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Quantification of Adulterant Residues in UHT Milk Products using ATR-FTIR Spectroscopy Coupled with Multivariate Analysis

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ABSTRACT

Milk adulteration is a dishonest act of some milk manufacturer, where they purposely combine or substitute the ingredient of milk with low quality substances which decrease the quality of the milk. The objectives of this study were to investigate the ATR-FTIR spectroscopy technique in detection of adulterant residues in milk products, and to classify and quantify the adulterant detected in milk sample using multivariate analysis. Ultra High-Temperature (UHT) milk sample was used with three common adulterants (melamine, formalin, anionic detergent) in five different concentrations (0.2 %, 0.8 %, 1.2 %, 1.5 % and 2.0 %). The ATR-FTIR spectroscopic analysis was used for the qualification of adulterants in the wavenumber range of 4000cm^{-1} to 500cm^{-1}. Then, principal component analysis (PCA), discriminant analysis (DA) and multiple linear regression (MLR) was applied for multivariate analysis. PCA was used to reduce the dimensionality of the data and to explore the similarities and differences among the unadulterated and adulterated milk samples. DA was used to confirm the significant variable obtain from PCA, and MLR was used to quantify the adulterants detected in the samples. The root mean squared error (RMSE), the coefficient of determination (R^2) value obtained for melamine data was 0.158% and 0.975 respectively with the mean square error (MSE) was obtained to be 0.025. Then, the RMSE, $R^2$and MSE value obtained for formalin were 0.308%, 0.904 and 0.095 respectively while the RMSE, R^2 and MSE value obtained for detergent were 0.639%, 0.632 and 0.408. These results show the potential of FTIR spectroscopy coupled with multivariate analysis as a rapid and sensitive technique for the qualification and quantification of adulterant residues in UHT milk products.

1. Introduction

Milk is widely known around the world to be a complete dairy product as it has various nutritional components. It consists of nutrients such as carbohydrates, proteins, fats, vitamins and even minerals

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that are crucial in maintaining good health for humans [1,2]. The wide consumption of milk by people around the globe leads to an increase in milk demand which is difficult to overcome due to limited production and supply. Other than that, the growth of the dairy market is also expected to continuously rising until the year of 2025, where the annual increase in production of skim milk powder is 2.0, 2.1 % increase for whole milk powder and 1.7 and 1.4 % production increase for butter and cheese respectively [3]. Thus, to meet the growing demand of the market, some irresponsible manufacturers have opted to use adulterants to increase the quantity of milk produced. By the adulteration of milk products, they increase their profit by using low-quality substances in milk, thus consequently decreasing the quality and safety of milk products produced.

Melamine is one of the most common adulterants used in milk products. It is a nitrogen-rich compound which is usually used in the industry in the making of materials such as plastics, cleansers, coating and adhesives. Melamine is added to milk as it can increase the protein content of the milk [4]. However, the addition of melamine into milk can pose some serious health risks to its consumers such as kidney failure and kidney stones. Thus, The Food Safety and Standards Authority of India (FSSAI) has set the limit for melamine content in milk and food products to 0.5 - 2.5 ppm [5].

Other than melamine, the addition of formalin which is a form of preservative, is also one of the common adulterants used in milk products. As a preservative, formalin is added to increase the self-life of milk as it helps prevent microbial contamination [6]. Meanwhile, addition of formalin in milk products can also cause the protein content, pH and fat percentage of the milk to be reduced [7]. Adulteration of milk with formalin is also dangerous to human health as they are carcinogenic and when ingested, formalin is quickly absorbed from the gastrointestinal tract. High levels of consumption of formalin can lead to abdominal pain, asthma, vomiting and even coma [8].

Then, the addition of detergent as an adulterants in milk is used to emulsify the oil in water and to give the milk a frothy solution. However, the unknown consumption of detergent in milk is detrimental to human health as it can cause toxic manifestations in the human body and lead to problems such as renal failure, cardiac dysfunction, ventricular tachycardia and haemolysis [9]. Thus, according to the World Health Organization (WHO), the safety limit for detergent in food should be less than 0.002 mg/kg [10].

As the unknown consumption of adulterants that exceeds the maximum permissible limit can lead to serious health problems, various methods can be used to detect and quantify the adulterants added to these milk products. Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopic analysis coupled with multivariate methods was applied in this study due to its accuracy, provides a rapid analysis, requires minimum sample preparation and is non-destructive [8]. In addition, FTIR spectrometry, which is a type of MIRS has also been authorized by the International Committee for Animal Recording, as a standardized system used in analyzing milk constituents [11]. Then, multivariate analysis authenticates and quantifies the levels of adulterant detected.

