Research Article

Detection of Adulterant Residues in UHT Milk Products using ATR-FTIR Spectroscopy

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ABSTRACT

Milk adulteration has been done to gain economic benefit by which the quality of milk is purposely compromised either by combining or substituting important ingredients of milk with adulterants. The objectives of this study were to determine the milk quality parameters of adulterated and unadulterated milk products through quality analysis and to investigate the ATR-FTIR spectroscopy technique in detection of adulterant residues in milk products. UHT milk samples were used with three common adulterants (melamine, formalin, anionic detergent) in five different concentrations (0.2%, 0.8%, 1.2%, 1.5% and 2.0%). Quality analysis was done on unadulterated milk and adulterated milk samples. Results showed that there were no significant differences between them. Thus, ATR-FTIR spectroscopic analysis was used for the qualification of adulterants in the wavenumber range of 4000cm^{-1} to 500cm^{-1}. These results show the potential of FTIR spectroscopy as a rapid and sensitive technique for the detection of adulterant residues in UHT milk products.

Keywords: ATR-FTIR spectroscopy; milk adulterant; milk quality

1. Introduction

Milk is an important source of dietary energy, high-quality proteins and fats which are needed for healthy growth and development in humans, especially newborns (Chalupa-Krebzdak *et al.*, 2018). Other than that, milk also contains calcium, magnesium, riboflavin, vitamin B12, pantothenic acid and selenium, which are required in nutrient intake of humans.

Although processed milk products have longer shelf life, there are many chemical and physical changes that can occur to milk during long periods of storage which could affect the overall quality of milk. The common parameters that determine the quality of milk are Solid-not-Fat (SNF) percentage, fat percentage, the protein content and the freezing point. The adulteration in milk is mainly to increase these parameters, thus compromising the milk quality in ways that could endanger the health of its consumers.

Adulteration of food can be defined as the replacement of valuable substances from natural food, the substitution of the food components with cheaper alternatives, or the addition of low-quality substances into the food (Farzaneh *et al*., 2023). One way of milk adulteration is by replacement of milk with dairy products of lower commercial value, mainly to reduce its manufacturing cost. For instance, a common problem in marketing of dairy products is the replacement of sheep milk with goat milk as sheep milk has a higher price compared to goat milk (Giglioti *et al*., 2022). Milk adulteration was reported back in 1958 where 8000 deaths involving infants was reported, due to the Swill Milk Scandal that happened in New York (Nagraik *et al*., 2021). Adulteration, not only in milk but other food products, is one of the global concerns and challenges in the food industry.

One of the common adulterants used in milk products is nitrogen rich compounds such as melamine. Melamine contains 66% of nitrogen by mass and it is added into milk to increase the protein content of the milk. One major incident involving the addition of melamine to milk is the widespread poisoning of 300,000 infants in China due to consumption of melamine adulterated infant formula which lead to the death of six infants (Farzaneh *et al*., 2023). As the unknown ingestion of melamine pose serious health risks such as kidney stones and even kidney failure to its consumers, the United States Food and Drug Administration (US FDA) has officially set the safety limit of melamine ingestion at 2.5 ppm for adults and 1 ppm for infant formula (Siddiquee *et al*., 2021). Furthermore, the addition of preservatives such as formaldehyde is also quite frequent in milk products. Preservatives are added to prolong the shelf life of milk by inhibiting the growth of microorganisms. The use of formalin as preservatives has been associated to be the cause of renal failure (Nagraik *et al*., 2021). In addition, according to the National Toxicology Program in conjunction with agencies such as Food and Drug Administration and The National Cancer Institute, formalin has been depicted as a human carcinogen and it could lead to myeloid leukemia (Bernard *et al*., 2023).

Other than that, detergents are also one of the common adulterants added into milk products to increase the rich frothy appearances of the milk and prevent milk spoilage (Azad & Ahmed, 2016; Tohidi *et al*., 2018). From a study done in Pakistan, detergents were the second most common adulterants found in 25% of milk samples (Ibrahim *et al*., 2023). Prolonged consumption of milk that has been adulterated with detergent may cause serious health problems such as gastrointestinal pain, heart problems and cancer.

Thus, various methods are used to detect and quantify the use of these adulterants in milk products. Physicochemical methods detect the presence of adulterants by observing the physicochemical changes, when adulterants in the milk react with chemical reagents used for the specific reactions. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopic analysis method is used due to its advantages in accuracy, low sample preparation, non-destructive nature and rapid detection (Ketty *et al.,* 2017; Tan *et al.,* 2023).

The aim of this study was to identify and quantify the use of three types of adulterants in UHT milk products which are melamine, formalin and anionic detergent, by using the ATR-FTIR spectroscopy technique. Unadulterated UHT milk was spiked with known concentration of adulterants in order to determine the ability of FTIR spectroscopy technique in detection of the adulterants in UHT milk products.

2. Materials and Methods

2.1 Quality Analysis

The unadulterated UHT milk sample and adulterated milk samples underwent an alcohol test, pH test, acidity test, specific gravity test and methylene blue reduction test to determine their milk quality parameters.

