation.[7] The fats in the human milk are the main source of dietary energy and bioactive molecules.[1] These fats are milk fat globules, which have a biological membrane named fat globule membrane (MFGM).[1] Human milk contains over 98% of it's lipids in the form of triacylglycerols(TAG).[8] These lipid particles come in different sizes. These size range between 0,3-15 μm[9, 1]. The mean diameter of these MFGMs are 3,5 to 5 μm. Human milk consists of many fatty acids. The three most important ones are 16:0, 18:1c9 and 18:2c9c12.[1]

2.2 Extracellular vesicles

Almost all cell types can create Extracellular vesicles (EVs). EVs are membrane-bound vesicles that are released into the extracellular space.[10] EVs travel through the body, they can cross physiological barriers and can be found in biological fluids, one of these is milk. EVs in milk can contain different components. These components exists of lipids, mRNAs, non coding RNAs and proteins.[11, 12] There are different types of EVs: Microvesicles (MVs), exosomes, oncosomes and apoptotic bodies.[12] Exosomes are found in human milk, these have a size between 40-100 nm. They contain RNA cargos. These cargoes have multiple roles, including cell-to-cell communication, metabolism and gene regulation and nutrition.

2.2.1 Microvesicles

MVs are involved with immune responses. They can have different origins, such as the placenta or maternal immune cells. The MVs activate leukocytes and help fight against external invading.[10] They have a diameter of 50-100 nm.[10, 12] MVs can be found in human milk.

2.2.2 Oncosomes

Malignant cells produce oncosomes[12]. Oncosomes can invade nearby tissue and destroy it. They have a diameter of 1-10 μm.[12]. This is not beneficial for an infant and these are not found in human milk.

2.3 Casein micelles

Casein micelles are colloidal particles containing proteins and calcium phosphates (figure 1.[13] They are a stable way of including calcium and phosphorus in human milk. Their main purpose is to increase the concentration of these minerals in human milk.[14] There still is no clear picture of the composition of casein micelles and their size.
It is known that a casein micelle is build up of multiple submicelles held together by hydrophobic bonds and salt bridges.[15] The casein micelles are restricted due to the structure. There exist two types of submicelles, with and without κ-casein. Micelles change their properties depending on the pH. Lowering the pH results in the formation of salt bridges. Another aspect that influences the micelles is temperature. By lowering the temperature, the micelles will increase in volume. If the temperature gets above 70°C, some parts of the molecule increases in flexibility.
Micelles (figure 1) has a size between 40- 300 nm in diameter [15].

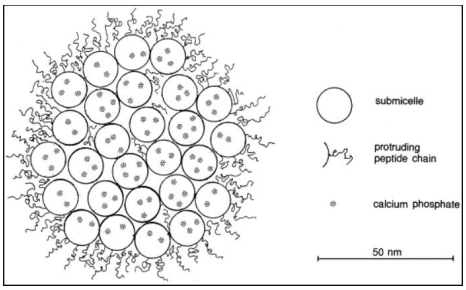


Figure 1: A model of Casein Micelles created by Walstra 1990[15] and 1999[16]. It shows the Casein Micells with it sub-casein Micelles, the peptide chains and calcium phosphates.

2.4 Raman scattering

Considering the use of Rayleigh-Raman scattering in this research, it is important to understand this. Raman spectroscopy is a nondestructive technique with fast analysis. Which requires a small sample volume. [17] With this technique the following components can be interpreted: DNA and RNA bases, Amino acids, Fatty acids and fats, Saccharides, Metabolites, Proteins and Crystals.
The principle is based on light of a frequency Vi that is scattered from a molecule (figure 2).[18] The scattered light consists of a strong component at frequency Vi and components of lesser strength at frequencies above or below Vi. This

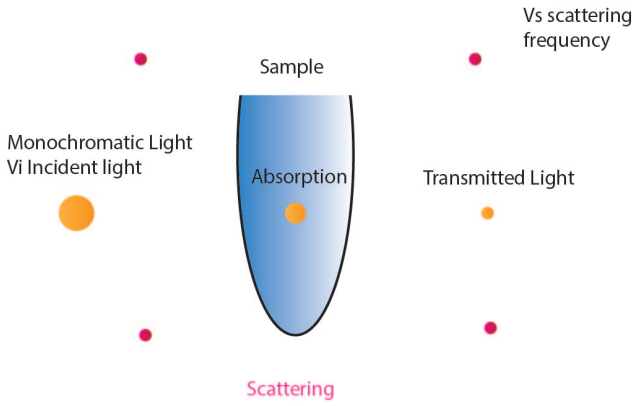


Figure 2: Raman principle: Incoming Monochromatic light with Vi (Incident frequency) goes through the sample. Some of the light is absorbed, the remaining light that leaves the sample is the transmitted light. From incident light some light is scattered. These are scattered with the scattering frequency Vs

scattered light at frequency Vi is called *elastic* or *Rayleigh scattering*. The scattered light can be *inelastic*, which is the result of light scattering at a different frequency from the incident light, this is named *spontaneous Raman scattering*. The inelastically scattered light with a frequency less than Vi is also known as *Stokes field*, the component with frequency greater than Vi is known as *anti-Stokes field*. The change in energy of a molecule results in energy loss or gain by the electromagnetic field. Spontaneous Raman scattering can act as seed photons, these are low energy photons that can stimulate additional Raman scattering. The characteristics of a molecule can be determined by the predominant frequencies in the Stokes and anti-Stokes fields. These can be detected and used to determine the composition of samples. Figure 3 displays the Stokes and anti-Stokes Raman scattering and the Rayleigh scattering.

Raman scattering is weak, Rayleigh scattering is strong. Within Raman spectroscopy the vibrational frequency is measured as a shift from the incident beam frequency[20].
There are different vibrational levels[19] of an electron(v_0-v_1 in figure 3). When absorption of incident light by a photon occurs, the electron is driven to the Virtual State. When the electron falls back to it the initial state, Rayleigh scattering occurs, in which a photon is emitted with the same energy as the incoming photon. When the photon falls back to a vibrational level higher than the groundstate (v_0), Stokes shift occurs. If the electron is driven to the virtual state from a vibrational level higher than v_0, then falls back to the ground state(v_0) or another state in a lower vibrational energy level, the anti-Stokes shift happens. With these spectra the rotational levels can be identified. A qualitative analysis can be performed. The intensity and the vibrational level of the spectral lines signify the concentration. The place of the spectral lines describe the constituent particles of a sample.
The Raman band positions are specific for molecule. The placement of these bands depend on the molecule, the saturation, the phase, geometrical isomerism and polymorphic/polytypic form and presence of hydrophilic groups. Fatty acids and fats can be distinguished from other groups by their typical bands 1300 and 1400cm^{-1}. In Appendix F a table with all the Raman bands are shown.
Figure 4 shows three figures with Raman spectra for fatty acids and fats. In

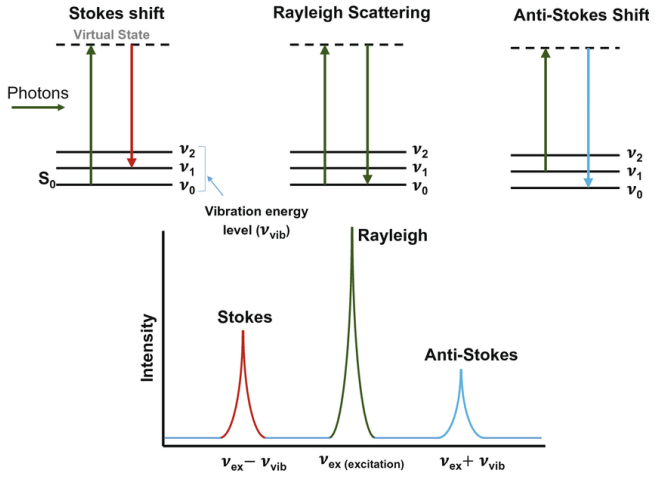
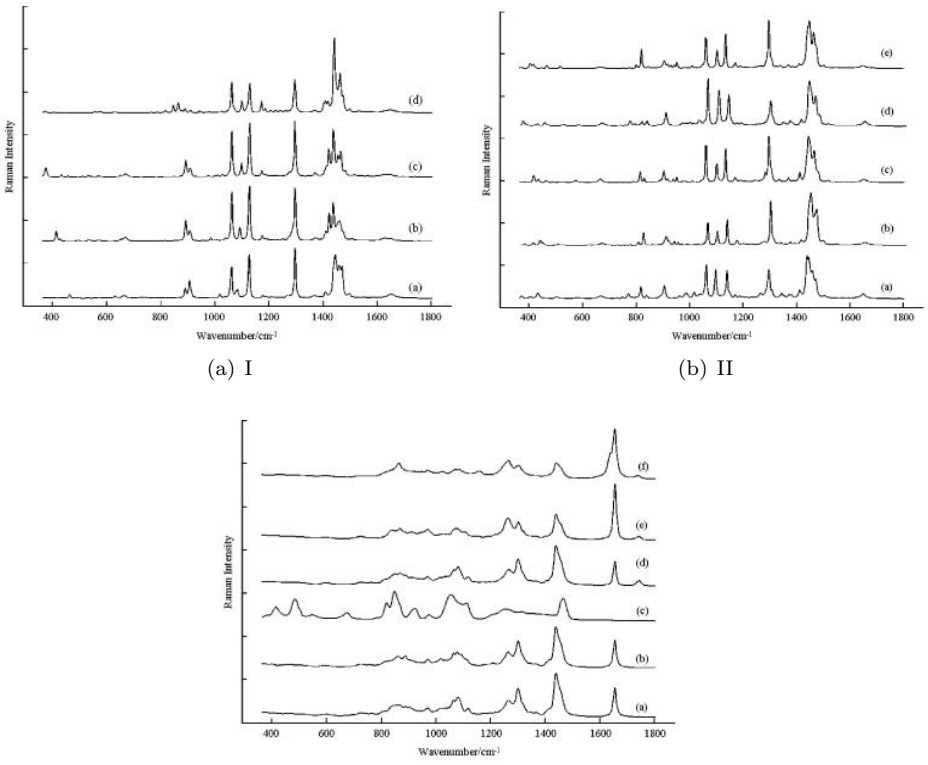


Figure 3: Figure shows the Stokes, Rayleigh and anti-Stokes diagrams[19]. There are different vibrational levels of an electron(v_0-v_1). When absorption of incident light by a photon occurs, the electron is driven to the Virtual State. When the electron falls back to it the initial state, Rayleigh scattering occurs, in which a photon is emitted with the same energy as the incoming photon. When the photon falls back to a vibrational level higher than the groundstate (v_0), Stokes shift occurs. If the electron is driven to the virtual state from a vibrational level higher than v_0, then falls back to the ground state(v_0) or another state in a lower vibrational energy level, the anti-Stokes shift happens.



(a) I

(b) II

(c) III

Figure 4: Raman spectra of pure fatty acids and fats.[17]
I) "Raman spectra of saturated linear fatty acids: (a) lauric acid, (b) myristic acid, (c) palmitic acid, (d) stearic acid." II) "Raman spectra of saturated branched fatty acids: (a) 12-methyltetradecanoic acid (15Aiso), (b) 13-methylmyristic acid (15iso), (c) 14-methylpentadecanoic acid (16iso), (d) 14-methylhexadecanoic acid (17Aiso), (e) 15-methylpalmitic acid (17iso)" III)" Sub figure 3: Raman spectra of the unsaturated fatty acids (a) oleic acid and (b) cis-vaccenic acid, of (c) glycerol and of the fats (d) triolein, (e) trilinolein and (f) trilinolenin." [17]

figure 8(a), three bands can be seen, between 1050 and 1150 cm^{-1}, at 1296 cm^{-1} and between 1400 and 1500 cm^{-1}. In figure 8(b) there are some differences noticable, there are two bands between 1400 and 1500 cm^{-1} these indicate branched fatty acids, linear fatty acids show four to five smaller bands. Branched fatty acids are mostly saturated fatty acids with one or more methyl branches on the carbon chain. In figure 4(c) the unsaturated fatty acids are shown.

2.4.1 Rayleigh scattering

Rayleigh scattering[21] is expressed by:
Figure 5 shows Rayleigh scattering and the difference between Rayleigh and Mie scattering[23]. Incident light comes in, reacts with particles and scatters,

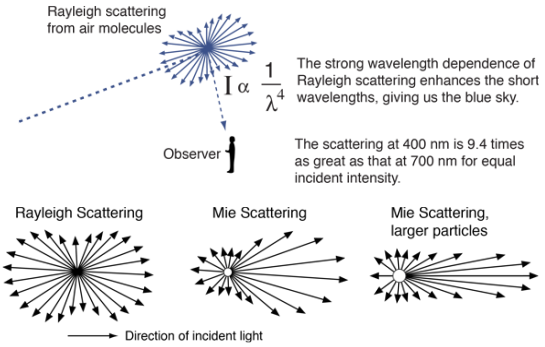


Figure 5: I = Intensity and λ= wavelength[22, 23]

which can be observed. For small particles (smaller than 1 /10 wavelength) , the scattering is mainly Rayleigh. Rayleigh back scattering is the event in which light that is reflected back to the origin. This is due to randomly-distributed variations in the refractive index of particles. Mie scattering is more dominant for particles of sizes larger than the wavelength.

In this research[6], it was possible to use a new method, based on synchronized Rayleigh and Raman light scattering from a single laser beam, to optically trap single Extracellular Vesicles.
The method uses a purge of an optical trap at regular time intervals. Each time it starts with and empty trap. The technique is applicable on diluted samples. For this technique to be successful a sample should have a concentration of 3,6 E8 -5 E10 particles/ mL[6] of milk.

3 Materials and methods

In this paragraph the materials and methods are discussed. Firstly, information on the milk will be provided. Secondly, the sample preparation will be discussed. Thirdly, the Rayleigh-Raman spectroscope set-up will be provided. Fourthly, the data processing will be described.

3.1 Human milk samples

There were 5 healthy volunteers between 24 and 34 years old, who donated milk samples between March and November of 2021. All volunteers lived in the Netherlands with a lactation stage between 2-15 months. An overview can be found in table 1 and in Appendix C. The study of Ms. De Wolf "Shedding light on human milk" was approved by the Ethical Committee at the University of Twente. That approval also applies to this study, the approval is included in Appendix A. The volunteers extracted the milk by using a breast pump. The samples were stored at -18°C withing 24 hours after donation.

Table 1: Details about the donated milk

Sample	**Day of lactation**	**Time of lactation**	**Total volume**	**Age donor**	**Gender Child**	**Lactation period (months)**
1	06-12-2021	06:00-07:00	60	30	V	15
2	15-04-2021	11:00 - 12:00	45	34	M	4
3 (T001A)	11-11-2021	21:00-22:00	100	30	V	14
4 (T002B)	08-05-2021	09:00-10:00	40	24	M	3
5 (T003C)	03-10-2021	20:00-21:00	95	28	V	4

3.1.1 Dilution sample

There were be two types of samples. The first batch (N) was pure milk and contains all the milk components. The second batch (V) contained the skim milk. To separate the fat globules from the other components in the milk, this batch was centrifuged in two steps. Firstly, each sample was centrifuged on 100G for 2 minutes at 20°C, than the supernatant was removed using a disposable scoop. The clear sample, semi-skimmed milk, was gently transferred with a pipette into a clean eppendorf. Secondly, the semi-skimmed milk was centrifuged again on 1000G for 10 minutes at 20°C, once more the supernatant was removed using a disposable scoop. The clear sample was gently transferred with a pipette into a clean eppendorf. In this sample most of the fat layer is

removed. Further, both batches went through the same remaining steps. The total volume of the dilution is 1 ml. The protocol is included in Appendix B.

3.2 Sample preparation

For the Raman trapping system to be functional and trap one particle at a time, the right dilution is needed. It was important to determine the concentration of the samples and the final sample. This is described in dilution sample and the milk samples, Appendix B. After some experiments it was concluded that a dilution of 75x (N3) and a dilution 20x (V2) were suitable. These samples often had a trapping event with one particle, which made them suitable for the experiment. The less diluted samples had many trapping events with multiple particles in the trap at a time, see table 2 The dilution series was made with sample 1. The dilutions were made with PBS, full overview can be found in Appendix E.

Table 2: Trapping-event per sample, Sample 1.

Sample	**Dilution**	**Number of Trapping-events over 15 measurements**
N1	25x	10
N2	50x	7
N3	75x	4
N4	100x	6
V1	10x	2
V2	20x	7
V3	30x	0
V4	40x	0

3.2.1 Sample preparation for measurement

For the Rayleigh-Raman set-up a concentration 3,6e8-5e-10 part/ml is desirable. The milk is divided in two types, pure and skim milk. Pure milk (i.e. N) contains approximately 40 g/l fat and skim milk (i.e. V) contains approximately 0.3 mass%, which is approximately 0,12g/l fat.[24] The samples were 75x diluted for the pure milk (N) and 20x diluted for the skim milk (V). The samples were heated to 37°C in a warmth bath for at least one hour.