This study aimed to classify and quantify the use of melamine, formalin and detergent in Ultra High-Temperature (UHT) milk products using the ATR-FTIR spectroscopy technique. After the adulterants were detected using the ATR-FTIR spectroscopy technique, quantification of the adulterants detected was done using multivariate analysis which consisted of Principal components analysis (PCA), discriminant analysis (DA) and multiple linear regression (MLR).

2. Methodology

2.1 Sample Preparation

The Dutch Lady Full Cream UHT milk samples were purchased from ABC supermarket at 1 Borneo Hypermall. The unadulterated control UHT milk sample was prepared in triplicate. For the preparation of an adulterated UHT milk sample, the sample was divided into three groups, and each group was spiked with different adulterants (melamine, formalin and anionic detergent) respectively. For each adulterant, the sample was divided and spiked with different percentages of adulterants which were 0.2 %, 0.8 %, 1.2 %, 1.5 % and 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 [12]. The amount of adulterant added for each concentration was calculated according to Eq. (1) and (2).

Melamine concentration:

$$\text{g of melamine} = \%\,\text{adulterant} \times \text{volume of solution} \quad (1)$$

Formalin and anionic detergent concentration:

$$\text{ml of adulterant} = \%\,\text{adulterant} \times \text{volume of solution} \quad (2)$$

2.2 Detection of Adulterant Residues using ATR-FTIR Analysis

In this study, the ATR-FTIR spectroscopy technique was used for the detection of adulterant residues. 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 [13].

2.3 Multivariate Analysis

PCA, DA and MLR methods were applied for the multivariate analysis and the data were analyzed using XLSTAT 2022. PCA was used to classify the samples based on their spectral differences and identify the important variables in the data set which was important for a more robust and less complex model.

DA then helped in determining whether significant differences exist among the groups of variables. It also helped in evaluating the accuracy of the classification. MLR was used to establish the linear relationship between multiple independent and dependent variables. It was also used to know how strong the relationship was between two or more independent variable and one dependent variables, and to quantify the value of the dependent variable at a certain value of the independent variables. The MLR performance was evaluated using coefficient of determination (R^2), root mean square error (RMSE) and mean square error (MSE) [14].

3. Results and Discussion

3.1 ATR-FTIR Spectra Analysis of UHT Milk Samples

A previous study by Julmohammad *et al*., [13] has reported on the quality tests and the FTIR transmittance spectra in the range of 4000 - 500 cm^{-1}, between unadulterated milk and milk adulterated with melamine, formalin and detergent.

3.1.1 FTIR spectra analysis of unadulterated UHT milk sample

The representative FTIR spectra of unadulterated UHT milk samples can be seen in the region 4000 - 500 cm^{-1}. Based on a previous study by Julmohammad *et al*., [13] this region comprises 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 a previous study by Ketty *et al.,* [15], the spectra located at the region of wavenumber between 3650 - 3000 cm^{-1} and the region of wavenumber between 1680 - 1600 cm^{-1} were described as indicative of water. In addition, the spectra located at the wavenumber range of 1680 - 1631 cm^{-1} correspond to amide I. These findings 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}.

3.1.2 FTIR spectra analysis of melamine

Also based on Julmohammad *et al*. [13], the results of FTIR spectra analysis of melamine showed there were 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. 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 [16].

3.1.3 FTIR spectra analysis of formalin

The transmittance spectra of the unadulterated UHT milk sample and formalin pure were characterized in the wavenumber of 4000 – 500 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 - 950 cm^{-1}. This peak was only present in pure formalin.

Again, Julmohammad *et al*., [13] showed that there were strong transmittance peaks and broad O-H stretch vibrations located in the region of 3700 - 3000 cm^{-1}. The appearance of broad O-H stretch vibrations also was in accordance with OH band appeared in study reported by Tajulruddin *et al.,* [17]. 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 - 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.

3.1.4 FTIR spectra analysis of anionic detergent

Julmohammad *et al*., [13] proposed the difference in transmittance spectra of the unadulterated UHT milk sample and detergent adulterated milk sample can be seen at FTIR transmittance of 1600 - 995 cm^{-1}. According to a previous study by Jaiswal *et al*., [18], the transmittance peak in the range of 1001 - 995 cm^{-1} was due to a weaker frequency of aromatic C-H in the plane bend. Then, the spectra at the wavenumber of 1343 - 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. However, in the result, 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.