2.1.1 Sample Preparation

The Dutch Lady Full Cream UHT milk samples were purchased from ABC supermarket at 1 Borneo Hypermall. The unadulterated UHT milk sample was prepared and underwent quality tests to determine

the milk quality parameters. For the preparation of the adulterated UHT milk sample, the sample was divided into three groups, and each group was spiked with different adulterants (melamine, formalin, anionic detergent). For each adulterant, the sample was spiked with different concentrations which were 2.5 mg/kg for melamine, 0.4 mL/100 mL for formalin and 10 mg/L for anionic detergent. The concentration of melamine and formalin adulterant was added according to their permissible limit, while the concentration of anionic detergent was added according to its lower detection limit, in order to see the change in milk quality when adulterants were added. The adulterated UHT milk samples were homogenized for 60 seconds using a vortex at 30 rpm amplitude.

2.1.2 Alcohol Test

1 mL of adulterated milk sample was put in a test tube. Then, 1 mL of 75% ethyl alcohol was added and the test tube was shaken. If the milk did not clot, then the milk was considered alcohol positive (accepted). If the milk clotted, the milk was considered alcohol-negative (rejected) (Rahman *et al.*, 2017).

2.1.3 pH Test

100 mL of adulterated milk sample was put in a beaker and its pH was checked using a digital pH meter. The pH of the sample was recorded.

2.1.4 Acidity Test

9 mL of adulterated milk sample was taken with 1 mL of phenolphthalein and was put in a beaker. Titration was done against 0.1M of NaOH until the solution turned to light pink colour. The amount of NaOH taken for the solution to turn to a light pink colour was recorded. Then, the acidity of the sample was calculated using the formula shown in equation (1) below:

$$\text{Lactic Acid } \% = \frac{9 \times V1 \times N}{V2} \quad (1)$$

V1 was the volume in mL of the standard NaOH required for titration. *N* was the normality of the standard NaOH solution and *V2* was the volume in mL of the milk sample used in the test (Awal *et al.*, 2016).

2.1.5 Specific Gravity Test

100 mL of adulterated milk sample was put in a measuring cylinder. The temperature of the sample was taken using a thermometer. Then, the lactometer was placed in the milk sample, and its reading was taken. The density of the sample was calculated using the formula in the equation (2) below:

$$\text{Density} = 1 + \frac{^{\circ}\text{L} + (\text{T} \times \text{F})}{1000} \quad (2)$$

Where $^{\circ}$L was the lactometer reading, *T* was the corrected temperature calculated by subtracting the standard temperature (20°C) from the sample temperature, and *F* was the temperature factor which was 0.2 (Awal *et al.*, 2016).

2.1.6 Methylene Blue Reduction Test (MBRT)

Milk samples were mixed thoroughly, and 10 mL of each milk sample was placed into test tubes. 1 mL of standard methylene blue solution was added into each test tube and the samples were mixed thoroughly. The test tubes were then placed in a thermostatically maintained water bath (37°C). The initial time was noted down and the test tubes were examined after half an hour. If no discolouration happened the test tubes were inverted once and then transferred to the water bath for further incubation. After another 30

minutes, the test tubes were observed again for the reduction of the dye. If the milk sample did not decolourize within 30 minutes, the milk was considered of good quality. The longer the time taken for the milk to decolourise, the better the quality of the milk (Anwer *et al.*, 2018).

2.2 Detection of Adulterant Residues

In this study, the ATR-FTIR spectroscopy technique was used for the detection of adulterant residues.

2.2.1 Sample Preparation

The Dutch Lady Full Cream UHT milk samples were purchased from ABC supermarket at 1 Borneo Hypermall, Kota Kinabalu, Sabah, Malaysia. The unadulterated control UHT milk sample was prepared in triplicate. For the preparation of adulterated UHT milk samples, the samples were divided into three groups, and each group was spiked with different adulterants (melamine, formalin, anionic detergent) respectively. For each adulterant, the sample was divided and spiked with different percentages of adulterants which are 0.2%, 0.8%, 1.2%, 1.5%, 2.0%. Then, each adulterated UHT milk sample that was spiked with a different percentage of adulterants was prepared in triplicate. The adulterated UHT milk samples were homogenized for 60 seconds using a vortex at 30 rpm amplitude (Jaiswal *et al.*, 2017). The amount of adulterant added for each concentration was calculated as shown in equation (3) and equation (4) below.

Melamine concentration:

$$\text{g of melamine} = \%\ \text{adulterant} \times \text{volume of solution} \quad (3)$$

Formalin and anionic detergent concentration:

$$\text{mL of adulterant} = \%\ \text{adulterant} \times \text{volume of solution} \quad (4)$$

2.2.2 ATR-FTIR Analysis

The absorption spectra of samples in the mid-infrared region (4000-500 cm^{-1}) were acquired using the Bruker Alpha II FTIR spectrometer with Diamond Crystal ATR at a resolution of 4 cm^{-1} with 16 scans for each sample. The samples were split into two sets, 70% for the calibration set and 30% for the validation set.

3. Results and Discussion

3.1 Quality test of adulterated and unadulterated milk

The results of the quality tests carried out between unadulterated milk and milk adulterated with melamine, formalin and detergent are shown in Table 1 and Table 2 respectively.

Insignificant differences in pH between unadulterated milk and adulterated milk were observed as pH obtain from adulterated milk were still within the normal pH range for milk. Slight increase in density was observed after the addition of adulterants in milk, while the titratable acidity was observed to have decreased. In addition, there was no significant difference between the results of unadulterated milk and adulterated milk for both alcohol test and MBRT.

Table 1 Physicochemical properties of an unadulterated UHT milk sample.