3.3 Rayleigh-Raman spectroscopy

The Rayleigh-Raman spectrometer contains a optical microscope BX41 Olympus, objective Olymp 40x NA0,95. To trap the fat globules a single laser beam of $\lambda = 640$ nm Cobolt was used. The Raman and Rayleigh spectra were simultaneously detected by a single CCD camera. A homebuild spectograph with a range 650-850 nm was used. In figure 6 the used setup is shown. The power measured at the sample was 35 mW. The objective was moved along the z-axis to a depth of 80 μm below the cover glass. A view samples (T001A N and T002B on 2022-06-08) were measured at 120μm below the cover glass. The spectra were collected over an acquisition time of 34,87 ms with 400 spectra over a time period of 13s. After the 13 s, the trapped particles were released by blocking the laser with a shutter for 5 seconds. Each cycle was repeated for 50 times, resulting in 400x50 = 20.000 Rayleigh-Raman spectra per measurement. Specific details about the measurements per sample can be found in Appendix E.

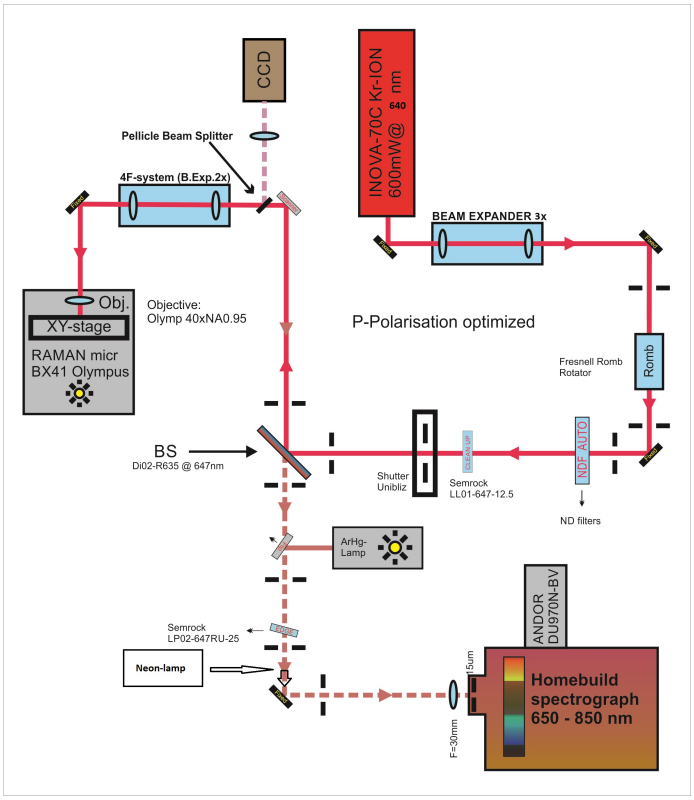


Figure 6: Raman Setup at MCBP lab

3.4 Measurement

The calibration was performed at the lab, measuring Toluene, White light source, ArHg-light and Neon light with known emission spectrum and background. After the calibration steps the real measurements were prepared as follows. The 50 µL of the sample after dilution was pipetted in a microscope well glass slide (BMS Microscope glass Art.nr. 12290 ISO 9001) and covered with a cover glass (VWR 22mm Coverglass Cat-No. 631-0158). This is done by placing the cover glass 2/3 over the well before the milk is pipetted into the well.

3.5 Data processing

The calibration measurements were needed for the pre-processing with the Quantitative Calibration Routine (QCR) program, this program performs Cosmic rays removal, noise reduction, axis calibration and amplitude correction. After following these steps a spectrum is obtained.
For the further analysis of the data two MATLAB files will be used. The first file reads the .sif-files, these are not calibrated, to determine the place of trapping, from start to end of a single trapping event. The second file uses the .asc-files, the calibrated files, to plot the mean Raman spectrum of the specific trapping event. Both MATLAB scripts are included in Appendix H.

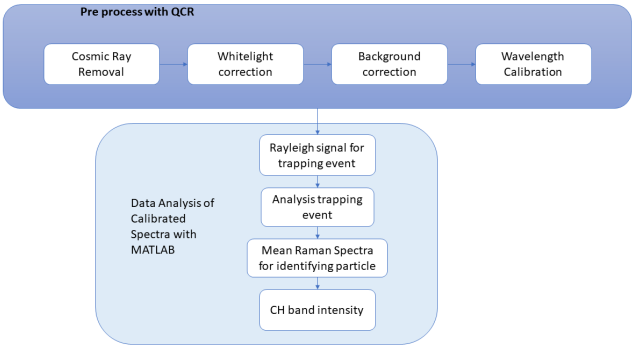
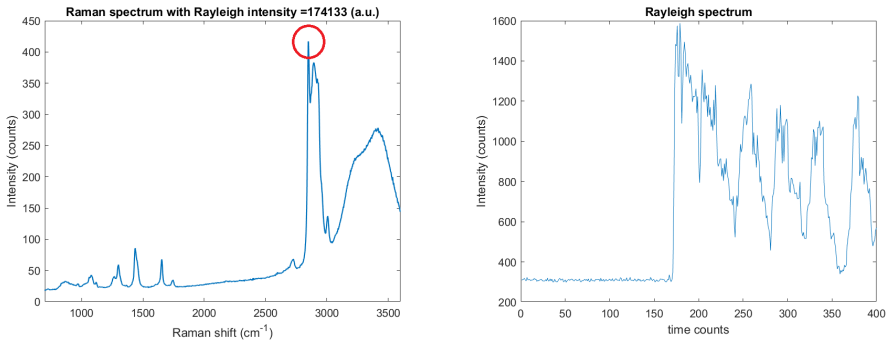


Figure 7: Flowchart of the data processing

The calculated Rayleigh signal is displayed in the title of figure 8, some were negative and had to be noted by hand. This Raman spectrum is the Calibrated Rayleigh signal intensity over the duration of the specific trapping event, this makes the Raman spectrum less noisy. The CH-band is determined by the highest intensity of the CH-band as shown by the Red marking in figure 8



(a) I (b) II

Figure 8: The mean Raman spectrum measured over the trapping event (spectra 168-400). Measurement happened on 2022-06-13 with sample T002B pure milk (N).

3.6 Band intensity ratio

Band intensity ratios of $\delta(1260)/\rho(1300)$ and $\nu(1655)/\delta(1440)$ was used. For these ratios, the Raman shift at that place was used for each specific trapping event. There is corrected for a baseline, specific for that measurement, usually between 20 and 30.

4 Results

In this paragraph the results of the measurements are presented. All spectra were collected following the data processing, paragraph 3.5. For the collected data the first five traps are displayed in this paragraph. Firstly, the relation between the intensity of the CH-band and the Rayleigh intensity will be discussed. Secondly, the CH-band and Rayleigh signal will be shown, to indicate whether there is a correlation between the two. Thirdly, the Raman fingerprint region and full Raman spectrum will be shown to determine the trapped particles. Lastly, a comparison is made between the smallest and biggest particle trapped in each sample.

4.1 CH-Band and Rayleigh

In this paragraph the relation between the intensity of the CH-band and Rayleigh will be discussed. Looking for a correlation can give an indication about the molecule composition. This can help by identifying bigger trapped particles. First, there is an overview of all the measurements. The sample of donor, which trapping event it is, this correlates with the trapping events in paragraph 4.3, the filename of the measurement, the spectra over which the trapping event took place, the CH-band intensity over the trapping event and the Rayleigh intensity over the trapping event.
The intensity of the measured CH-band and Rayleigh intensity can be found in table 3 for the pure milk and table 4 for the skim milk. The intensity varies between the samples.

Table 3: Overview of the Ch-band and Rayleigh signal measured of pure milk (N) (*all filenames start with Image)

Sample	**Trapping event**	**Filename***	**spectra #**	**CH-band intensity**	**Rayleigh intensity**
1	1	2022-05-30@145034	340-400	315	57932
	2	2022-05-30@144139	363-365	53	1762
	3	2022-05-30@144948	92-309	299	45276
	4	2022-05-30@144948	322-346	259	34419
	5	2022-05-30@145052	258-269	185	2732
2 (T004)	1	2022-06-08@140425	371-400	722	152590
	2	2022-06-08@140447	190-279	770	42528
	3	2022-06-08@140447	280-400	1065	106967
	4	2022-06-08@140509	189-400	880	42929
	5	2022-06-08@140531	182-400	936	47808
3 (T001A)	1	2022-06-13@134657	4-80	452	102604
	2	2022-06-13@134657	252-391	521	150161
	3	2022-06-13@134720	385-400	157	20792
	4	2022-06-13@134742	170-400	669	142911
	5	2022-06-13@134804	170-400	658	69771
3 (T001A)	1	2022-06-14@140214	172-174	53	6766
	2	2022-06-14@140214	204-212	59	10025
	3	2022-06-14@140236	132-136	60	6384
	4	2022-06-14@140236	145-148	66	8600
	5	2022-06-14@140045	51-53	80	3088
4 (T002B)	1	2022-06-13@143442	325-400	508	186439
	2	2022-06-13@143526	133-400	672	283814
	3	2022-06-13@143548	168-400	416	174133
	4	2022-06-13@143610	171-298	507	24617
	5	2022-06-13@143504	150-400	685	287289
4 (T002B)	1	2022-06-14@142459	258-262	90	2686
	2	2022-06-14@142521	129-133	101	3017
	3	2022-06-14@142627	98-103	120	7410
	4	2022-06-14@142755	209-214	101	1521
	5	2022-06-14@142839	23-24	196	15258
5 (T003C)	1	2022-06-14@144802	172-400	171	15680
	2	2022-06-14@144824	212-400	178	17692
	3	2022-06-14@144846	186-400	186	18840
	4	2022-06-14@144908	171-400	194	20774
	5	2022-06-14@145248	239-245	106	19220

Table 4: Overview of the Ch-band and Rayleigh signal measured of skim milk (V) (*all filenames start with Image)

Sample	**Trapping event**	**Filename***	**spectra #**	**CH-band intensity**	**Rayleigh intensity**
1	1	2022-05-30@161248	350-354	27	937
	2	2022-05-30@161354	237-246	65	22403
	3	2022-05-30@161448	176-185	31	504
	4	2022-05-30@161646	135-142	29	314
	5	2022-05-30@161552	20-27	270	13388
2 (T004)	1	2022-06-08@165904	276-400	677	2343
	2	2022-06-08@165928	151-214	585	1448
	3	2022-06-08@165928	218-400	623	3780
	4	2022-06-08@165950	207-400	518	3224
	5	2022-06-08@170012	209-400	617	3625
3 (T001A)	1	2022-06-13@145620	169-190	266	23727
	2	2022-06-13@145727	4-9	44	1940
	3	2022-06-13@145749	135-143	42	694
	4	2022-06-13@150425	281-289	215	21235
	5	2022-06-13@145705	323-328	272	8562
4 (T002B)	1	2022-06-13@154349	283-304	276	10793
	2	2022-06-13@154814	38-49	190	18114
	3	2022-06-13@155512	398-400	187	8491
	4	2022-06-13@155238	9-15	88	1917
	5	2022-06-13@155428	267-271	63	645
5 (T003C)	1	2022-06-14@153747	7-9	99	7527
	2	2022-06-14@153747	30-35	86	3820
	3	2022-06-14@153959	236-245	166	6605
	4	2022-06-14@154127	334-356	341	83867
	5	2022-06-14@153451	23-29	300	29917

4.1.1 Correlation between the CH-band and Rayleigh intensity

In this paragraph the correlation between the CH-band and the Rayleigh intensity are displayed. The given figures, have the Rayleigh intensity on the y-axis and the CH-band intensity on the x-axis. Figure 9 is a graph with the collected data points for the pure milk (N) and figure 10 is a graph with the collected data points for the skim milk (V). In the graphs a fitted line is plotted.

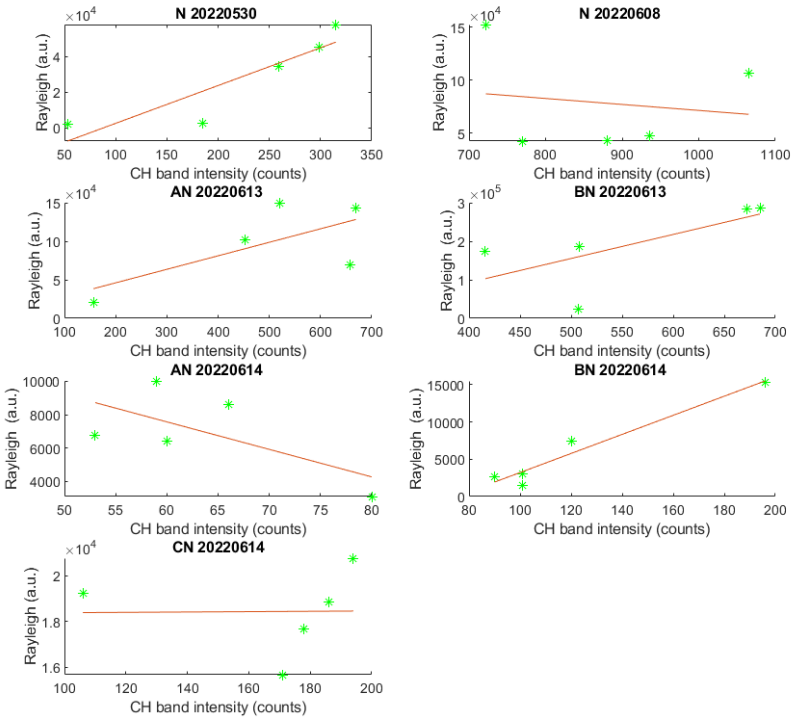


Figure 9: The correlation between CH-band and Rayleigh signal for the N-samples. Only sample 2 (graph N 20220608)and sample T001A (graph AN 20220614) displays a negative correlation.

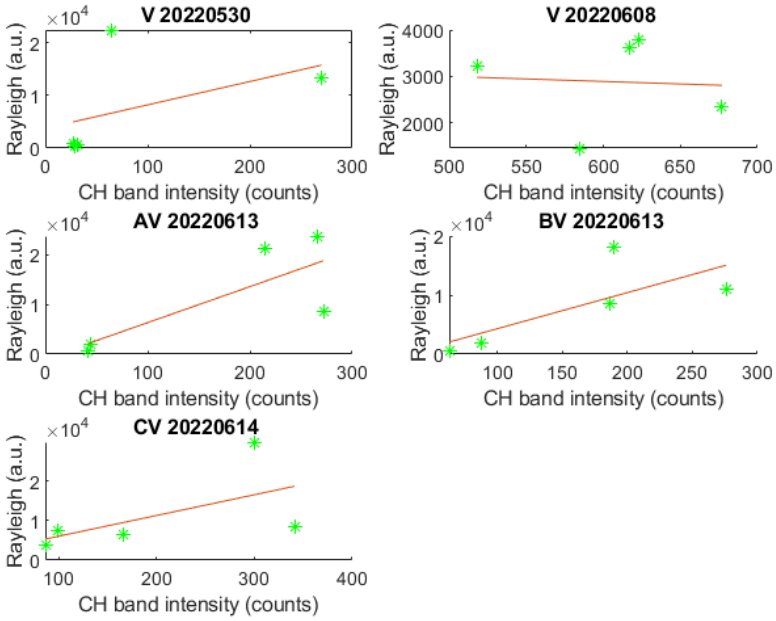


Figure 10: The correlation between CH-band and Rayleigh signal for the V-samples. All samples, except sample 2 (graph 'V 20220608'), show a positive correlation.

For the pure milk, five out of seven graph show an increase, while there are two graphs with a decrease, sample T001A (N) (graph 'AN 20220614') and sample 2 (graph 'N 20220608'). For the skim milk, one of five graphs shows a decline, sample 2 (graph 'V 20220608'), while the other graphs show an incline.

All the previous points from figures 9 and 10 have been collected and added into one graph, figure 11. There is a line fitted through the data points, with an increase.

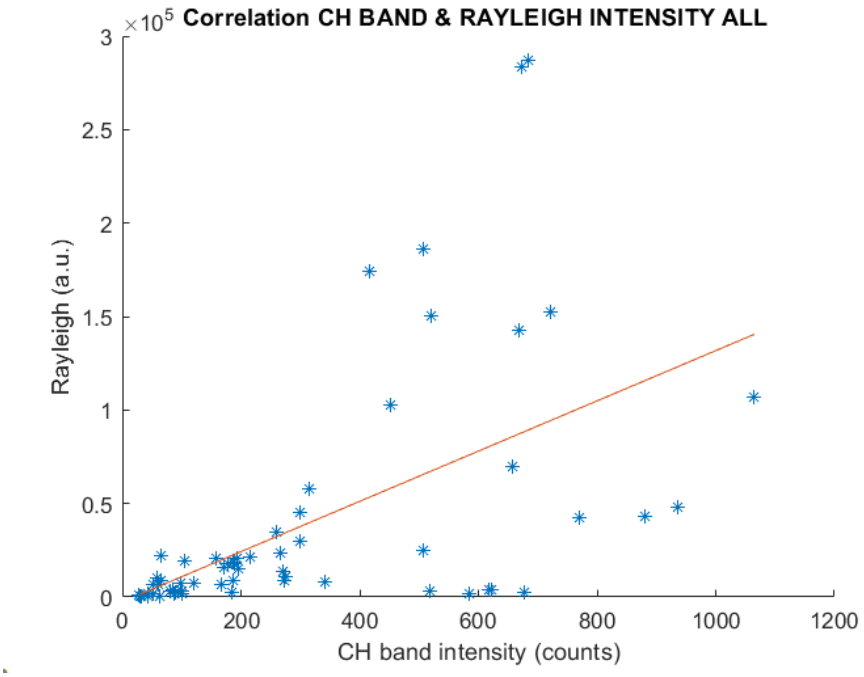
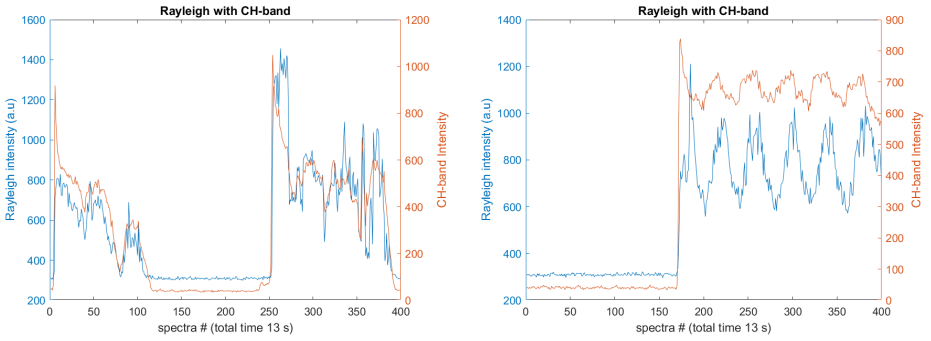


Figure 11: The correlation between CH-band and Rayleigh signal for all collected data points.