3.2 Multivariate Analysis

Multivariate analyses were applied to develop qualitative and quantitative models with the FTIR data sets as FTIR peaks itself was not enough to classify and differentiate between unadulterated and adulterated milk samples. Classification and quantification of unadulterated and adulterated milk samples were evaluated using PCA, DA and MLR [14].

3.2.1 Classification of unadulterated and adulterated milk samples

PCA was performed for possible clustering of samples. The samples lying closer together in the scores plot was more similar while the samples far away from each other were considered different from each other [18]. PCA was run using variables obtained from variable transformation. Then, the significant variable obtained was confirmed using DA.

3.2.1.1 PCA of melamine

From Figure 1 below, it can be seen that variables in wavenumber of 1560.9076 cm^{-1}, 772.2385 cm^{-1}, 1429.4627 cm^{-1}, 1634.8453 cm^{-1}, 2998.5856 cm^{-1}, 1659.4912 cm^{-1}, 1667.7065 cm^{-1} and 3335.4130 cm^{-1} were used for PCA modelling due to its capability in providing good separation among the evaluated samples. Then, the unadulterated milk sample was separated from the other groups as they were not adulterated with melamine. The group of 0.2 % of melamine were seen close to the unadulterated group due to the low concentration of melamine. This may also indicate that the FTIR used was not sensitive enough to detect such low concentrations of melamine. Then, it can also be seen that the groups for 0.8 %, 1.2 % and 1.5 % of melamine were overlapped indicating the correlation between them. Meanwhile, the group of 2.0 % melamine were widely separated from the unadulterated sample indicating the potential of FTIR in discriminating the unadulterated and adulterated milk sample. In addition, variable 1560.9076 cm^{-1} lies in the direction of unadulterated milk, indicating that unadulterated milk samples were detected in that variable. Next, variables 2998.5856 cm^{-1}, 1659.4912 cm^{-1}, 1667.7065 cm^{-1} and 3335.4130 cm^{-1} lay near the melamine 2.0 % which means melamine can be detected in those wavenumbers. This might be due to the NH_2 stretch of melamine that was detected by the FTIR spectra. PC-1 (F1) described a 68.35 % variance while PC-2 (F2) described a 22.91 % variance. Thus, the sum of the variances explained by PC-1 and PC-2 was 91.26 % with a significance level of 5 %, i.e., a confidence interval of 95.5 % which indicated the potential of spectroscopy in discriminating unadulterated and adulterated milk samples.