Milk Sample	pH	SG (g/mL)	Acidity	Alcohol (75%)	MBRT
Dutch Lady UHT Full Cream	6.57	1.0260	1.9	Accepted	Pass

Table 2 Physicochemical properties of the adulterated UHT milk sample.

Milk Sample	Adulterant (Concentration)	pH	SG (g/mL)	Acidity	Alcohol (75%)	MBRT
Dutch Lady UHT Full Cream	Melamine (2.5 mg/kg)	6.65	1.0314	1.5	Accepted	Pass
	Formalin (0.4 mL/100 mL)	6.52	1.0304	1.4	Accepted	Pass
	Detergent (10 mg/L)	6.53	1.0302	1.3	Accepted	Pass

3.1.1 pH

The acidity of milk is a good indicator of the freshness of milk as its pH changes over time. As milk gets less fresh and turns sour, its pH decreases and becomes more acidic. This is due to the increase of lactic acid bacteria in milk, that converts lactose sugar into lactic acid, which leads to a decrease of pH in milk and the change in its taste (Marouf *et al.*, 2017). Based on Table 1 and Table 2 it can be seen that the pH for the unadulterated milk sample is 6.57, while the pH for UHT milk adulterated with melamine, formalin and detergent are 6.65,6.52, and 6.53 respectively. The pH of milk determines whether the milk is considered an acid or base. From these results, while the change is small, it appears that the adulteration of milk with melamine increased the pH of milk while adulteration with formalin and detergent decreased the pH of milk. Overall, the result shows that the adulteration of milk with melamine, formalin and detergent does not have a huge effect on the milk's pH, as the pH is still in the normal pH range which is 6.5 to 6.7.

3.1.2 Specific gravity (SG)

The specific gravity of milk indicates the density of milk when compared with water. According to the FAO, the standard range of specific gravity for UHT milk is 1.028 to 1.033 (Awal *et al.*, 2016). Based on Table 1 the recorded specific gravity of the unadulterated milk sample appeared to be slightly lower than the standard range which is 1.026. From Table 2, UHT milk adulterated with melamine, formalin and detergent has the specific gravity of 1.0314, 1.0304 and 1.0302 respectively, which are well within the standard range. This is because one of the factors that can lead to a decrease of the specific gravity of milk is the addition of fat. As melamine, formalin and detergent do not add to the content of fat in milk, the specific gravity of milk was not affected negatively.

3.1.3 Acidity

The acidity of milk is determined using titratable acidity, which is the percentage of lactic acid in milk. It is a rapid test that indicates the quality of milk and gives an indirect measure of the acid content in milk. In general, when the content of acid in milk increases, so does its titratable value (Fauziah *et al*., 2020). Based on Table 1 the recorded acidity of the unadulterated UHT milk sample was 1.9, while the acidity of UHT milk adulterated with melamine, formalin and detergent was 1.5, 1.4 and 1.3, respectively. These results show a slight decrease in the acidity of milk when adulterant was added.

3.1.4 Alcohol Test

The alcohol test is a simple test that is used in milk to indicate whether the milk will coagulate upon processing. In theory, milk that contains more than 0.21% acid will coagulate when alcohol is added (Kumssa, 2018). If the milk coagulates, fine particles of curd will be visible in the sample. In this study, both unadulterated and unadulterated milk samples had undergone the alcohol test, and none of the samples were found to coagulate after the addition of alcohol.

3.1.5 Methylene Blue Reduction Test (MBRT)

MBRT is a quick method used to assess the microbiological quality of milk. This test works based on the fact that the blue colour of the dye solution added to the milk is decolourized when oxygen present in the milk is exhausted due to microbial activity (Anwer *et al*., 2018). In this study, both unadulterated and adulterated samples had passed the MBRT test, which indicate good quality of milk with a low amount of microbial contamination.

3.2 ATR-FTIR Spectra Analysis

In this study, the FTIR transmittance spectra in the range of 4000 cm^{-1} to 500 cm^{-1} were used.

3.2.1 FTIR Spectra Analysis of Unadulterated UHT Milk Sample

The representative FTIR spectra of unadulterated UHT milk samples in the region 4000 cm^{-1} to 500 cm^{-1} are shown in Figure 1. This region comprises of various peaks that correspond to distinct chemical bonds of milk constituents interacting with the FTIR transmittance. Major spectra can be seen at 3323 cm^{-1}, 1640 cm^{-1}, 1443 cm^{-1} and 1025 cm^{-1} were assigned to -OH stretching vibration, C=O stretching, CH_2-CH_3 bending, and C-O stretching, respectively. According to Ketty *et al.* (2017) the spectra located at the region of wavenumber between 3650 cm^{-1} to 3000 cm^{-1} and the region of wavenumber between 1680 cm^{-1} to 1600 cm^{-1} were described as indicative to water. In addition, the spectra located at the wavenumber range of 1680 cm^{-1} to 1631 cm^{-1} correspond to amide I. These findings appear to correspond with those of the unadulterated milk sample in this study. Meanwhile, carbonyl groups (C=O) of milk fat were seen at a wavenumber of 1747 cm^{-1}, and hydroxyl groups of lactose were seen at a wavenumber of 1039 cm^{-1}.