4.2 Oscillations

Samples T001A pure milk and T002B pure milk were measured on 13-06-2022 at a depth of $120\mu m$. These measurements show the intensity per spectra over time. There are oscillations visible in figure 12 for T001A pure milk and figure 13 for T002B pure milk.



(a) (b)

Figure 12: The Rayleigh intensity on the left y-axis in comparison CH-band on right axis over the spectra of one measurement. Both figures show an oscillation in the trapping event for sample AN . Both measurements were on 13-06-2022 (a) Measurement at 13:46:57 (b) Measurement at 13:47:42

Figure 12 shows that the Ch-band intensity is oscillating with the Rayleigh intensity.

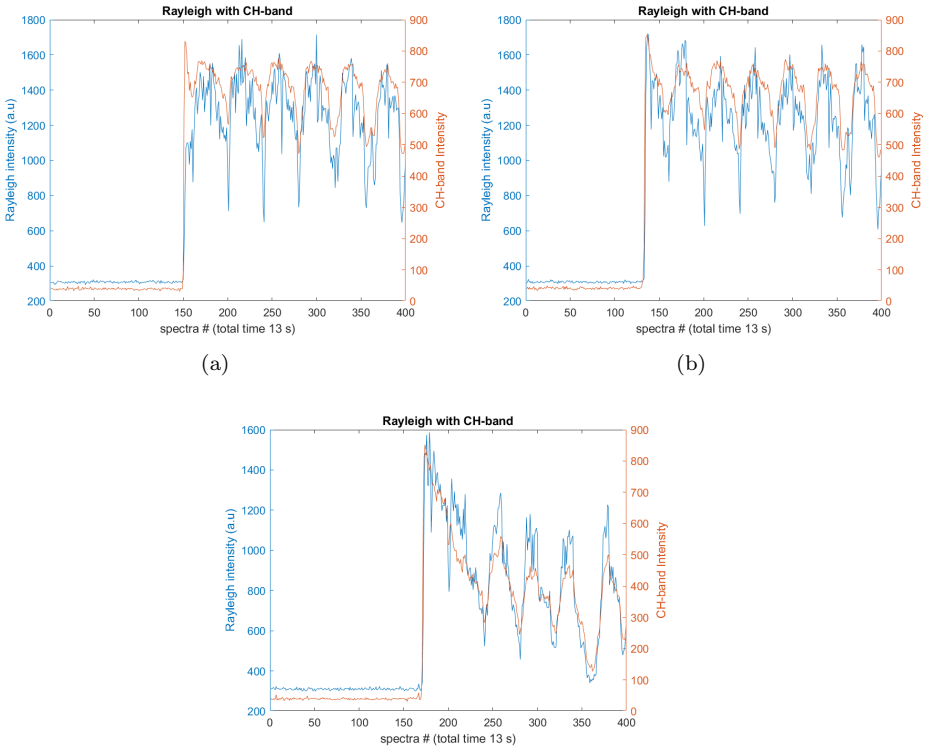


Figure 13: The Rayleigh intensity on the left y-axis in comparison CH-band on right axis over the spectra of one measurement. All the figures show oscillation in the trapping event for sample BN. The measurements were on 13-06-2022 (a) Measurement at 14:35:04 (b) Measurement at 14:35:26 (c) Measurement at 14:35:48

The CH-band of sample T002B pure milk is oscillating with the Rayleigh intensity, figure 13.
The oscillations were visible in these specific samples, due to the measuring depth. The particles were close to the cover glass.

4.3 Types of particles

In this paragraph all the figures of trapping events are displayed per measurement. The figures show the first 5 trapping events. The figures on the left show the fingerprint region, the figures on the right show the full Raman spectrum.
The Rayleigh signal of the measurements is included in Appendix G to show the trapping event duration.

4.3.1 Measurement 1

Measurement 1 was measured at 80 μm in the well with sample 1. The results are shown in figure 14.

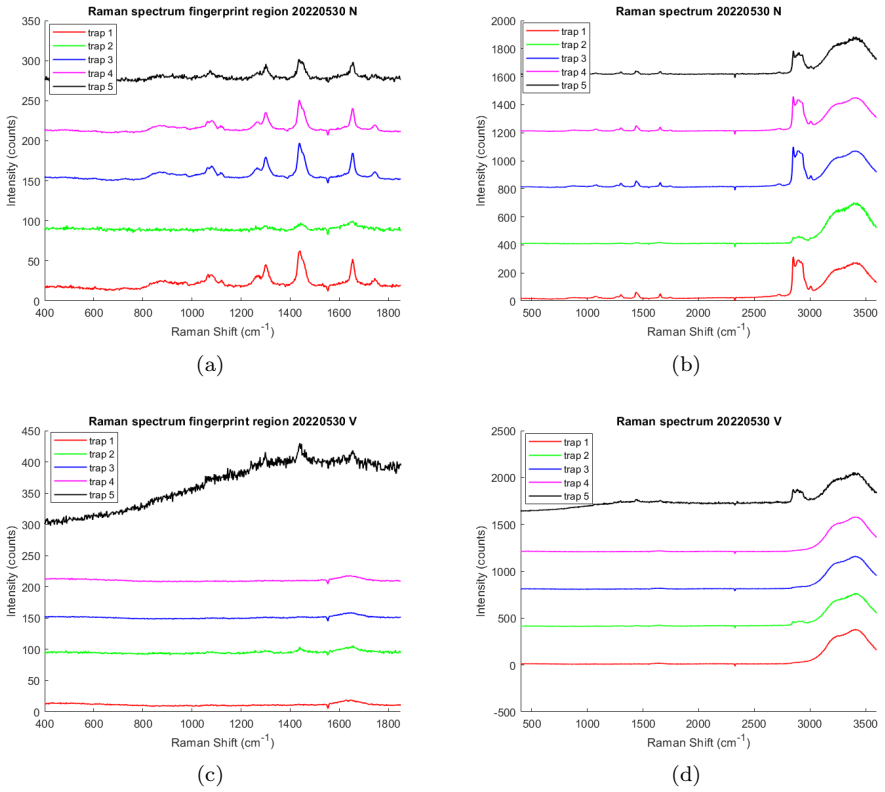


Figure 14: The measured spectra of measurement 1 pure milk: (a) Fingerprint Region (b) Complete spectrum. The measured spectra of measurement 1 skim milk: (c) Fingerprint Region (d) Complete spectrum

Measurement 1N (figure 14(a) and 14(b)): trap1, trap3, trap 4, trap5 are similar in fingerprint peaks. Trap2 seems to have less notable fingerprint peaks. Trap 1, 3 to 5 are fat particles (TAG, triolein (TOA)) and trap 2 is unclear. Measurement 1V (figure 14(c) and 14(d)): Trap 5 has an increase in intensity over the fingerprint region, this makes it a fluorescent particle. Trap 1 has barely trapped anything. Trap 1, 2, 4 and 5 have a small peak at the 2800 band, which indicates the presence of CH binding. Further, trap 2 has a small peak around 1400, this indicates vibrations in the CH_2 -groups. Trap 1 to 4 are unclear.

4.3.2 Measurement 2

Sample 2 (T004) was measured at 80μm in the well. The results are presented in figure 15.

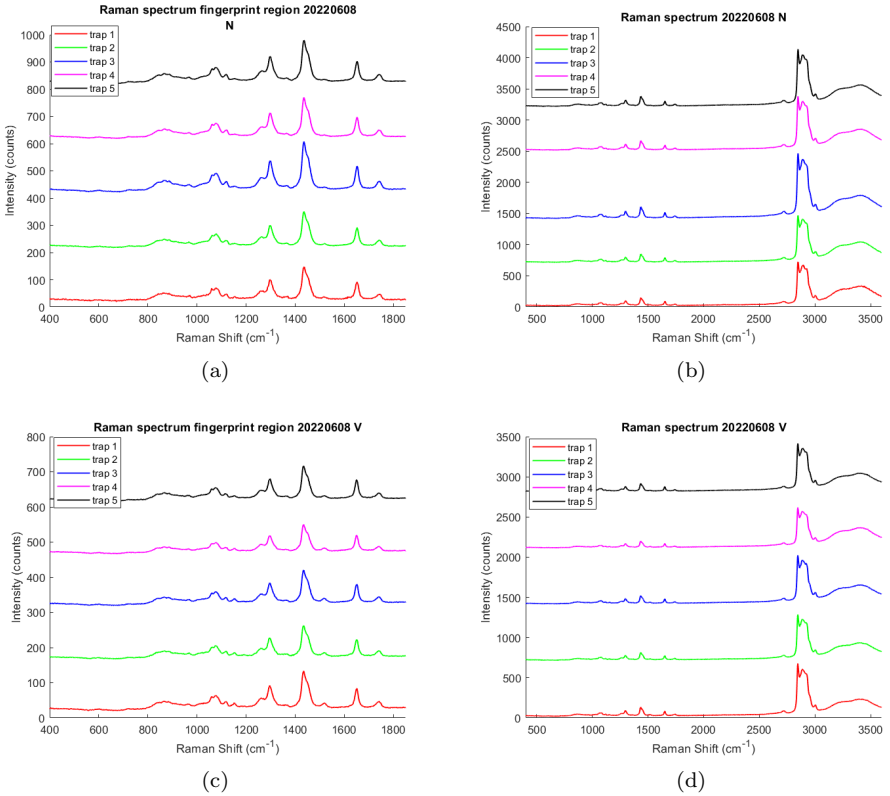


Figure 15: The measured spectra of measurement 2 pure milk: (a) Fingerprint Region (b) Complete spectrum. The measured spectra of measurement 2 skim milk: (c) Fingerprint Region (d) Complete spectrum

Measurement 2N (figure 15(a) and 15(b)): Trap 1 to trap 5 appear to have the same fingerprint regions present, around 900, 1100, 1300, 1500, 1660. These 5 traps all have a higher intensity at 2800, which indicates that CH binding is present. Trap 3 appears to have a higher intensity CH-band than trap 1 and 2. The particles trapped are Fat (TAG, triolein (TOA)).
Measurement 2V (figure 15(c) and 15(d)): Trap 1 to 5 appear to have the same fingerprint regions present, around 900, 1100, 1300, 1500, 1550, 1660, 1760. These 5 traps all have a higher intensity at 2800, which indicates that CH binding is present. The particles trapped are fat particles (TAG, tripalmitolein (TPO)).

4.3.3 Measurement 3

Samples T001A (N) and T002B (N) were measured at 120μm below the cover glass. Samples T001A (V) and T002B (V) were measured at a depth of 80μm in the well. The results are presented in figures 16 and 17, respectively.

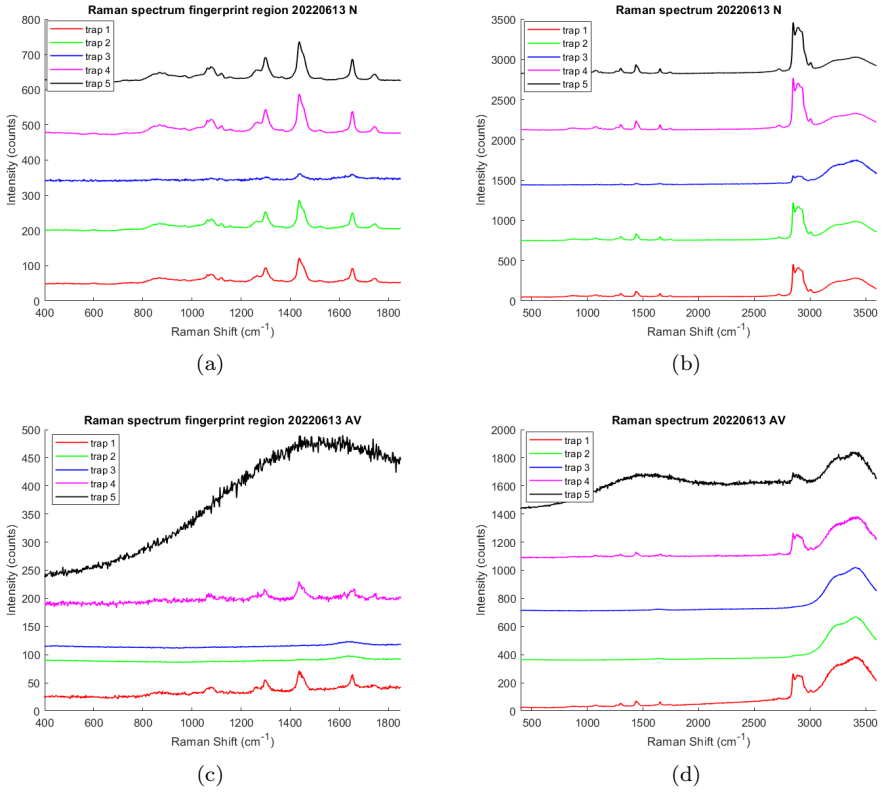


Figure 16: The measured spectra of measurement 3 (T001A) pure milk: (a) Fingerprint Region (b) Complete spectrum. The measured spectra of measurement 3 (T001A) skim milk: (c) Fingerprint Region (d) Complete spectrum

Measurement 3 T001A pure milk (figure 16(a) and 16(b)): trap 1, trap 2 appear to be similar, they have a higher intensity at 850, 1100, 1200-1300, 1440 and 1650, 1700. Trap 4 and trap 5 also appear to be similar, they have a higher intensity at 850, 1100, 1200-1300, 1440 and 1650, 1700. Trap 4 and 5 appear to have a higher intensity than trap 1 and 2. Trap 3 appears to have some higher intensities around 1300, 1500 and 1660. Trap 4 and 5 also have a higher intensity at 2800 than trap 1 to 3. Trap 1 and 2 have a higher intensity than trap 3. The trapped particles are fat particles (TAG, triolein (TOA)). Measurement 3 T001A skim milk (figure 16(c) and 16(d)): Trap 1 has a higher intensity in the fingerprint region around 880, 1100, 1300, 1440, 1650. Trap 1

also has a higher intensity around 2800. This particle is a fat particle (TAG, tripalmitolein (TPO)). Trap2 and 3 do not appear to have higher intensity peaks in the fingerprint region except for around 1630, which indicates the presence of water. Further, trap 2 and 3 have a low increase in intensity around 2800. Which means the trapped particle is unclear. Trap 4 has a higher intensity at 880, 1300, 1440, 1650 and it has a higher intensity around 2800. This particle is a fat particle (TAG, tripalmitolein (TPO)). Trap 5 has a high intensity from 900 to 2000 and a higher intensity around 2800 which indicates the CH-band. Trap 5 are fluorescent particles.