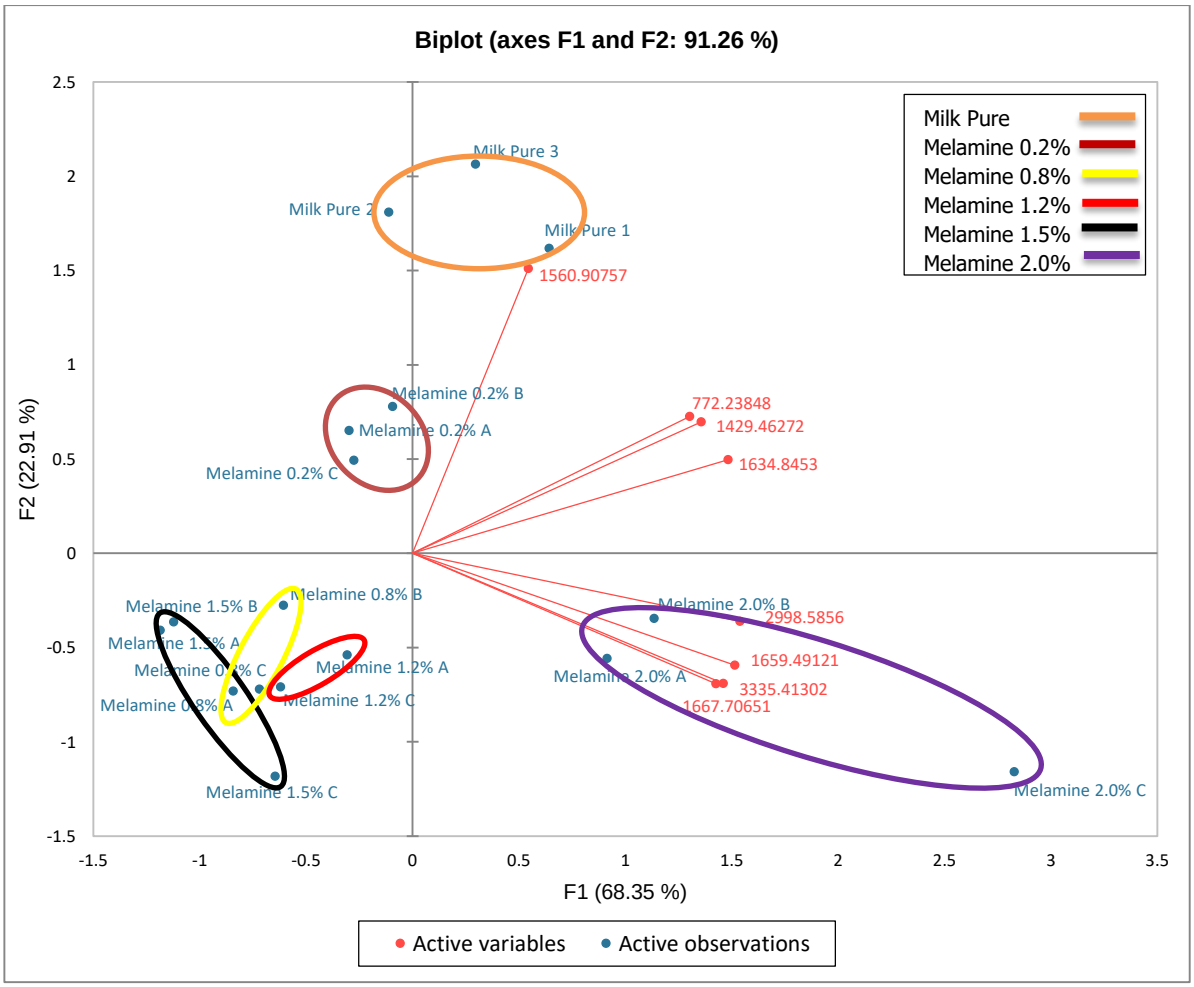

Fig. 1. Biplot for pure and adulterated milk samples of melamine

3.2.1.2 PCA of formalin

From Figure 2 below, the variables show (2982.1550 cm^{-1}, 1577.3382 cm^{-1}, 1536.2617 cm^{-1}, 1519.8311 cm^{-1}, 1281.5873 cm^{-1}, 1092.6353 cm^{-1}, 1043.3435 cm^{-1} and 1010.4823 cm^{-1}) were all significant with *p*-value < 0.05. All these variables were used for PCA modelling as it provided good classification among the evaluated samples. All the significant variables were also positively correlated with each other. From this biplot, variable 1577.3382 cm^{-1} was seen lying at formalin 1.5 % and formalin 2.0 % which indicates that formalin in those concentrations can be detected around the wavenumber of 1577.3382 cm^{-1}. PC-1 (F1) described a 76.16 % variance while PC-2 (F2) described a 9.36 % variance. Thus, the sum of the variances explained by PC-1 and PC-2 was 85.52 % with a significance level of 5 %, i.e., a confidence interval of 95.5 %.