3.2.2 FTIR Spectra Analysis of Melamine

The FTIR transmission peaked at 3500 cm^{-1} to 3000 cm^{-1} and 1700 cm^{-1} to 1300 cm^{-1} in the melamine spectrum and this was attributed to the stretching and bending vibrations of amino groups commonly present in melamine, typically absent in milk. Furthermore, the spectra located at 3333.7 cm^{-1} and 3132.0 cm^{-1} of melamine were attributed to the asymmetric NH_2 stretch and symmetric NH_2 stretch respectively. Jawaid *et al*. (2014) described that the transmission peaks at 1653.7 cm^{-1} of pure melamine were caused by NH_2 deformation and the transmission peaks at 1559.2 cm^{-1} and 1438.2 cm^{-1} were attributed to quadrant stretching of 1,3,5-s-trianzine ring and semicircle stretching of 1,3,5-s-triazine ring respectively. Additionally, the transmittance peak of 1027.5 cm^{-1} was also described to be attributed by C-N stretching

of primary amines. These results correspond to this study as melamine powder was used in both studies. Next, the transmittance peaks at 814 cm^{-1} were a characteristic of out-of-plane bending of 1,3,5-s-triazine ring of melamine, which was also absent in the pure milk spectrum.

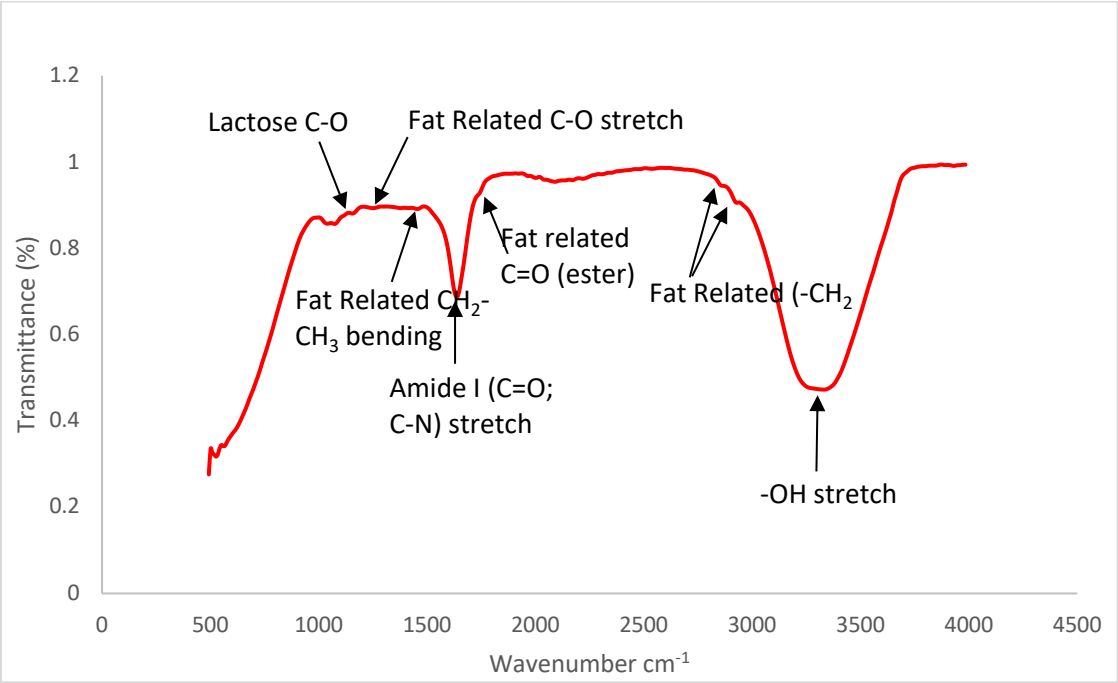

Figure 1 Transmittance spectra of unadulterated UHT milk sample.