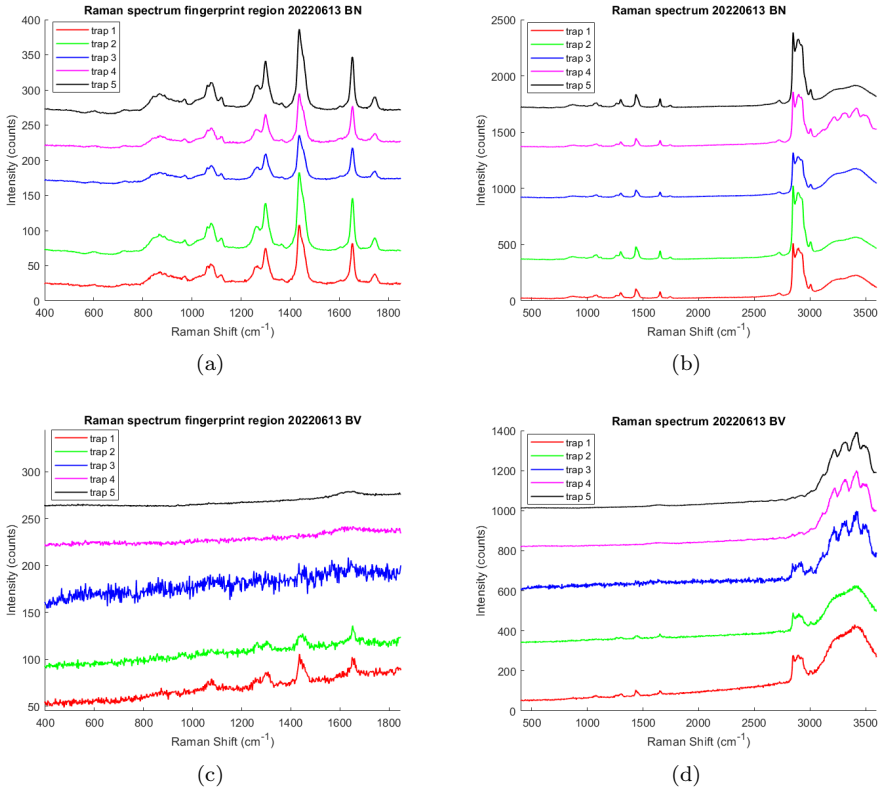


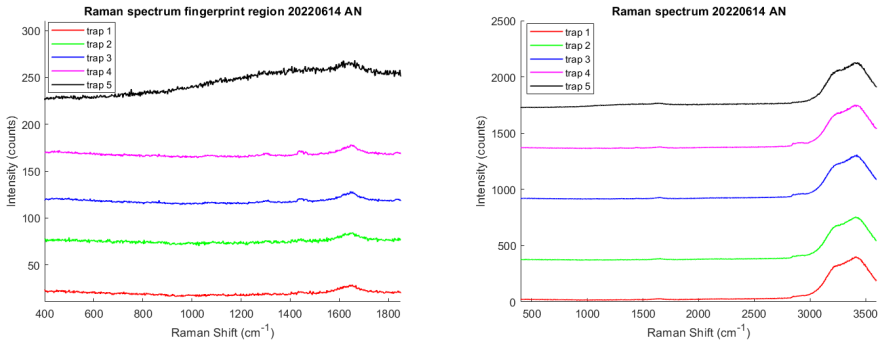
Figure 17: The measured spectra of measurement 3 (T002B) pure milk: (a) Fingerprint Region (b) Complete spectrum. The measured spectra of measurement 3 (T002B) skim milk: (c) Fingerprint Region (d) Complete spectrum

Measurement 3 T002B pure milk (figure 17(a) and 17(b)): Trap 1 to 5 look similar and have a high intensity in the fingerprint region at 800-900, a double peak at 1100, a double peak at 1240-1300, 1440, 1640 and 1760. It also shows a high intensity around 2800, with trap 5 and 2 having a higher intensity than trap 1, 3 and 4.It also appears that the waterband (3000-3600) of trap 4 is

spiky. The trapped particles are fat particles (TAG, triolein (TOA)). Measurement 3 T002B skim milk (figure 17(c) and 17(d)): Trap 1 and 2 appear to be similar, trap 1 has a higher intensity than trap 2. There are peaks visible at 1050, 1230-1300, 1440 and 1640. Trap 3 is too noisy to distinguish peaks from the noise. Trap 4 and 5 do not appear to have peaks in the fingerprint region. Trap 1 and 2 also have a peak at 2800 which appears to be the CH-band. Trap1 and 2 are fat globules (fatty acid, Elaidic acid (OA)). Trap 3 also has a higher intensity at the 2800 and it has a spiky waterband. Tap 3 contains an unclear particle. Trap 4 and 5 have a low intensity at 2800. Trap 4 and 5 are unclear particles.

4.3.4 Measurement 4

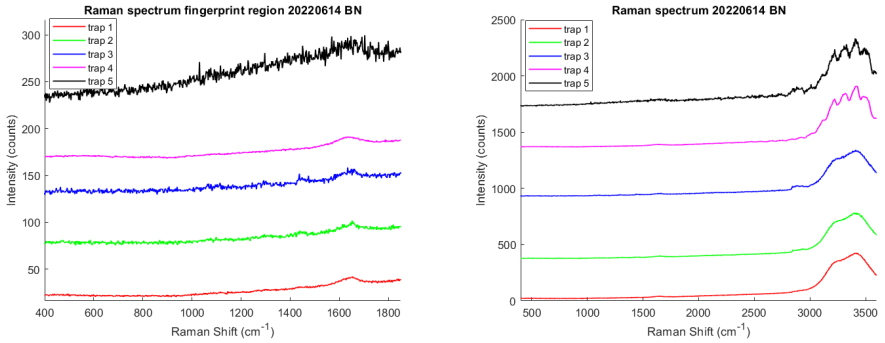
For these measurements the samples T001A pure milk and T002B pure milk of measurement 3 have been at room temperature for a day. They were measured at 80 μm in the well in measurement 4. Sample T003C is measured at a depth of 80μm in the well. The results of Sample T001A (N) and T002B (N) are presented in figure 18 and 19, respectively. The results of sample T003C are presented in figure 20.



(a) (b)

Figure 18: The measured spectra of measurement (T001A) pure milk, one day outside the fridge: (a) Fingerprint Region (b) Complete spectrum

Measurement 4 T001A pure milk (figure 18): Trap 5 is noisy and has a high intensity for most of the fingerprint region, it also has a low increase in intensity at 2990 (CH-band), which means this is a fluorescent particle. Traps 3 and 4 have the same peaks present in the fingerprint region, 1300, 1400, 1640. They both have a low increase in intensity at 2800 which indicates the presence of CH binding. These trapped particles are unidentified. Trap 1 and 2 do not have a significant increase except for around 1640, both signals appear to have some noise. Furthermore they both have a small increase in intensity around 2900. These particles are unclear.



(a) (b)

Figure 19: The measured spectra of measurement 4 (T002B) pure milk, one day outside the fridge: (a) Fingerprint Region (b) Complete spectrum

Measurement 4 T002B pure milk (figure 19): Trap 3 and 5 are noisy. Traps 1, 2 and 4 appear to only have a higher intensity around 1640 which is part of the waterband. Trap 5 has a higher intensity over the whole fingerprint region, it also has a higher intensity around 2800, it appears to be a fluorescent particle. Traps 1 to 4 have a small increase in intensity around 2800, with trap 3 having a higher intensity than trap 1, 2 and 4. These particles are unidentified.

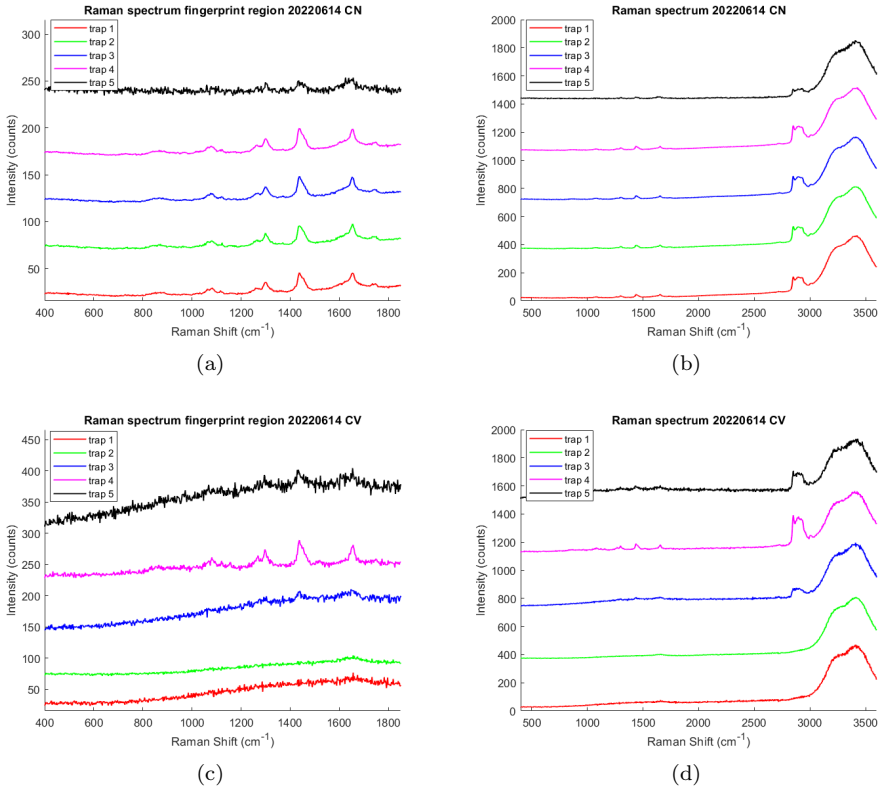


Figure 20: The measured spectra of measurement 4 (T003C) pure milk: (a) Fingerprint Region (b) Complete spectrum. The measured spectra of measurement 4 (T003C) skim milk: (c) Fingerprint Region (d) Complete spectrum

Measurement 4 T003C pure milk(figure 20(a) and 20(b)): Trap 1 to 4 are similar in the fingerprint region. They have higher intensity at 900, 1100, 1300, 1460, 1680 and 1720. They all have a higher intensity at 2900 which is the CH band, the waterband is also visible at 3200-3600. Trap 5 is noisy and appears to have a higher intensity at 1300 and 1460. It also has a higher intensity at the CH-band. The trapped particles in trap 1 to 4 are fat globules (TAG, triolein (TOA)), trap 5 is unclear.
Measurement 4 T003C skim milk (figure 20(c) and 20(d)): Traps 1, 3 and 5 are noisy and appear to have a higher intensity in the full fingerprint region. Trap 5 has a higher intensity than trap 3 and 1 at 2900. Trap 2 has a small increase in intensity at 1640 and 2900. Trap 4 has an increase in intensity at 1100, 1300, 1460, 1550 and 1700. It also has an increase at 2900. Trap 1, 3 and 5 are fluorescent particles. Trap 2 is unclear. Trap 4 is fat globule (TAG, triolein (TOA)).

4.4 Particle size

The intensity of the CH-band is a good indicator for the particle size, where a higher CH-band intensity corresponds to a larger particle. These intensities can be found in tables 3 and 4. In table 5, the biggest particle and smallest particle per sample is displayed. For particle size the degree of saturation is discussed. There will be looked at the $\delta(1260)/\rho(1300)$ and $v(1655)/\delta(1440)$, these are also included in the table. A high value of the ratio reveals a high degree of unsaturation.

Table 5: Overview of the highest and lowest intensity of the Ch-band measured of skim milk (*all filenames start with Image)

Sample	**Trapping event**	**Filename***	**CH-band intensity**	**Pure milk (N) or Skim milk (V)**	**δ(1260) /ρ(1300)**	**v(1655) /δ(1440)**
1	2	2022-05-30@144139	53	N	1.01	1.04
	1	2022-05-30@145034	315	N	0.59	0.59
1	1	2022-05-30@161248	27	V	1.1	1.6
	5	2022-05-30@161552	270	V	0.92	0.89
2 (T004)	2	2022-06-08@140447	770	N	0.57	0.55
	3	2022-06-08@140447	1065	N	0.52	0.53
2 (T004)	4	2022-06-08@165950	518	V	0.68	0.74
	1	2022-06-08@165904	677	V	0.67	0.55
3 (T001A)	3	2022-06-13@134720	157	N	0.75	0.9
	4	2022-06-13@134742	669	N	0.59	0.61
3 (T001A)	3	2022-06-13@145749	42	V	1	1.4
	5	2022-06-13@145705	272	V	0.95	1.02
3 (T001A)	1	2022-06-14@140214	53	N	1	1.33
	5	2022-06-14@140045	80	N	0.96	1.15
4 (T002B)	3	2022-06-13@143548	416	N	0.64	0.82
	5	2022-06-13@143504	685	N	0.61	0.73
4 (T002B)	5	2022-06-13@155428	63	V	0.94	1.38
	1	2022-06-13@154349	276	V	1.06	0.92
4 (T002B)	1	2022-06-14@142459	90	N	0.93	1.3
	5	2022-06-14@142839	196	N	1.04	1.18
5 (T003C)	5	2022-06-14@145248	106	N	0.88	1.03
	4	2022-06-14@144908	194	N	0.82	1
5 (T003C)	2	2022-06-14@153747	86	V	0.98	1.3
	4	2022-06-14@154127	341	V	0.77	0.96

4.4.1 Sample 1

Sample 1 (N) shows that trap 2 has the lowest CH-band intensity and trap 1 has the highest CH-band intensity. It is clear that trap 1 is triolein (TOA), it has both ratios lower than trap 2. This means that trap 1 is more saturated than trap 2. Trap 2 is an unclear particle of which has low intensity in the fingerprint region.
Sample 1 (V) shows trap 1 has the lowest CH-band intensity and trap 5 the highest CH-band intensity. Trap 1 has a low intensity in the fingerprint region and a small increase in the CH-band intensity. Trap 5 is a fluorescent particle with a higher intensity of the CH-band. Trap 1 has both ratios higher than trap 5, which means that trap 5 is more saturated than trap 1.

4.4.2 Sample 2

Sample 2 (N) (T004) shows that trap 2 has the lowest CH-band intensity and trap 3 has the highest CH-band intensity. Trap 2 and 3 are triolein (TOA), both traps have around the same ratios, trap 2 has a little higher ratio, which means that trap 2 is more unsaturated.
Sample 2 (V) (T004) shows that trap 4 has the lowest CH-band intensity and trap 1 has the highest CH-band intensity. Trap 1 and 4 are triolein (TOA). Trap 1 has a lower ratio for v(1655) /δ(1440) than trap 4, ratio δ(1260) /ρ(1300) is a little lower. Trap 1 is more saturated than trap 4.

4.4.3 Sample 3 (T001A)

Sample 3 (N) (T001A) 2022-06-13 shows that trap 3 has the lowest CH-band intensity and trap 4 has the highest CH-band intensity. Trap 3 and 4 are Triolein (TOA). trap 3 has higher ratios than trap 4, which means that trap 3 is less saturated than trap 4.
Sample 3 (V) (T001A) 2022-06-13 that trap 3 has the lowest CH-band intensity and trap 5 has the highest CH-band intensity. Trap 3 is unclear, there is water in the trap present. Trap 5 is a fluorescent particle with a higher intensity of the CH-band. Trap 3 is more saturated than trap 5.
Sample 3 (N) (T001A) 2022-06-14 that trap 1 has the lowest CH-band intensity and trap 5 has the highest CH-band intensity. The particle in trap 1 is unclear, there is water present. Trap 5 is a fluorescent particle with a higher intensity of the CH-band. Trap1 is less saturated than trap 5.

4.4.4 Sample 4 (T002B)

Sample 4 (N) (T002B) 2022-06-13 that trap 3 has the lowest CH-band intensity and trap 5 has the highest CH-band intensity. Trap 3 and 5 are triolein (TOA). Trap 5 has a higher intensity than trap 3. Trap 3 is less saturated than trap 5.
Sample 4 (V) (T002B) 2022-06-13 that trap 5 has the lowest CH-band intensity and trap 1 has the highest CH-band intensity. Trap 5 has no clear

intensity increase in the fingerprint region. Trap 1 is a Elaidic acid (OA). Trap 1 has a higher ratio $\delta(1260)$ /$\rho(1300)$, but a lower ratio v(1655) /$\delta(1440)$ than trap 5.
Sample 4 (N) (T002B) 2022-06-14 that trap 1 has the lowest CH-band intensity and trap 5 has the highest CH-band intensity. Trap 1 is unidentified. It has a small increase of the CH-band intensity. There is water present Trap 5 is a fluorescent particle with a higher intensity of the CH-band. Trap 5 has a higher ratio $\delta(1260)$ /$\rho(1300)$, but a lower ratio v(1655) /$\delta(1440)$ than trap 1.

4.4.5 Sample 5 (T003C)

Sample 5 (N) (T003C) that trap 5 has the lowest CH-band intensity and trap 4 has the highest CH-band intensity. Trap 5 is unclear. Trap 4 triolein (TOA). Trap 5 has both ratios higher than trap 4, this means that trap 5 is less saturated than trap 4.
Sample 5 (V) (T003C) that trap 2 has the lowest CH-band intensity and trap 4 has the highest CH-band intensity. Trap 2 is unclear, there is a small increase in CH-band intensity. Trap 4 is triolein (TOA). Both ratios of trap 2 are higher than trap 4, this means that trap 2 is less saturated than trap 4.

5 Discussion

This chapter contains a discussion of the results and suggestions for further research. First, the sample handling could be improved by keeping samples at 37°C up to right before measurement. At time of arrival, the samples had cooled down to around room temperature. The move from the warmth bath is approximately 5-10 minutes. Some samples depending on the time of measurement had been removed from the warmth bath for at least 10 minutes up to an hour.
Secondly, while the samples were underneath the microscope, some of the liquids vaporized, due to the power of the laser. The Vaporizing has an influence on the concentration of the sample, making it less diluted over time. The measurements took approximately 15 minutes. This is something that can be solved by gluing the cover glass to the microscope glass.
Thirdly, there were more measurements, but it was decided to only use the first 5 measurements with a trapping event. This means that the data provided in this thesis is a subset of all the measurements. Fourth, some particles were trapped for a short period of time, only a couple of spectra. In order to get the best results it is best if a particle is trapped for as long as possible, this will reduce the noise in the signal. With these noisy signals it is hard to distinguish higher intensities for the Raman bands. There were also incidents in which over time there were less trapping events per sample, this could come from the low measuring depth. The fat particles have a habit of floating. In a next study, a depth of closer to the cover glass could be beneficial, for example 40μm might be better. Lastly, there were no EVs and Casein Micelles trapped, this could be because the power of the laser was too low. In a future study, at least twice the power, 70 mW at the sample, would be recommended if there is an interest in these particles [6].