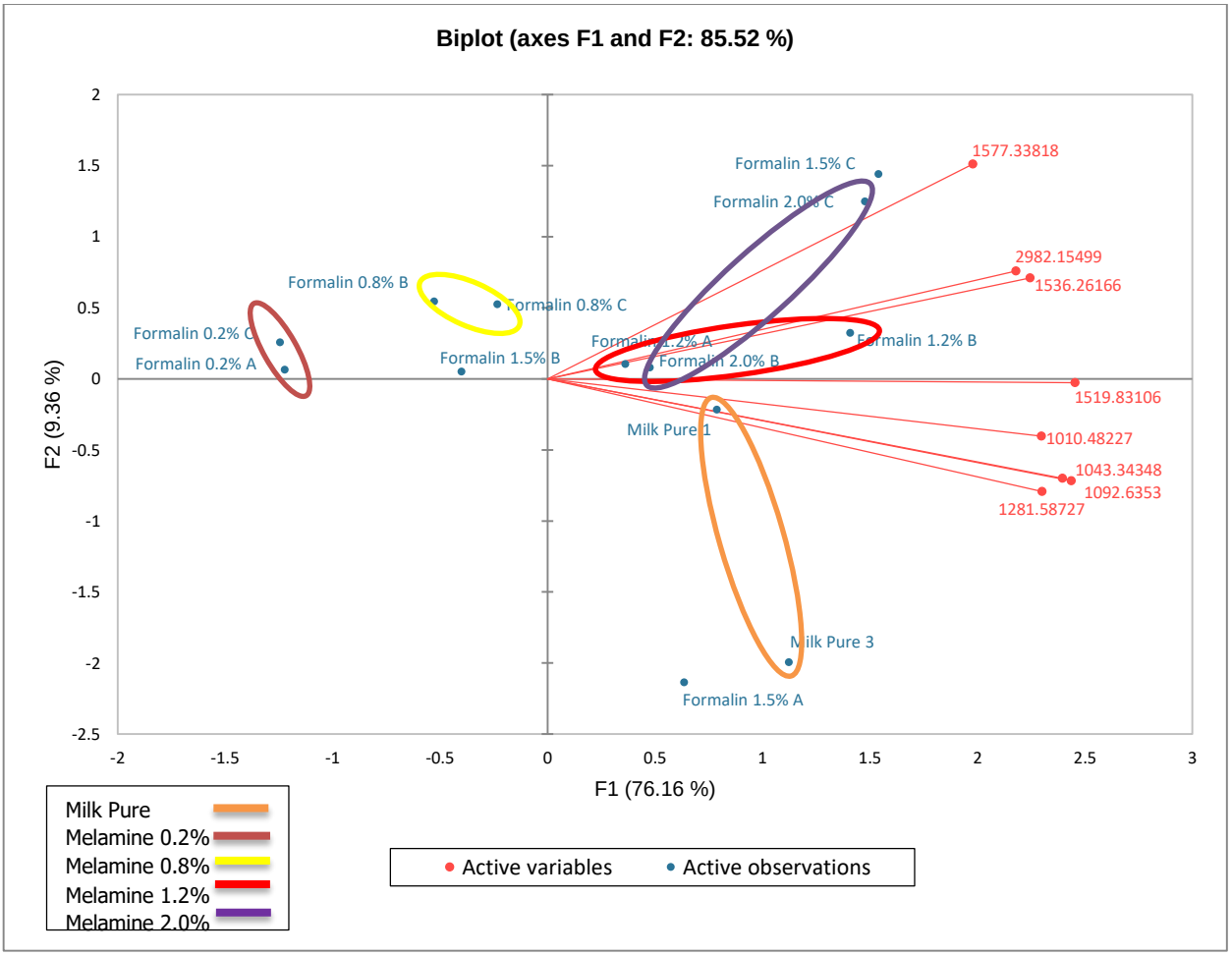

Fig. 2. Biplot for pure and adulterated milk samples of formalin

3.2.1.3 PCA of anionic detergent

Figure 3 below shows the significant variables and active observation of samples. From this biplot, positive correlation was seen between all the significant variables (3023.2315 cm^{-1}, 2637.1123 cm^{-1}, 2505.6674 cm^{-1}, 2431.7297 cm^{-1}, 1552.6923 cm^{-1}, 1528.0464 cm^{-1}, 1306.2332 cm^{-1}, 952.9752 cm^{-1} and 780.4538 cm^{-1}) involved as their vectors were close to each other. Variable 780.4538 cm^{-1} was observed to lay close to detergent 2.0 % which indicates the detergent 2.0 % can be detected around that wavenumber. This may be due to the detection of weak frequency of aromatic C-H in-plane bend [18]. As for the observation of unadulterated and adulterated milk samples, high overlapping can be seen especially for detergents 0.2 %, 0.8 %, 1.2 % and 1.5 %, which indicated the high correlation between the samples thus leading to failure of classification by PCA.