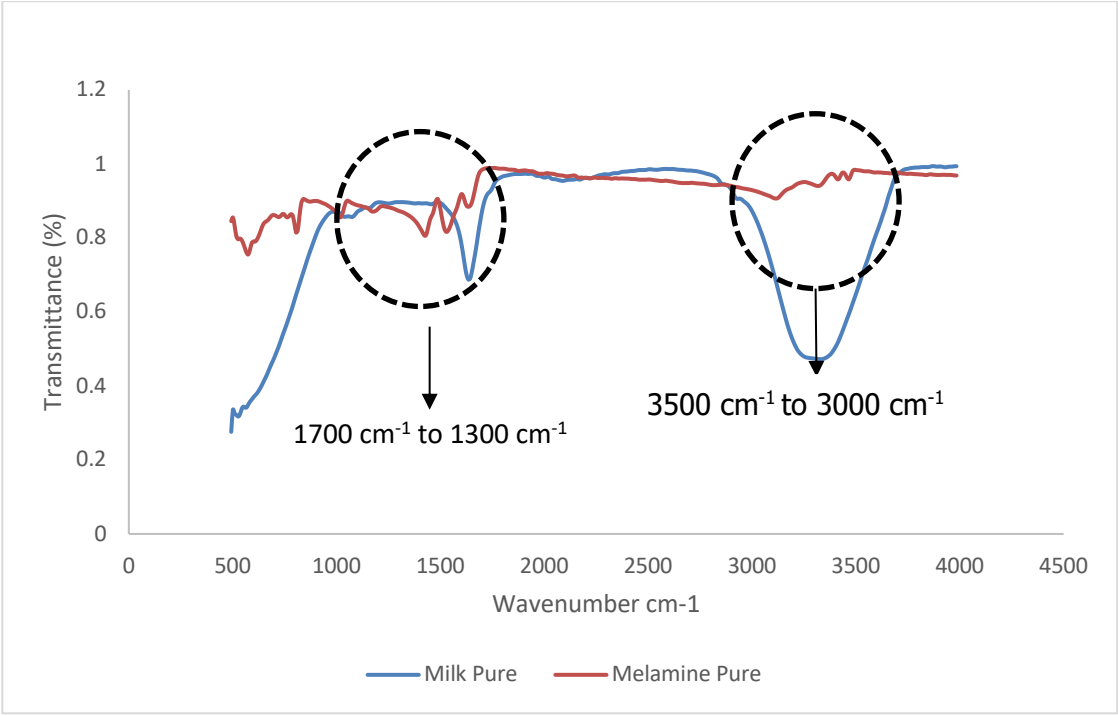

Figure 2 Transmittance spectra of pure UHT milk sample and pure melamine sample.

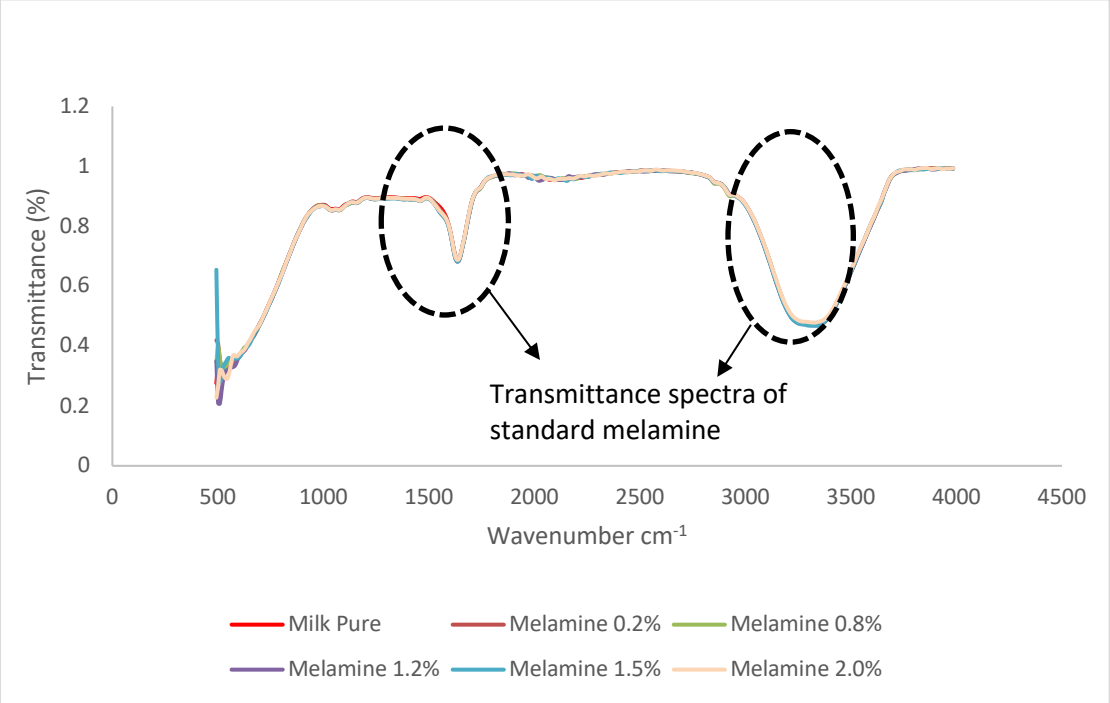

Figure 3 Transmittance spectra of unadulterated UHT milk sample and melamine with different concentration (0.2%,0.8%,1.2%,1.5%,2.0%).

As can be seen in Figure 3, there are some spectral differences between the unadulterated milk sample and the milk sample adulterated with melamine, but the difference was too small to be seen by the naked eye. As melamine was added into the UHT milk sample, the transmittance spectra of standard melamine (3500 cm^{-1} to 3000 cm^{-1} and 1700 cm^{-1} to 1300 cm^{-1}) were difficult to be seen as they happened to overlap with the -OH stretch of milk at peak 3000 cm^{-1} to 3650 cm^{-1}, and the C=O stretch of Amide I and fat related CH_2-CH_3 bending at peak 1640 cm^{-1} and 1443 cm^{-1} respectively. However, the difference between an unadulterated UHT milk sample and a melamine-adulterated UHT milk sample still exists and thus can be used for quantitative and qualitative purposes (Wu *et al.*, 2016).

3.2.3 FTIR Spectra Analysis of Formalin

From Figure 4, the transmittance spectra of the unadulterated UHT milk sample and formalin pure were characterized in the wavenumber of 500 cm^{-1} to 4000 cm^{-1}. In both samples, major transmittance spectra can be seen at the wavenumber of 3323 cm^{-1} and 1640 cm^{-1}, which correspond to the -OH stretching vibration and C=O stretching of Amide I which corresponds to the stretching vibrations of peptide linkages. A most distinct difference between the unadulterated UHT milk sample and formalin pure was observed at the transmittance peak of 1080 cm^{-1} to 950 cm^{-1}. This peak was only present in pure formalin.