5.1 CH-band and Rayleigh signal

To determine the size of a particle in the trap, the CH-band intensity was used. In measuring the CH-band intensity only the highest point of the intensity was taken, this was at point 1322 of the the spectrometer and the matrix in MATLAB. For a better analysis the surface underneath the CH-band could give a better indication for the intensity of the CH-band, since the band increases in intensity at different Raman shifts depending on the particle. Also some Raman spectra contained noise, which makes it hard to find the right intensity. This can also be solved by analysing the surface underneath the CH-band.
The Rayleigh signal stated in tables 3 and 4, was calibrated. These Rayleigh signals are not good representatives of the actual Rayleigh signal. For a following study, it is advised to use the uncalibrated Rayleigh signal. The uncalibrated signal is not corrected and gives the Rayleigh intensity of a particle, the calibration reduces the Rayleigh signal.

5.1.1 Correlation

A positive correlation can be seen between CH-band intensity and Rayleigh signal, which would mean a bigger particle has a higher Rayleigh intensity and a higher intensity CH-band. This can be seen in V1, V3a V4b V5c, V2 has a negative correlation (figure 10). Further N1, N3A1, N4B1, N4B2 and N5C have a positive correlation (figure 9). N2 and N3A2 have a negative correlation. The positive correlation was expected, since a bigger particle would have more CH bonds present in the molecule. Since these individual samples only contain 5 measurements per sample, all of the measurements are shown in one graph, figure 11. In this figure it is clear that there is a positive correlation between the CH-band intensity and the Rayleigh intensity. Most of the particles close to the origin come from the data collected of the skim milk. The more spread data points are mostly data points of the pure milk sample. Figure 11 contains more data points, this makes it more reliable than all the separate figures with 5 data point. One abnormality in those 5 points can cause the fitted line to change the slope drastically.

5.2 Oscillations

While working on the Rayleigh signals of the trapping events, some interesting results came up. There were oscillations visible in the Rayleigh intensity for specific trapping events (120 μm T001A (N), T002B (N)). Figures 12 and 13 show a correlation between the Rayleigh intensity and the CH-band intensity. The CH-band oscillates with the Rayleigh signal. This happened because the measurements happened close to the surface of the cover glass, to which particles seemed to stick. It is thought that these molecules move inside the laser beam and move in and out of the laser beam focus. There is the possibility that the heat of the laser causes the particle to stretch in one direction than in the other direction. This would change the surface of the particle and cause different intensity of the Rayleigh signal.

5.3 Type of particles

For this study it was of importance to investigate human milk composition. This was done by studying the Raman spectra. The particles that were trapped are visible in the figures in paragraph 4.3. The trapped particles differed per sample. In the same figure multiple traps are plotted, within these figures some traps are similar. In figure 14 it is visible that the fifth trap is more noisy, this is due to less spectra over which the trapping event took place. All fluorescent particles show noise, more than the fat particles or the traps with less noticeable particles. For sample 5 T003C more of the Raman spectra are noisy, this is due to the low number of spectra during the trapping event. In the spectra 2 types of particles were clearly visible; Fluorescent and fat globules. Off the smaller traps, it is unclear what these particles are. These particles could have been EVs or Casein Micelles. Since the power of the laser

was 35 mW at the sample, it is advised to increase the power. Increasing the power will give EVs a better chance of being noticeable. The low power setting was chosen in order to not destroy fat globules.
In figure 19(a) trap 1 and 2 show a spiky waterband. This can also be seen in figure 17(b) and 17(d). For these specific spectra, something could have gone wrong with the calibration, which is unlikely, since the other measurements of day 2022-06-14 were calibrated using the same file. What is suspected is that the water had a different temperature. What truly happened is unclear.
The following particles were clearly visible in the trapping events. The Raman spectra were compared to the spectra in the literature.[25]
Volunteer 1 has triolein (TOA) in the pure milk. Volunteer 1 has fluorescent particle in the skim milk.
Volunteer 2 has triolein (TOA) in the pure milk. Volunteer 2 has tripalmitolein (TPO) in the skim milk.
Volunteer 3 (T001A) has triolein (TOA) in the pure milk. Volunteer 3 (T001A) has tripalmitolein (TPO) and a fluorescent particle in the skim milk.
Volunteer 4 (T002B) has triolein (TOA) in the pure milk. Volunteer 4 (T002B) has Elaidic acid (OA) in the skim milk.
Volunteer 5 (T003C) has triolein (TOA) in the pure milk. Volunteer 5 (T003C) has triolein (TOA) and fluorescent particles in the skim milk.
It is also not advised to use Milk that has been at room temperature for one day. The measurements of 2022-06-14 samples T001A(N) and T002B(N) show not enough increase in intensity to distinguish the particle type. There are many particles of which it is unclear what particle it is. These particles might have been out of focus or simply too small for this setup to properly measure them.

5.4 Particle size

From the experiment it is clear that the size of the particles in the milk between volunteers differs. It differs within a sample and between samples. Some ratios were above 1 in table 5, sample 1 trap 2 (N) and trap 1 (V), sample 3 trap 3 (V) and trap 1 (N one day old), sample 4 (V) and trap 1 and 5 (N one day old) and sample 5 (N) and trap 2 (V). Sample 1 (N) had a big difference between the high CH-band (315) and the lower CH-band (53) approximately 0.4 in ratio. While sample 2 (N) differed in CH-band intensity with 295 and ratio 0.05 and 0.02. Overall the ratios between the traps of the skim milk are close to each other. While the ratios of the pure milk seem to have more variation. The smaller particles, lower CH-band intensity, have higher ratios. The smaller particles are more unsaturated than the bigger particles.

6 Conclusion

The aim of this research is to firstly, find a correlation between the size of the CH-band and the Rayleigh signal of a trapped particle. Secondly, determine the different types of particles present in the human milk. Lastly, comparing the sizes of fat globules per mother. All using Rayleigh-Raman scattering. This lead to the following research questions. Is there a correlation between the size of the CH-band intensity and the Rayleigh signal of a trapped particle? Can the different types of particles in the human milk be determined? Can the sizes of the particles be compared by the CH-band intensity?
The results are in agreement with the hypotheses, there is a positive correlation between the CH-band intensity and the Rayleigh signal, following figure 11. It is partially possible to determine trapped particles. The identified particles are triolein, tripalmitolein, Elaidic acid and fluorescent particles. However there were many unidentified particles trapped. It was expected to also trap EVs and Casein Micelles, these were not identified. By measuring the CH-band intensity it is possible to determine bigger and smaller particles. However, it is not possible to determine the exact measurements of the particle using this method. Lastly, the CH-band intensity resonates with the Rayleigh intensity.
Concluding, it is possible to trap fat globules in human milk with this Raman setup. There is a positive correlation between the Rayleigh intensity and the CH-band intensity. It was also found that CH-band intensity resonates with the Rayleigh intensity. Furthermore, it is possible to determine fat globules and fluorescent particles with this setup. Lastly, the intensity of the CH-band gives an indication of the size of a trapped particle. Smaller particles are more unsaturated than bigger particles.

7 Acknowledgements

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A Approval ethical commission

Considering the use of human milk for this research, there had to be an evaluation performed by the ethical commission at the University of Twente. The approval of Mrs. De Wolf "Shedding light on human milk" also applies to this research.

Dear Mrs. De Wolf,

The Natural Sciences and Engineering Sciences Ethics committee has reviewed your submission for "Shedding light on human milk" and based on the submitted material has formulated a positive advice for the dean.

On the basis of this advice I approve your application and leave the responsible execution of this project in your hands trusting that you will conduct this research in a manner worthy of the University of Twente.

The request has been registered under **reference number 2021.118**

I wish you good luck with your research.

Yours sincerely,

Prof.dr. J.L. Herek
Dean faculty of Science and Technology
University of Twente

Figure 21: Approval granted by the Natural Sciences and Engineering Sciences Ethics committee for "Shedding light on human milk"

B Protocol Dilution

Goal: having a concentration of approximately 3,6 E8 -5 E10 particles/ mL of milk.
MATERIALS:

- 0.25 mL Milk
- Water bath set 37°C [6]
- 8 ml PBS
- Centrifuge
- 10 Eppendorf tubes

Protocol:

1. Let the milk desolidify and homogenize the milk sample by inverting a couple of times. Pipet the sample up and down until visually homogeneous (about 5 times).
2. Batch one (N) will not be centrifuged.
   Batch two (V), centrifuge sample for two sessions. Firstly, centrifuge the sample at 100G for 2 minutes at 20°C, remove the top skim layer and pipet the bottom volume into a different eppendorf tube. Then centrifuge this eppendorf tube at 1000G for 10 minutes at 20°C, take away the skim top layer and pipet the bottom volume into a different eppendorf tube, this is the final centrifuged milk sample.
3. Dilute each sample from batch N with Phosphate Buffered Saline (PBS) following table 6 and batch V following table 7. Batch V will be diluted shortly before measuring.
4. Warm the samples in a water bath at 37 °C for one hour.[6]
5. Fill the microscope well glass slide (BMS Microscope glass Art.nr. 12290 ISO 9001) with 50 µL of Batch N and batch V and cover with a cover glass (VWR 22mm Coverglass Cat-No. 631-0158). This is done by placing the cover glass 2/3 over the well before the milk is pipetted into the well.

Table 6: Dilution protocol for determining the accurate concentration 25x-100x.

Sample	Milk (ml)	PBS (ml)	Dilution	Total Volume (ml)
1	0.04	0.96	25x	1
2	0.02	0.98	50x	1
3	0.0133	0.9867	75x	1
4	0.01	0.99	100x	1

Table 7: Dilution protocol for determining the accurate concentration 10x-40x.

Sample	Milk (ml)	PBS (ml)	Dilution	Total Volume (ml)
1	0.1	0.99	10x	1
2	0.05	0.95	20x	1
3	0.0333	0.9667	30x	1
4	0.025	0.975	40x	1

C Details samples

In table 8 more details can be found relating to the donated milk.

Table 8: Details about the donated milk

Sample	Day of lactation	Time of lactation	Total volume	Age donor	Gender Child	Lactation period (months)
1	06-12-2021	06:00-07:00	60	30	V	15
2 (T004)	15-04-2021	11:00 - 12:00	45	34	M	4
3 (T001A)	11-11-2021	21:00-22:00	100	30	V	14
4 (T002B)	08-05-2021	09:00-10:00	40	24	M	3
5 (T003C)	03-10-2021	20:00-21:00	95	28	V	4

D Milk sample protocol

From the dilution (table 6 and 7) and test Rman are the following procedures final. MATERIALS:

- 0.35 mL Milk
- Water bath set 37°C [6]
- 11 ml PBS
- Centrifuge
- 41 Eppendorf tubes

Protocol:

1. Let the milk desolidify and homogenize the milk sample by inverting a couple of times. Pipet the sample up and down until visually homogeneous (about 5 times).
2. Batch one (N) will not be centrifuged.
   Batch two (V), centrifuge sample for two sessions. Firstly, centrifuge the sample at 100G for 2 minutes at 20°C, remove the top skim layer and pipet the bottom volume into a different eppendorf tube. Then centrifuge this eppendorf tube at 1000G for 10 minutes at 20°C, take away the skim top layer and pipet the bottom volume into a different eppendorf tube, this is the final centrifuged milk sample.
3. Dilute each sample from batch N with Phosphate Buffered Saline (PBS) following table 6 and batch V following table 7. Batch V will be diluted shortly before measuring.
4. Warm the samples in a water bath at 37 °C for one hour.[6]
5. Fill the microscope well glass slide (BMS Microscope glass Art.nr. 12290 ISO 9001) with 50 µL of Batch N and batch V and cover with a cover glass (VWR 22mm Coverglass Cat-No. 631-0158). This is done by placing the cover glass 2/3 over the well before the milk is pipetted into the well.

Resulting in the following samples in table 9

Table 9: Details about the used milk samples

Donor	Sample	Dilution	Date of sample preparation	Time of removal from freezer	In Warmth-bath	Removed from warmth-bath
1	N0	75	30-05-2022	9:00	11:52	13:43
	V0	20	30-05-2022	9:00	12:54	15:06
T004	N4	75	08-06-2022	9:00	11:40	13:00
	V4	20	08-06-2022	9:00	11:53	15:37
T001A	N1	75	13-06-2022	9:26	10:43	13:33
	V1	20	13-06-2022	9:26	10:54	14:20
T002B	N2	75	13-06-2022	9:26	10:43	13:33
	V2	20	13-06-2022	9:26	10:54	14:20
T003C	N3	75	14-06-2022	10:00	11:11	14:30
	V3	20	14-06-2022	10:00	11:50	15:17

E Raman Protocol

For the measurements the following settings are used: 34,870 ms, 400 points with a total time is 13 seconds. In the measurement time a particle is trapped and released by the shutter closing for 1 second in case of N0 V0 for the others the shutter time is increased to 5 seconds to make sure that a particle is released from the trap. The sample in table 9 correspond with the samples in table below. For more details about the measuring settings see table10

Table 10: Raman setup per sample

Sample	Exposure time (ms)	Shutter time	Measuring depth	Ramanlab Temperature	Number of spectra	Repeats
N0	34.87	1	80 μm	20.3-20.5°C	400	3x5
V0	34.87	1	80 μm	20.3-20.5°C	400	3x5
N4	34.87	5	80 μm	21°C	400	50
V4	34.87	5	120 μm	21°C	400	50
N1	34.87	5	80 μm	20-22°C	400	50
V1	34.87	5	80 μm	20-22°C	400	50
N2	34.87	5	80 μm	20-22°C	400	50
V2	34.87	5	80 μm	20-22°C	400	50
N3	34.87	5	80 μm	20.5-21°C	400	50
V3	34.87	5	80 μm	20.5-21°C	400	50

F Raman Bands

Group	Acronym	Region I					Region II		Region III		
		ν(=CH)	$\nu_{as}(=CH_3)$	$\nu_s(=CH_3)$	$\nu_{as}(=CH_2)$	$\nu_s(=CH_2)$	ν(C = O)	ν(C = C)	$\beta(CH_2/CH_3)$	$\alpha(CH_2/CH_3)$	β(CH₂)
Fatty acids	MA		2943	2909	2869	2832			1457	1433	1419
	PA		2967	2925	2881	2848			1467	1443	1426
	SA			2928	2882	2848			1466	1445	1409
	ARA			2922	2879	2845			1466	1442	1421
	POA	3005		2924	2895	2849		1655		1442	
	OA	3004		2920	2891	2852		1657		1444	
	EA			2921	2880	2844		1672	1464	1441	1420
	LA	3002		2929	2885	2845		1654		1438	
	ALA	3002		2923	2888	2848		1654		1437	
	AA	3003		2923	2886	2862		1653		1438	
Triacylglycerols	TCA			2937	2876		1744			1444	
	TCY				2875	2858	1745			1442	
	TCI		2965	2934	2883	2847	1743, 1729		1454	1443	
	TLU		2957	2935	2882	2848	1743, 1729		1459	1442	
	TMA		2957	2933	2882	2846	1742, 1729		1465	1443	
	TPA		2957	2934	2882	2846	1743, 1729		1466	1442	
	TSA		2957	2934	2881	2847	1742, 1727		1465	1443	
	TAR		2957	2933	2881	2847	1742, 1729		1467	1444	
	TBH		2959	2931	2881	2847	1742, 1727		1466	1444	
	TPO	3007			2896	2854	1746	1656		1444	
	TPE	3001		2933	2883	2848	1743, 1730	1655	1456	1444	
	TOA	3007			2896	2853	1749	1655		1440	
	TEA	3000		2934	2884	2847	1743, 1728	1672	1463	1438	
	TLA	3011			2898	2855	1745	1657		1440	
	TLN	3013		2933	2904	2856	1744	1656		1442	
	TEI	3007			2893	2852	1743	1656		1440	
	TER	3005	2958	2934	2883	2848	1738, 1727	1660	1462	1441	
Cholesterol and	CHL			2930		2864		1672		1442	
cholesterol ester	CPA		2957	2937	2881	2846	1739	1668	1464	1442	
	CSA				2885	2852	1741	1671	1468	1444	1428
	COA	3009				2866, 2846	1739	1670, 1659		1441	
	CLA	3013		2937		2865	1742	1662		1442	
Membrane lipids	PC	3007	2959	2925	2882	2847	1737	1657		1442	
	PE	3007	2959	2920	2882	2847	1737	1657		1442	
	SM		2959		2880	2847		1670, 1654		1437	

α, scissoring; β, bending; δ, deformation; τ, twisting; υ, stretching (s, symmetric; as, asymmetric).