In Figure 5, the FTIR transmittance spectra of the unadulterated UHT milk sample and formalin adulterated milk sample at different concentrations (0.2%, 0.8%, 1.2%, 1.5%, 2.0%) were observed in the spectral range of 500 cm^{-1} to 4000 cm^{-1}. Again, the strong transmittance peak and broad O-H stretch vibrations were located in the region of 3700 cm^{-1} to 3000 cm^{-1}. Then, Balan *et al.* (2020) described the peaks at 2924 cm^{-1} and 2852 cm^{-1} to be assigned as fat regions that correspond to the asymmetric and symmetric CH_2 stretching. A typical spectrum of lactose where C-O stretching was also noted in the wavenumber region of 1100 cm^{-1} to 1000 cm^{-1} with a maximum peak at 1075 cm^{-1}. Finally, the formalin peak around 1025 cm^{-1} was observed. However, the formalin peak in adulterated milk samples was less prominent due to the small concentration of formalin used.

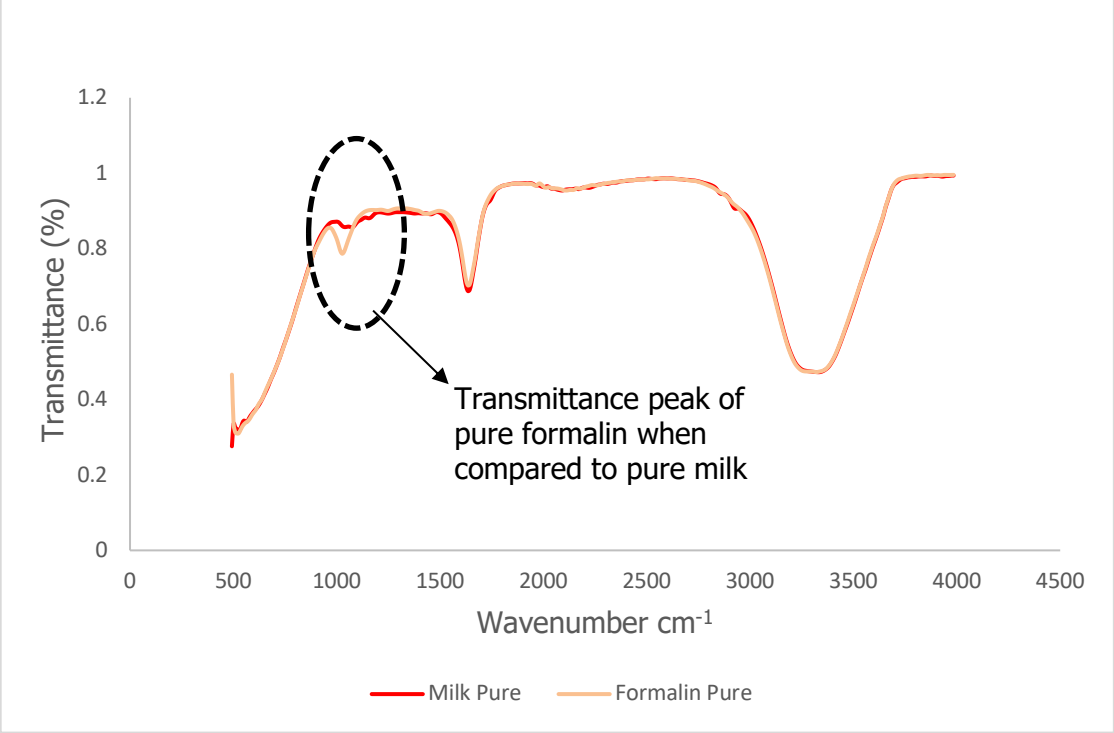

Figure 4 Transmittance spectra of pure formalin and unadulterated UHT milk sample.