Region IV		Region V				Region VI			Region VII				
$\tau(CH_2)$	δ(=CH)		ν(C — C)		ν(P — O)	β(CH)	(C — O — O) skel.	$\nu_{as}N^+(CH_3)_3$	Ethanolamine	$\nu_sN^+(CH_3)_3$	cholesterol ring def.	δ(C = O — C)	$\beta(CH_2)$ in ring
1294		1175	1125	1091, 1062		958	891					661, 674, 570	
1300		1179	1132	1103, 1067		1034, 980, 912	898					674	
1300		1178	1133	1105, 1067		984, 915	896						
1298		1176	1132	1111, 1066		1029, 975 912	898					671, 574	
1305	1265		1118	1086		975	894						
1306	1265		1120	1084		976	866						
1303	1287	1166	1124	1098, 1065		909							
1300	1262		1107	1087, 1075		972, 904	872						
1300	1263			1086, 1068		971	867						
1296	1260	1159	1111	1064		974, 935							
1306		1116		1064			847						
1305		1118	1077	1065			867						
1299		1125	1075	1063			891						
1297		1127	1085	1063			892						
1297		1129	1094	1062			891						
1297		1130	1100	1062			890						
1297		1130	1106	1061			891						
1297		1131	1109	1061			890						
1296		1131	1113	1061			890						
1304	1266	1114	1079	1067			890						
1297	1267, 1252	1127	1075	1063			892						
1302	1268	1118	1082	1069			870						
1297	1263	1123	1098	1062			891						
1303	1266		1108	1077									
1301	1267		1090	1071			868						
1303	1275, 1254			1065			871						
1297	1276, 1249	1111		1062			891						
		1178	1130	1087							701		548, 424
1298			1131	1066							702		429
1301			1133	1068							702		430
1306	1261										704		429
1306	1263										703		431
1300	1267		1125	1089	1096			876		719			
1300	1267		1125	1085	1096				760				
1295			1129	1090, 1104	1096			882		723			

α, scissoring; β, bending; δ, deformation; τ, twisting; υ, stretching (s, symmetric; as, asymmetric).

Figure 22: An overview of all the Raman spectra with types of lipids. [25]

G Rayleigh spectra

All the Rayleigh figures are corresponding with their traps.

G.1 Measurement 1

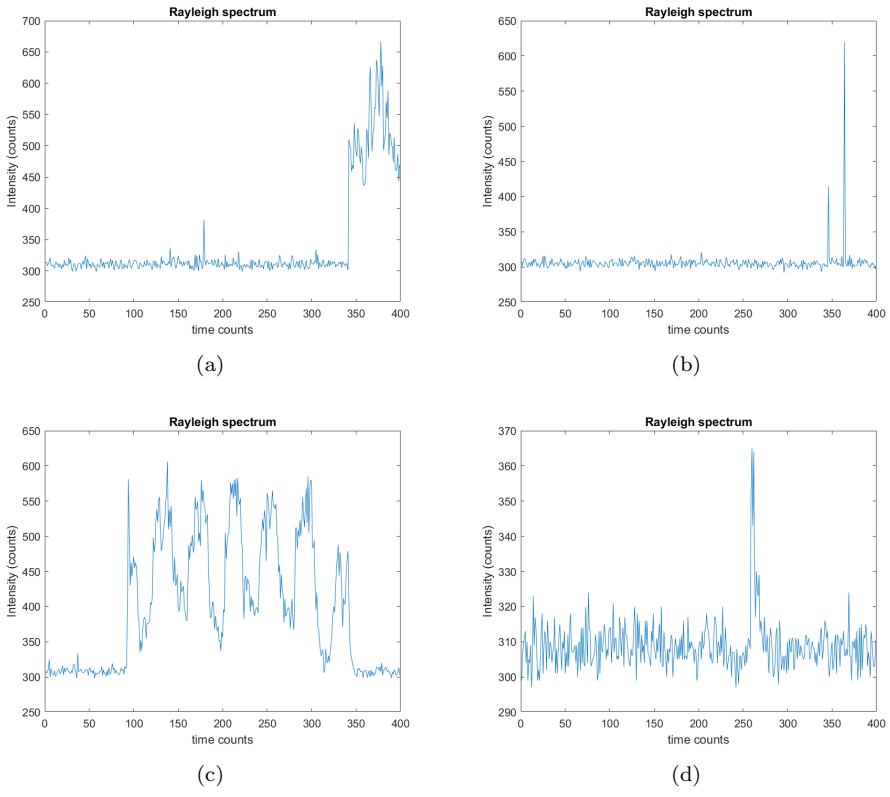


Figure 23: The measured Rayleigh signal on 20220530 pure milk (a) trap1 (b) trap2 (c) trap3 and 4 (d) trap5

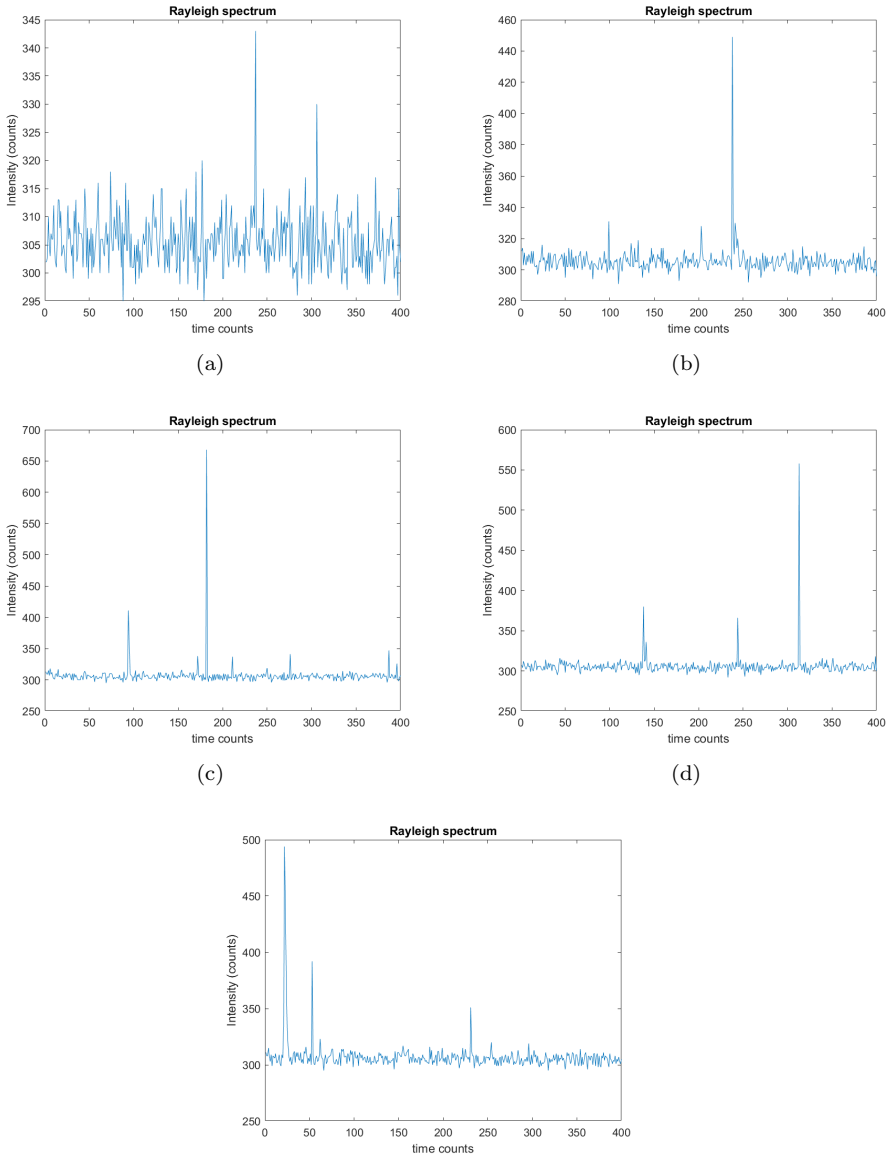


Figure 24: The measured Rayleigh signal on 20220530 Skim milk (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

G.2 Measurement 2

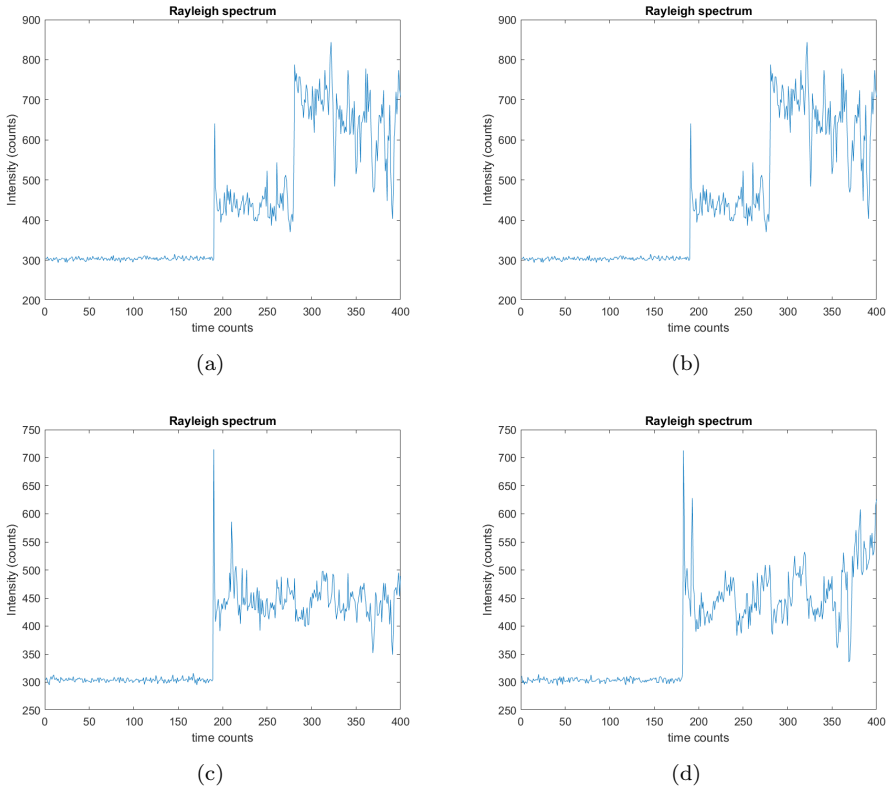


Figure 25: The measured Rayleigh signal on 20220608 pure milk (a) trap1 (b) trap2 and trap3 (c) trap4 (d) trap5

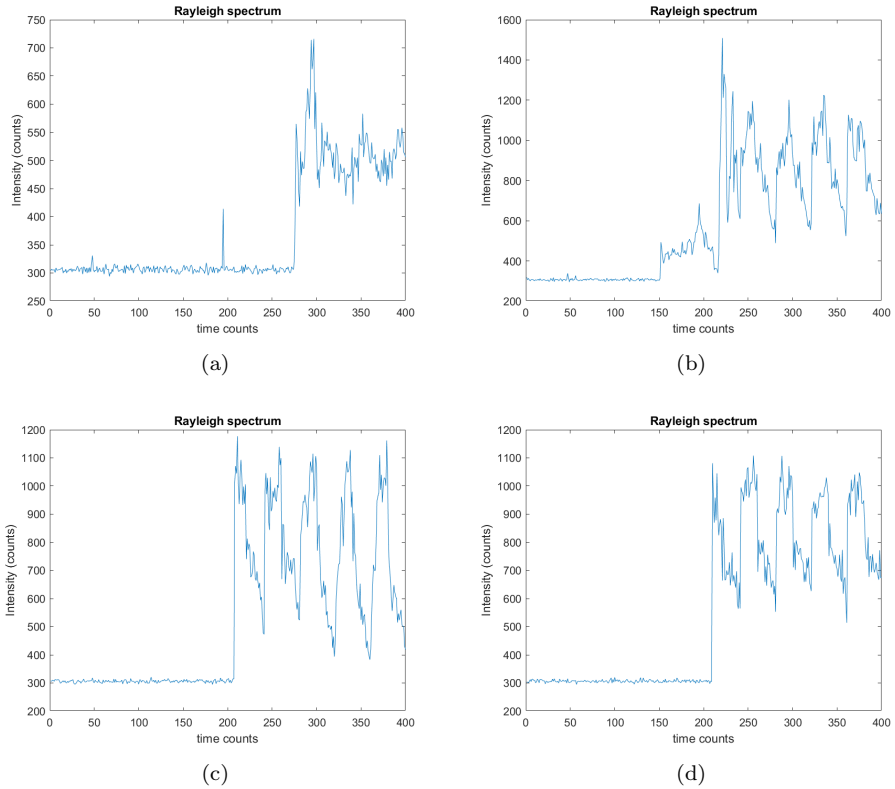


Figure 26: The measured Rayleigh signal on 20220608 skim milk (a) trap1 (b) trap2 trap3 (c) trap4 (d) trap5

G.3 Measurement 3

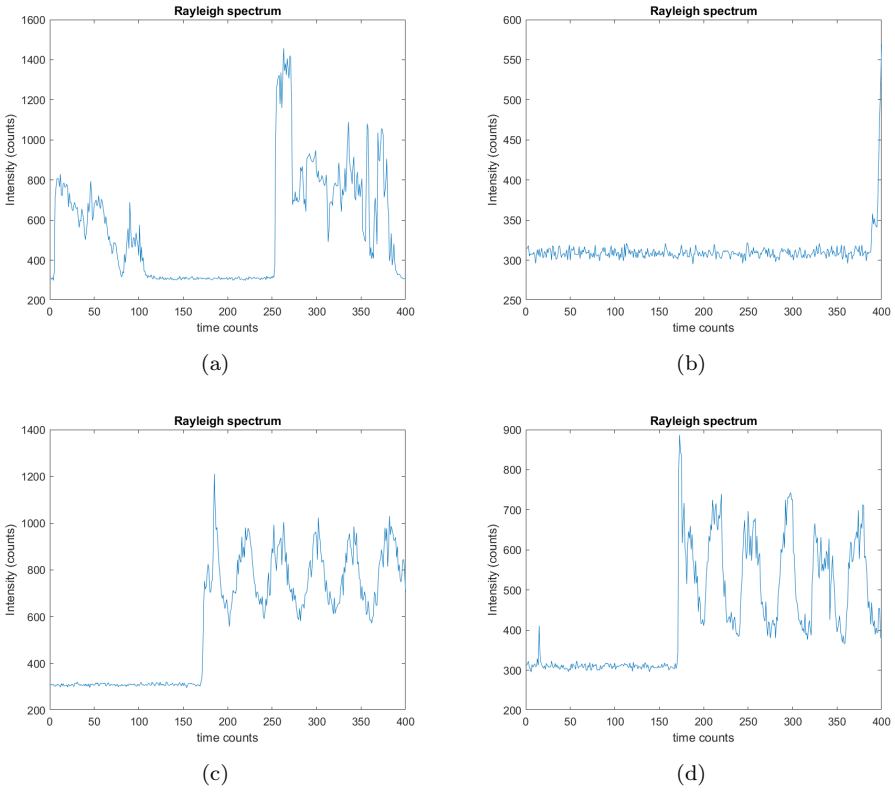


Figure 27: The measured Rayleigh signal on 20220613 purem milk sample 3(T001 A) (a) trap1 and trap2 (b) trap3 (c) trap4 (d) trap5

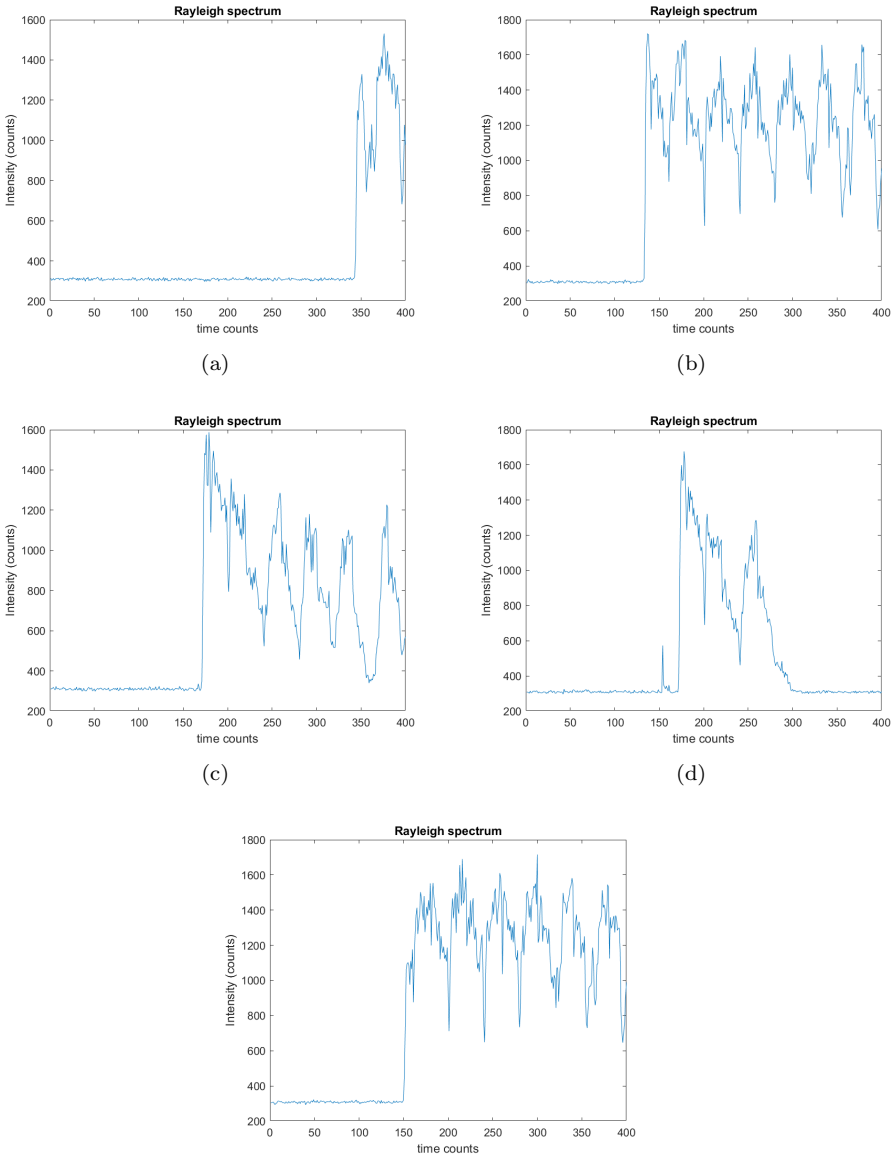
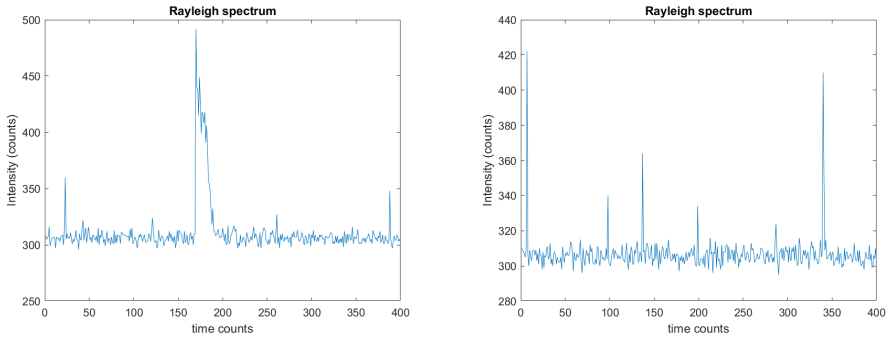
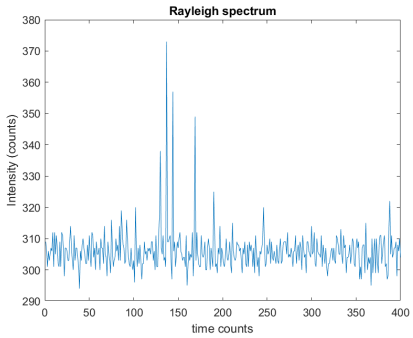


Figure 28: The measured Rayleigh signal on 20220613 pure milk sample 4 (T002B) (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

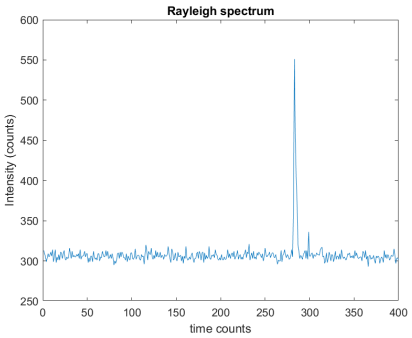


(a)

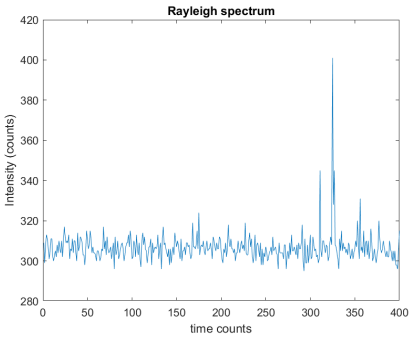
(b)



(c)



(d)



(e)

Figure 29: The measured Rayleigh signal on 20220613 skim milk sample 3 (T001A) (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

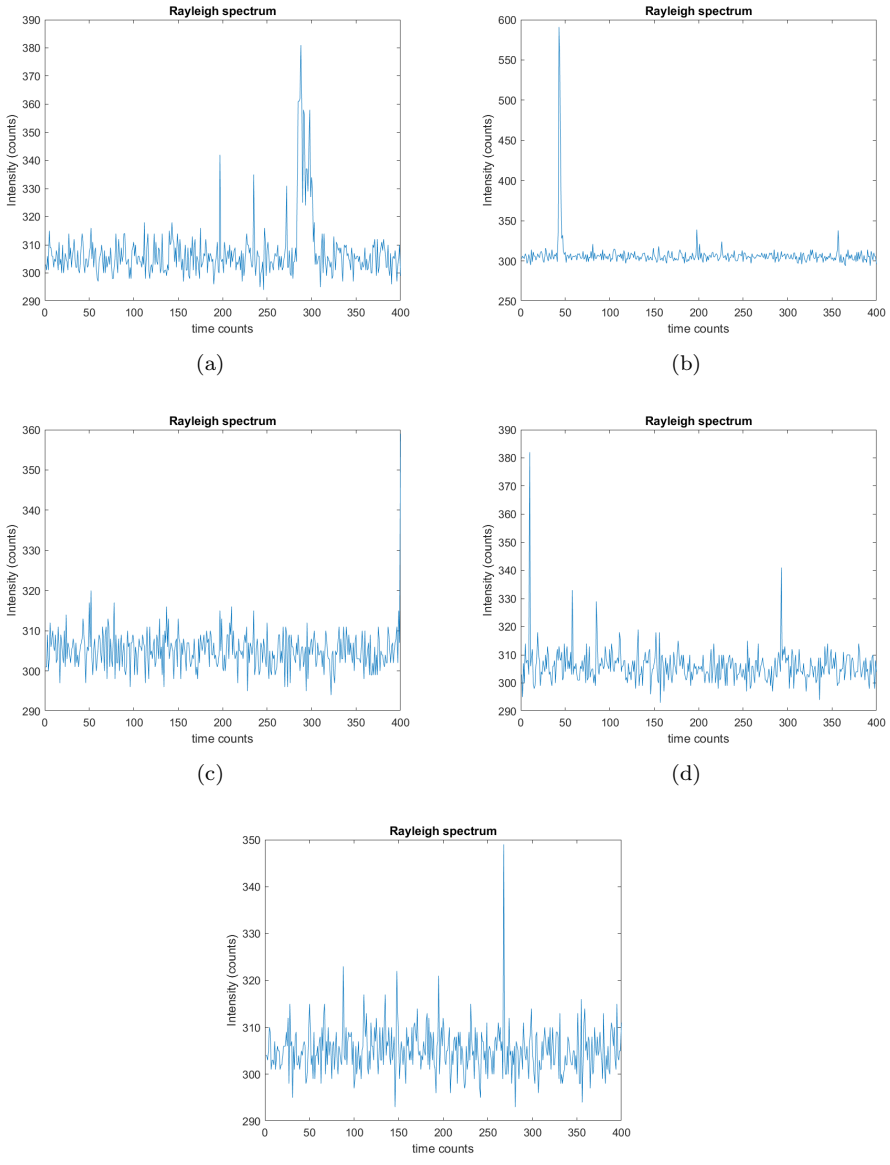


Figure 30: The measured Rayleigh signal on 20220613 skim milk sample 4 (T002B) (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

G.4 Measurement 4

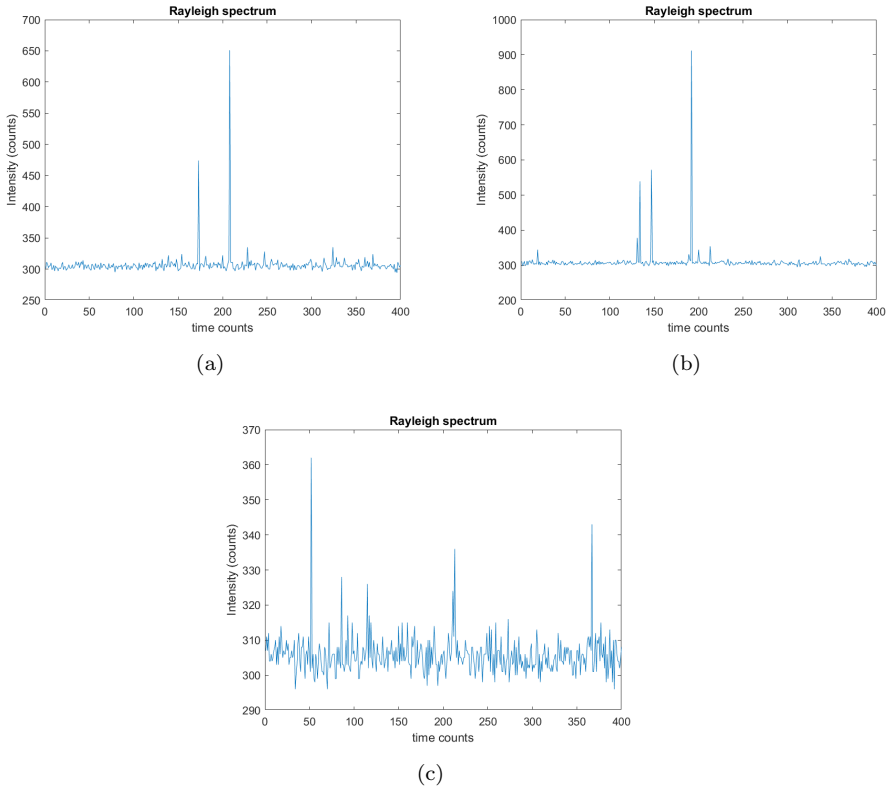


Figure 31: The measured Rayleigh signal on 20220614 pure milk sample 3 (T001A) one day old (a) trap1 and trap2 (b) trap3 and trap4 (c) trap5

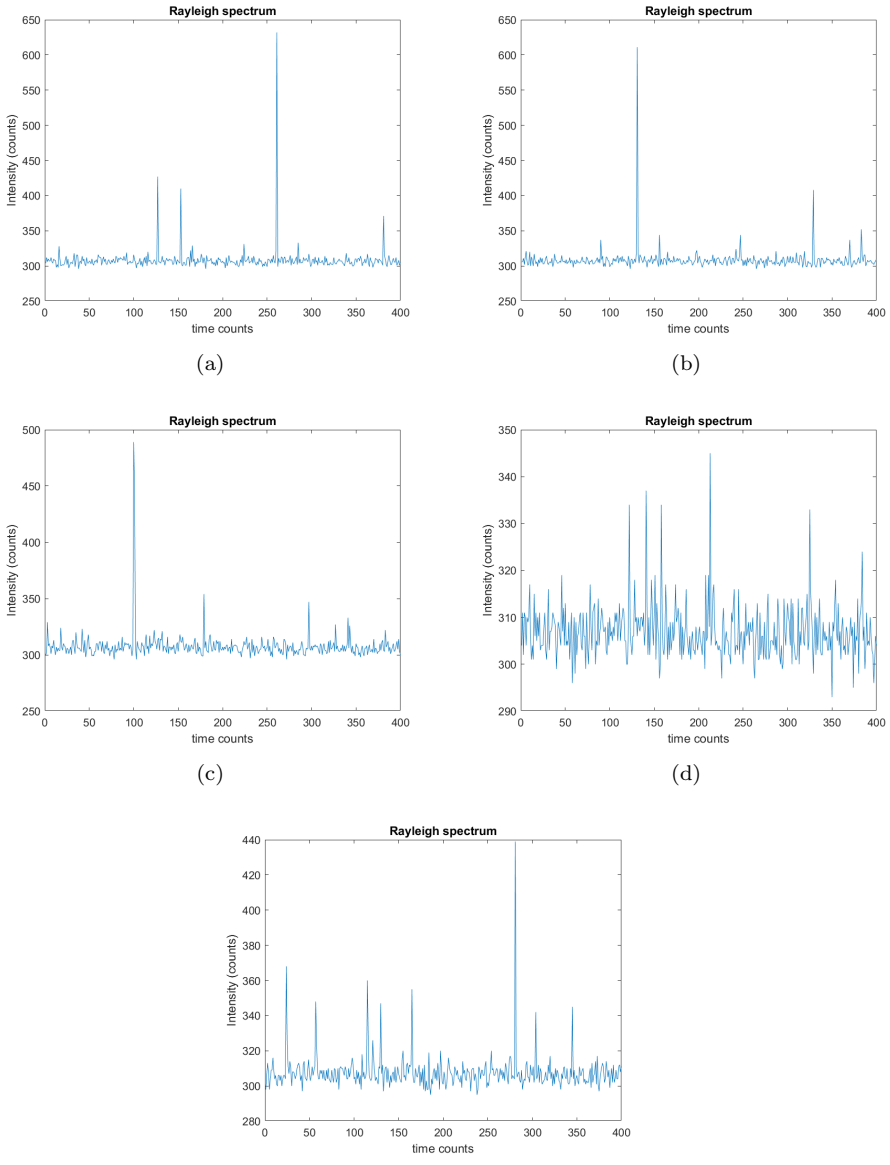


Figure 32: The measured Rayleigh signal on 20220614 pure milk sample 4 (T002B) one day old (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

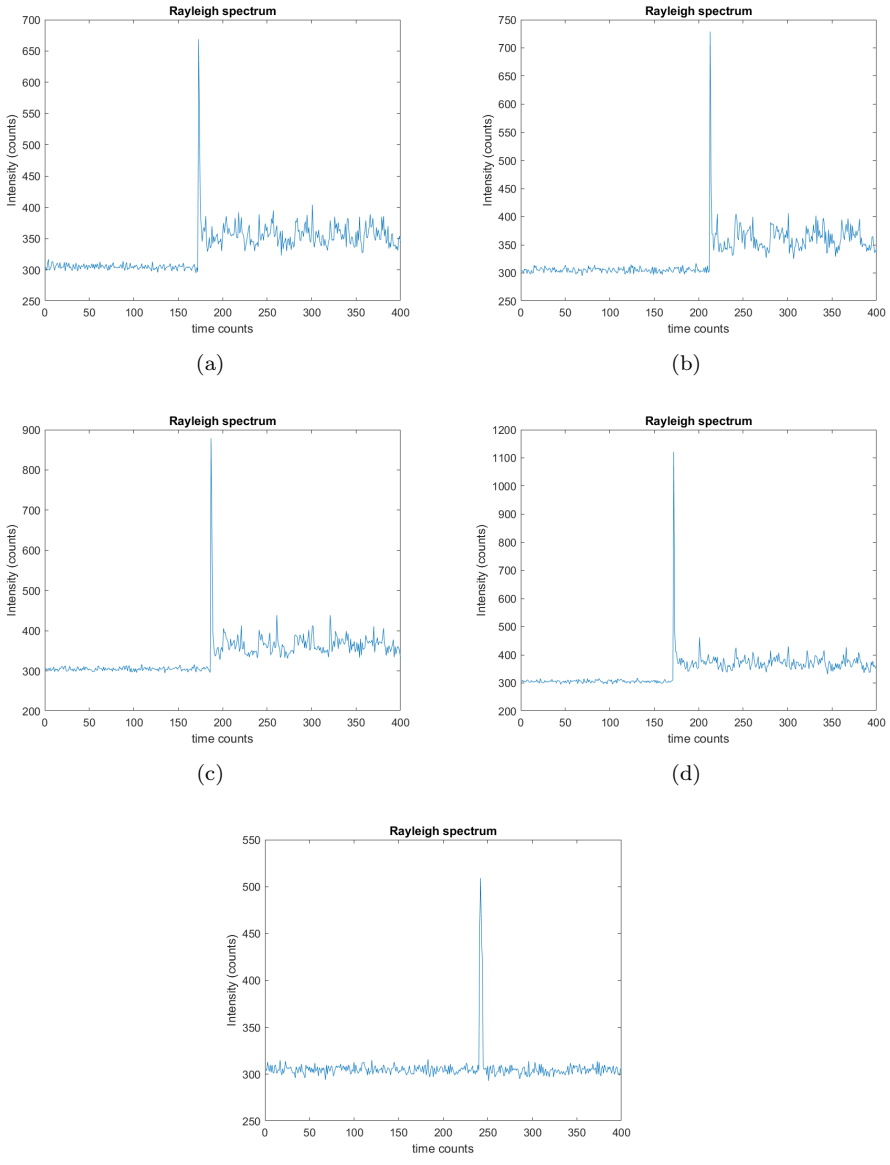


Figure 33: The measured Rayleigh signal on 20220614 pure milk sample 5 (T003C) (a) trap1 (b) trap2 (c) trap3 (d) trap4 (e) trap5

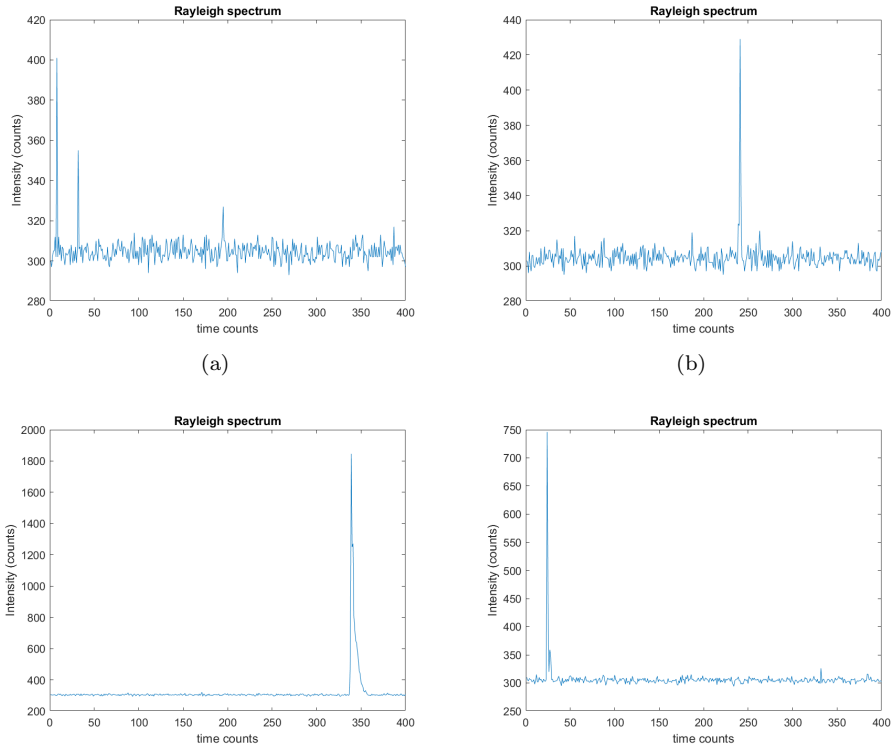


Figure 34: The measured Rayleigh signal on 20220614 pure milk sample 5 (T003C) (a) trap1 and trap2 (b) trap3 (c) trap4 (d) trap5

H MATLAB CODE

In this appendix all the used Matlabfiles can be found.