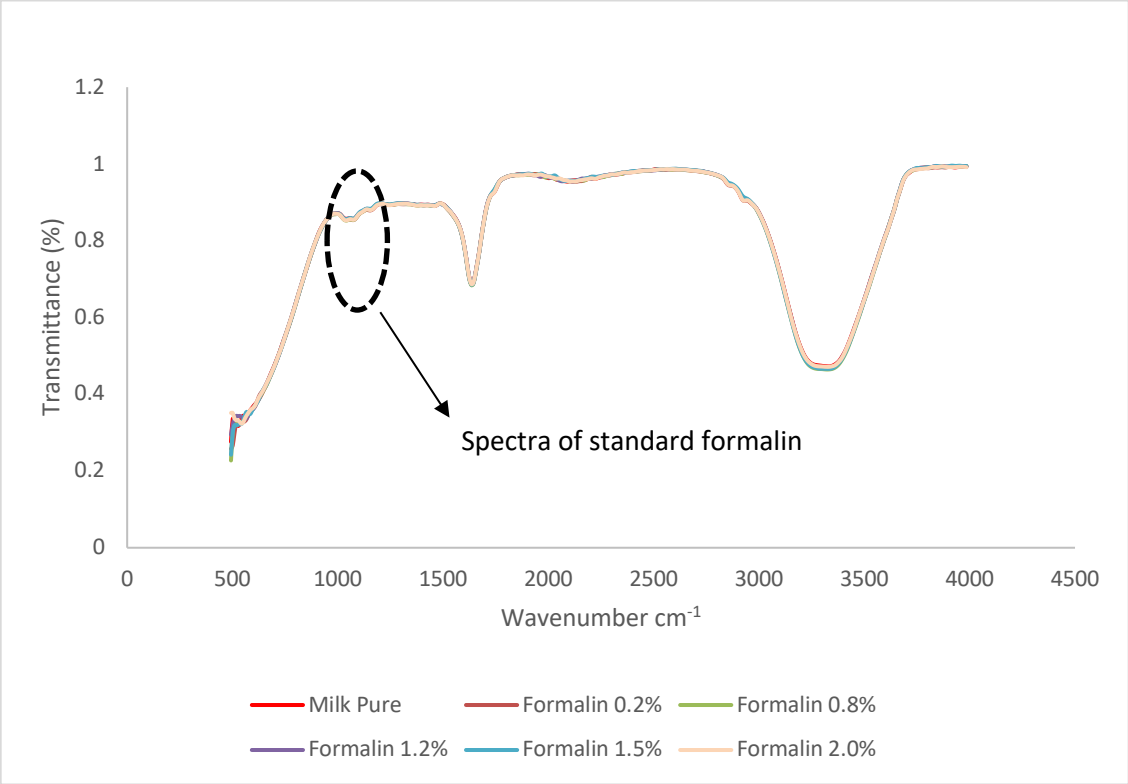

Figure 5 Transmittance spectra of unadulterated milk sample and formalin adulterated milk sample with different concentration (0.2%, 0.8%, 1.2%, 1.5%, 2.0%).

3.2.4 FTIR Spectra Analysis of Anionic Detergent

From Figure 6, it was observed that there were noticeable differences between the FTIR transmittance spectra of the unadulterated UHT milk sample and pure anionic detergent. The differences were more noticeable in the transmittance peak of 1600 cm^{-1} to 955 cm^{-1}, which may be due to the difference of chemical composition of the anionic detergent. The spectral range of 1600 cm^{-1} to 955 cm^{-1} include five smaller spectral windows which are 1507 cm^{-1} to 1456 cm^{-1}, 1343 cm^{-1} to 1333 cm^{-1}, 1333 cm^{-1} to 1313 cm^{-1}, 1086 cm^{-1} to 1056 cm^{-1} and 1001 cm^{-1} to 995 cm^{-1}. Anionic detergents that are commonly used were prepared using linear alkyl benzene sulphonate being the major ingredient, caustic soda, tri-sodium phosphate, urea and water. The alkylbenzene part is hydrophobic in nature while the sulphonate part is hydrophilic in nature (Jaiswal *et al.,* 2016). The transmittance peak observed in the wavenumber range of 1086 cm^{-1} to 1056 cm^{-1} corresponds to the group frequency of phosphate and sulphonate ions present in the detergent.

From Figure 7, the difference in transmittance spectra of the unadulterated UHT milk sample and detergent adulterated milk sample can be seen at FTIR transmittance of 1600 cm^{-1} to 995 cm^{-1}. According to Jaiswal *et al*. (2016), the transmittance peak in the range of 1001 cm^{-1} to 995 cm^{-1} was due to a weaker frequency of aromatic C-H in the plane bend. Moreover, the spectra at the wavenumber of 1343 cm^{-1} to 1333 cm^{-1} might be caused by the stretching vibration of aryl sulfones in alkyl benzene sulphonate, C-N stretching vibration of aromatic primary amine (urea) and the wagging mode of vibration of CH_2. In addition, detergent adulterated milk samples were also prone to have a peak in the wavenumber range of 3040 cm^{-1} to 2851 cm^{-1} that was caused by aromatic vibrations of benzene sulphate. However, in the result shown in Figure 7, the peak cannot be seen as clearly as they overlapped with the -OH stretch and the fat-related stretch of UHT milk. Other than that, the peak may also not be seen clearly due to the small concentration of anionic detergent used.

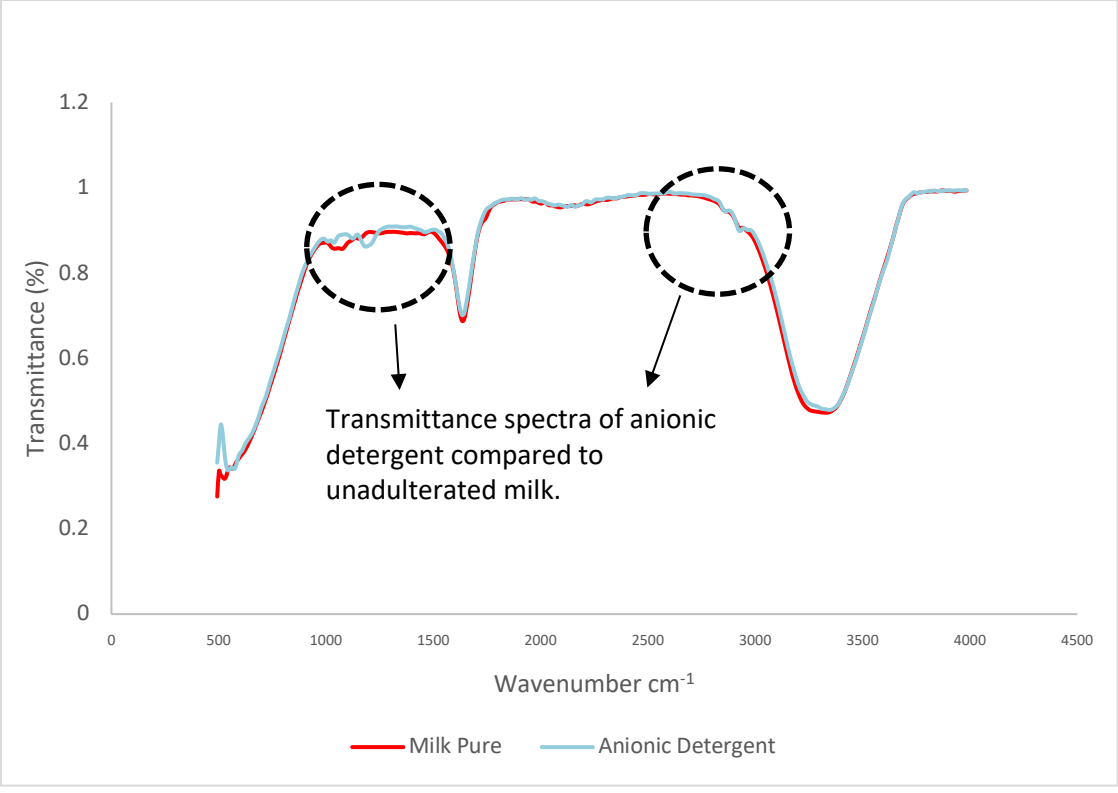

Figure 6 FTIR transmittance of unadulterated UHT milk sample and pure anionic detergent.

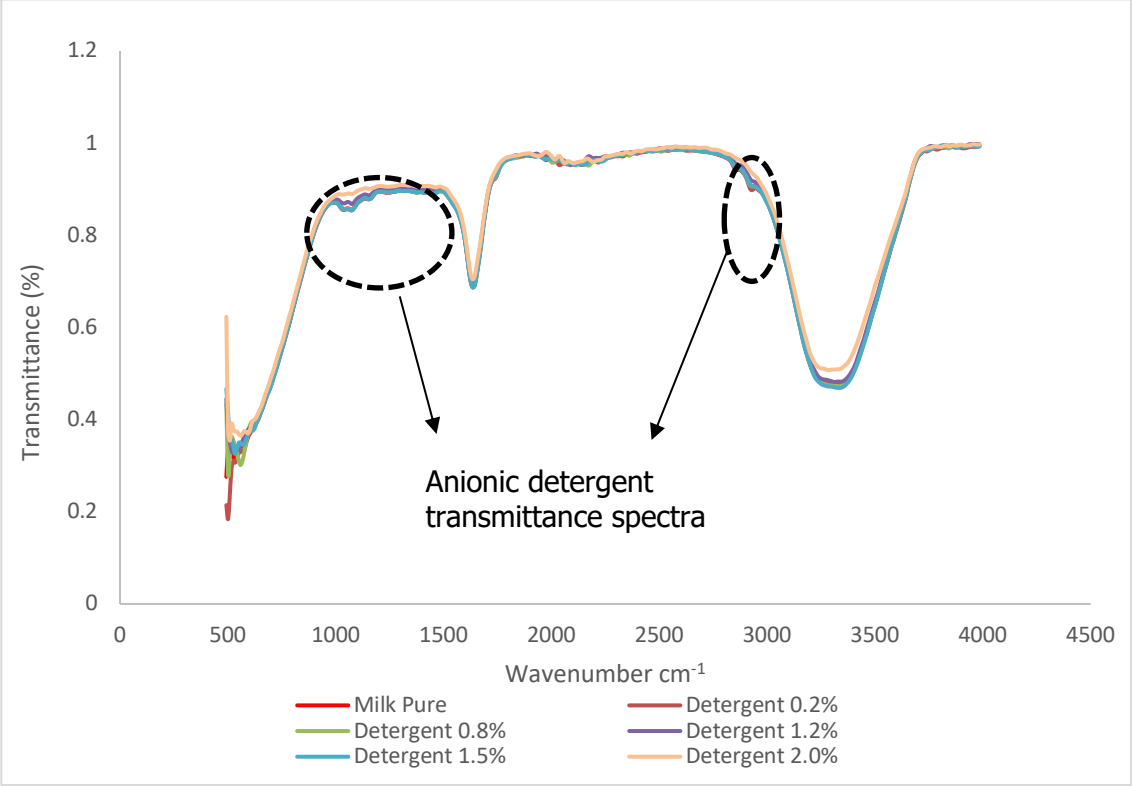

Figure 7 Transmittance spectra of unadulterated UHT milk sample and anionic detergent adulterated milk sample with different concentration (0.2%, 0.8%, 1.2%, 1.5%, 2.0%).

4. Conclusion

In conclusion, the addition of adulterants in milk products is still a growing concern due to its negative effect on human health. A quality test was done to see the difference in the quality of the milk when adulterants were added. Results from the quality test did not indicate significant changes to the quality of the milk. This might be due to the low concentration of adulterants used which was still within the permissible limit. As low concentration did not change the milk quality, other detection methods that can detect the addition of adulterants even at low concentrations were used. FTIR spectroscopy was used in this study for the detection of adulterants added to milk. The most common adulterants used in milk such as melamine, formalin and anionic detergent were used at concentrations of 0.2%, 0.8%, 1.2%, 1.5% and 2.0%. The detection of adulterants by FTIR spectroscopy was shown based on its transmittance spectra in the wavenumber range of 4000 cm^{-1} to 500 cm^{-1}. Thus, from this study, it can be concluded that ATR-FTIR spectroscopy is a rapid, sensitive and non-destructive technique that can be used for the detection of adulterant residues in milk products.

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