H.1 LoadSifFiles

With this file, the Rayleigh signal can be displayed. This file is used to determine the spectra in which a particle was trapped.

```
close all

%clc
% load all sif files
File_name = ('Image2022-06-14@142459--200p000um no6.sif')
    ;
%The air background spectrum
%%
filenames = input('FileName to write data to :     ','s');
info = sifread_ac(File_name,0);
NumberofSpectra = info.NumFrames;
air = zeros(NumberofSpectra,1600);
for i = 1:NumberofSpectra
    data = sifread_ac(File_name,i);
    air(i,:) = reshape(data.imageData', 1, []);
end
%% plot Raman of one spectrum
figureRaman = figure (1);
plot((1:1600),air(319,:))
title('Raman spectrum ')
ylabel('Intensity (counts)'), xlabel('Pixels')
% saveas(figureRaman, strcat(filenames, 'Raman_spectrum.
    fig'))
% saveas(figureRaman, strcat(filenames, 'Raman_spectrum.
    png'))
%% plot rayleigh of the spectrum
Rayleighpnt= air(:,7);
figureRayleigh = figure (3);
plot((1:400),Rayleighpnt)
title('Rayleigh spectrum')
ylabel('Intensity (counts)'), xlabel('time counts')
mean_rayleigh= mean(Rayleighpnt);
saveas(figureRayleigh, strcat(filenames, 'Rayleigh.fig'))
saveas(figureRayleigh, strcat(filenames, 'Rayleigh.png'))
%% Plot the meanRaman over its trapping time
meanRaman = mean(air(135:143,:));
FiguuremeanRaman = figure (2);
plot((1:1600),meanRaman)
```

```
36 title('Raman Noise removed')
37 ylabel('Intensity (counts)'), xlabel('Pixels')
38 % saveas(FiguuremeanRaman, strcat(filenames, 'MeanRaman.
       fig'))
39 % saveas(FiguuremeanRaman, strcat(filenames, 'MeanRaman.
       png'))
40 %
41 %
```

H.2 Calibrated Raman Spectra

This file uses asc-files obtained with the QCR-program. It displays the calibrated Raman spectrum for the specific spectra.

```
%close all

%% read in the asc-file
fprintf('Loading dataset ...\n');
[FileName,PathName]=uigetfile({'*.asc';'*.txt';'*.bin'},'
    MultiSelect','on','Select the data');
% choose the name bv. time_S#_range#_#
filenames = input('FileName to write data to :     ','s');
output_name = [PathName,'output_', date];
if ~exist(output_name,'dir')
    mkdir(output_name);
end
% In case one file was selected FileName is a char string
    and NMeasurements acquires the number of char in the
    string.
% FileName(N) for N=1 is then interpreted as the first
    character in that string.
% In case of single selection the character string
    FileName must be converted to a cell
if iscellstr(FileName)
    FileName=cellstr(FileName);
else
    FileName=cellstr(FileName);
end
Nmeasurements=size(FileName,2);     % which number of
    measurements did you select to load?

 for N=1:Nmeasurements
        fprintf('Loading "%s"\n',char(FileName(N)));
        DataName=fullfile(PathName,FileName(N));
        commapoint(char(DataName));  % replaces comma's
            by points. Avoids double counting of the
            number of columns after a comma.
        Intensity=load(char(DataName));
        dim = size(Intensity);
        [PixelXnumber(N),PixelYnumber(N)]=
            PreparePixelnumber(dim);
        wnr_orig=Intensity(:,1);
        Intensity=Intensity(:,2:dim(2));
 end
    %%

```

```
34  % write the trapping time from the rayleigh spectrum.
35 beginTrap = 257;
36 eindeTrap = 264;
37
38 meanRaman = mean(Intensity(:,beginTrap:eindeTrap),2);
39 Rayleigh_mean = mean(Intensity(7,beginTrap:eindeTrap),2);
40
41 % plot the Calibrated meanRaman spectrum over the
       trappingtime
42 figureSpectrum = figure(4);
43 plot(wnr_orig,meanRaman, 'linewidth', 1.1)
44 xlim([700 3600])
45 title(strcat('Raman spectrum with Rayleigh intensity = ',
        num2str(round(Rayleigh_mean)), ' (a.u.)'))
46 ylabel('Intensity (counts)'), xlabel('Raman shift (cm
       ^{-1})')
47
48 %saves the file in .mat, .fig, .png
49 save(strcat(filenames, '.mat'), 'Rayleigh_mean', '
       meanRaman')
50 saveas(figureSpectrum, strcat(filenames, '.fig'))
51 saveas(figureSpectrum, strcat(filenames, '.png'))
52
53 %% Oscillerend Rayleigh with CH-band
54
55 Osc=figure();
56 title ('Rayleigh with CH-band')
57 yyaxis left
58 plot(1:400,Rayleighpnt)
59 ylabel('Rayleigh intensity (a.u)')
60
61 yyaxis right
62 plot(1:400,Intensity(1322,:))
63 ylabel ('CH-band Intensity')
64 xlabel('spectra # (total time 13 s)')
65 saveas(Osc, strcat(filenames, 'osc.fig'))
66 saveas(Osc, strcat(filenames, 'osc.png'))
```

H.3 Multiplot

With the collected files from AscfileReaderNetter, the spectra was plotted in one figure.

```
%{
Makes a plot with multiple trapspectra in it. Load .mat
    file in and write
in command window:
trap1=meanRaman;
Load .mat file in
trap2=meanRaman;
Load .mat file in
trap3=meanRaman;
Load .mat file in
trap4=meanRaman;
Load .mat file in
trap5=meanRaman;
%}


%% fingerprintregion
filenames = input('FileName to write data to :    ','s');

figureSpectrum = figure(1);
hold on
plot(wnr_orig,trap1, 'r','linewidth', 1.1)
plot(wnr_orig,trap2+75, 'g','linewidth', 1.1)
plot(wnr_orig,trap3+100, 'b','linewidth', 1.1)
plot(wnr_orig,trap4+150, 'm','linewidth', 1.1)
plot(wnr_orig,trap5+ 200, 'k','linewidth', 1.1)


xlim([400 1850])
hold off
title('Raman spectrum fingerprint region 20220614 CV')
xlabel('Raman Shift (cm^{-1})')
ylabel('Intensity (counts)')
legend('trap 1', 'trap 2', 'trap 3', 'trap 4', 'trap 5')

saveas(figureSpectrum, strcat(filenames, '.fig'))
saveas(figureSpectrum, strcat(filenames, '.png'))

%% Full Raman spectrum
filenames = input('FileName to write data to :    ','s');

figureSpectrum2 = figure(2);
```

```
42 hold on
43 plot(wnr_orig,trap1, 'r','linewidth', 1.1)
44 plot(wnr_orig,trap2+350, 'g', 'linewidth', 1.1)
45 plot(wnr_orig,trap3+700,'b', 'linewidth', 1.1)
46 plot(wnr_orig,trap4+1050, 'm','linewidth', 1.1)
47 plot(wnr_orig,trap5+1400, 'k','linewidth', 1.1)
48 xlim([400 3600])
49 hold off
50 title('Raman spectrum 20220614 CV')
51 xlabel('Raman Shift (cm^{-1})')
52 ylabel('Intensity (counts)')
53 legend('trap 1', 'trap 2', 'trap 3', 'trap 4', 'trap 5')
54
55 saveas(figureSpectrum2, strcat(filenames, '.fig'))
56 saveas(figureSpectrum2, strcat(filenames, '.png'))
```

H.4 Correlations

All the points for Rayleigh_peak, CHPEAK and Waterpeak were collected from the spectra collected with AscfileReaderNetter.m The following file was used to plot all the seperate correlation graphs of the pure and skim milk samples

```
%% N

Rayleigh_peak= [1762 45276 34419 57932 2732];     % 20220530
CHPEAK = [53 299 259 315 185];

Rayleigh_peak2 = [152590 42528 106967 42929 47808]; % 20220608
CHPEAK2 = [722 770 1065 880 936];

Rayleigh_peak3 = [102604 150161 20792 142911 69771]; % 20220613 A
CHPEAK3 = [452 521 157 669 658];
Rayleigh_peak4 = [186439 287289 283814 174133 24617]; % 20220613 B
CHPEAK4 = [508 685 672 416 507];

Rayleigh_peak5 = [3088 6766 10025 6384 8600 ]; %20220614 A
CHPEAK5 = [80 53 59 60 66];
Rayleigh_peak6 = [2686 3017 7410 15258 1521]; % 20220614 B
CHPEAK6 = [90 101 120 196 101];

Rayleigh_peak7 = [15680 17692 18840 20774 19220];% 20220614 C
CHPEAK7 = [171 178  186 194 106];




p=polyfit(CHPEAK, Rayleigh_peak ,1);
yfit = polyval(p,CHPEAK);

p2=polyfit(CHPEAK2, Rayleigh_peak2 ,1);
yfit2 = polyval(p2,CHPEAK2);

p3=polyfit(CHPEAK3, Rayleigh_peak3 ,1);
yfit3 = polyval(p3,CHPEAK3);

p4=polyfit(CHPEAK4, Rayleigh_peak4 ,1);
```

```
yfit4 = polyval(p4,CHPEAK4);

p5=polyfit(CHPEAK5, Rayleigh_peak5 ,1);
yfit5 = polyval(p5,CHPEAK5);

p6=polyfit(CHPEAK6, Rayleigh_peak6 ,1);
yfit6 = polyval(p6,CHPEAK6);

p7=polyfit(CHPEAK7, Rayleigh_peak7 ,1);
yfit7 = polyval(p7,CHPEAK7);

figure (1)
hold on
subplot(4,2,1)
plot(CHPEAK, Rayleigh_peak , 'g*')
hold on
plot(CHPEAK, yfit , '-')
title('N 20220530 ')
ylabel('Rayleigh (a.u.)')
xlabel ('CH band intensity (counts)')
subplot(4,2,2)
hold on
title('N 20220608 ')
ylabel('Rayleigh (a.u.)')
xlabel ('CH band intensity (counts)')
plot(CHPEAK2, Rayleigh_peak2 , 'g*')
hold on
plot(CHPEAK2, yfit2 , '-')
subplot(4,2,3)
hold on
title('AN 20220613 ')
ylabel('Rayleigh (a.u.)')
xlabel ('CH band intensity (counts)')
plot(CHPEAK3, Rayleigh_peak3 , 'g*')
hold on
plot(CHPEAK3, yfit3 , '-')
subplot(4,2,4)
hold on
title('BN 20220613 ')
ylabel('Rayleigh (a.u.)')
xlabel ('CH band intensity (counts)')
plot(CHPEAK4, Rayleigh_peak4 , 'g*')
hold on
plot(CHPEAK4, yfit4 , '-')
subplot(4,2,5)
hold on
```

```
81  title('AN 20220614 ')
82  ylabel('Rayleigh  (a.u.)')
83  xlabel ('CH band intensity (counts)')
84  plot(CHPEAK5, Rayleigh_peak5 , 'g*')
85  hold on
86  plot(CHPEAK5, yfit5 , '-')
87
88  subplot(4,2,6)
89  hold on
90  title('BN 20220614 ')
91  ylabel('Rayleigh  (a.u.)')
92  xlabel ('CH band intensity (counts)')
93  plot(CHPEAK6, Rayleigh_peak6 , 'g*')
94  hold on
95  plot(CHPEAK6, yfit6 , '-')
96  subplot(4,2,7)
97  hold on
98  title('CN 20220614')
99  ylabel('Rayleigh (a.u.)')
100 xlabel ('CH band intensity (counts)')
101 plot(CHPEAK7, Rayleigh_peak7 , 'g*')
102 hold on
103 plot(CHPEAK7, yfit7 , '-')
104
105
106
107 %% V
108
109  Rayleigh_peak = [937 22403 504 13388 314]; %20220530
110 CHPEAK = [27 65 31 270 29];
111
112  Rayleigh_peak2 = [2343 1448 3780 3224 3625]; %20220608
113 CHPEAK2 = [677 585 623 518 617];
114
115  Rayleigh_peak3 = [23727 8562 1940 694 21235]; %20220613 A
116 CHPEAK3 = [266 272 44 42 215];
117
118  Rayleigh_peak4 = [10973 18114 1917 645 8491]; %20220613 B
119 CHPEAK4 = [276 190 88 63 187];
120
121
122  Rayleigh_peak5 = [29917 7527 3820 6605 8387];
123 CHPEAK5 = [300 99 86 166 341];
124
125
126 p=polyfit(CHPEAK, Rayleigh_peak ,1);
```

```
127 yfit = polyval(p,CHPEAK);
128
129 p2=polyfit(CHPEAK2, Rayleigh_peak2 ,1);
130 yfit2 = polyval(p2,CHPEAK2);
131
132 p3=polyfit(CHPEAK3, Rayleigh_peak3 ,1);
133 yfit3 = polyval(p3,CHPEAK3);
134
135 p4=polyfit(CHPEAK4, Rayleigh_peak4 ,1);
136 yfit4 = polyval(p4,CHPEAK4);
137
138 p5=polyfit(CHPEAK5, Rayleigh_peak5 ,1);
139 yfit5 = polyval(p5,CHPEAK5);
140
141 figure (1)
142 hold on
143 subplot(3,2,1)
144 plot(CHPEAK, Rayleigh_peak , 'g*')
145 hold on
146 plot(CHPEAK, yfit , '-')
147 title('V 20220530 ')
148 ylabel('Rayleigh (a.u.)')
149 xlabel ('CH band intensity (counts)')
150 subplot(3,2,2)
151 hold on
152 title('V 20220608 ')
153 ylabel('Rayleigh (a.u.)')
154 xlabel ('CH band intensity (counts)')
155 plot(CHPEAK2, Rayleigh_peak2 , 'g*')
156 hold on
157 plot(CHPEAK2, yfit2 , '-')
158 subplot(3,2,3)
159 hold on
160 title('AV 20220613 ')
161 ylabel('Rayleigh (a.u.)')
162 xlabel ('CH band intensity (counts)')
163 plot(CHPEAK3, Rayleigh_peak3 , 'g*')
164 hold on
165 plot(CHPEAK3, yfit3 , '-')
166 subplot(3,2,4)
167 hold on
168 title('BV 20220613 ')
169 ylabel('Rayleigh (a.u.)')
170 xlabel ('CH band intensity (counts)')
171 plot(CHPEAK4, Rayleigh_peak4 , 'g*')
172 hold on
```

```
173 plot(CHPEAK4, yfit4 , '-')
174 subplot(3,2,5)
175 hold on
176 title('CV 20220614 ')
177 ylabel('Rayleigh (a.u.)')
178 xlabel ('CH band intensity (counts)')
179 plot(CHPEAK5, Rayleigh_peak5 , 'g*')
180 hold on
181 plot(CHPEAK5, yfit5 , '-')
```

The correlatie.m plots the correlation with all the collected data.

```
%Correlation
%% ALL

Rayleigh_peak= [1762 45276 34419 57932 2732 152590 42528
    106967 42929 47808 102604 150161 20792 142911 69771
    186439 287289 283814 174133 24617 3088 6766 10025 6384
     8600 2686 3017 7410 15258 1521 15680 17692 18840
    20774 19220 937 22403 504 13388 314 2343 1448 3780
    3224 3625 23727 8562 1940 694 21235 10973 18114 1917
    645 8491 29917 7527 3820 6605 8387];
CHPEAK = [53 299 259 315 185 722 770 1065 880 936 452 521
     157 669 658 508 685 672 416 507 80 53 59 60 66 90 101
     120 196 101 171 178  186 194 106 27 65 31 270 29 677
    585 623 518 617 266 272 44 42 215 276 190 88 63 187
    300 99 86 166 341];

p=polyfit(CHPEAK,Rayleigh_peak,1);
yfit = polyval(p,CHPEAK);
figure()
hold on
plot(CHPEAK,Rayleigh_peak, '*')
hold on
plot(CHPEAK,yfit, '-')
title('Correlation CH BAND & RAYLEIGH INTENSITY ALL ')
ylabel('Rayleigh (a.u.)')
xlabel ('CH band intensity (counts)')
hold off
```