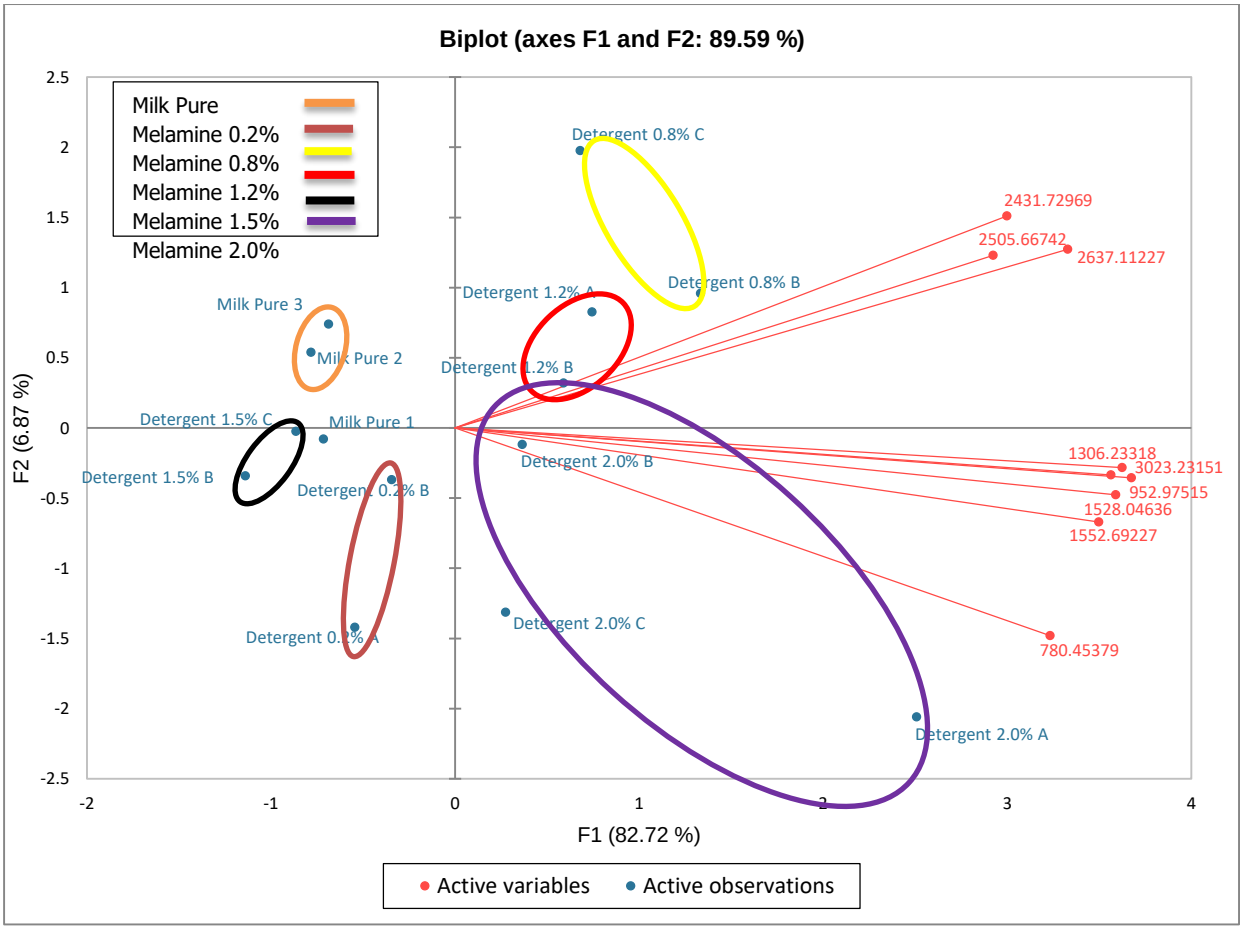

Fig. 3. Biplot for pure and adulterated milk samples of anionic detergent

3.2.2 MLR

MLR was pragmatic to predict multiple outcome variables using one or more variables. It also helped in determining the numerical relationship between the set of variables and the others. In this study, linear relationships between the variables and the observations were also obtained through MLR. Significant variable obtained from PCA and DA was applied for MLR to predict the level of adulteration. In addition, the RMSE, R^2 and MSE were attained to evaluate the performance of MLR in this study.

3.2.2.1 MLR for melamine

The predicted levels of adulteration were compared with the actual level of adulteration and the results are shown in Figure 4. It can be observed that MLR was successful in predicting the actual adulteration levels [19]. For the unadulterated milk sample, MLR predicted melamine concentration to be -0.1 - 0.16 %, while for melamine adulterated milk sample of 0.2 %, 0.8 %, 1.2 %, 1.5 % and 2.0 %, the predicted level was 0.068 - 0.271 %, 0.809 - 0.956 %, 1.097 - 1.284 %, 1.429 - 1.585 % and 1.695 - 2.079% respectively, which were close to the actual adulteration levels.

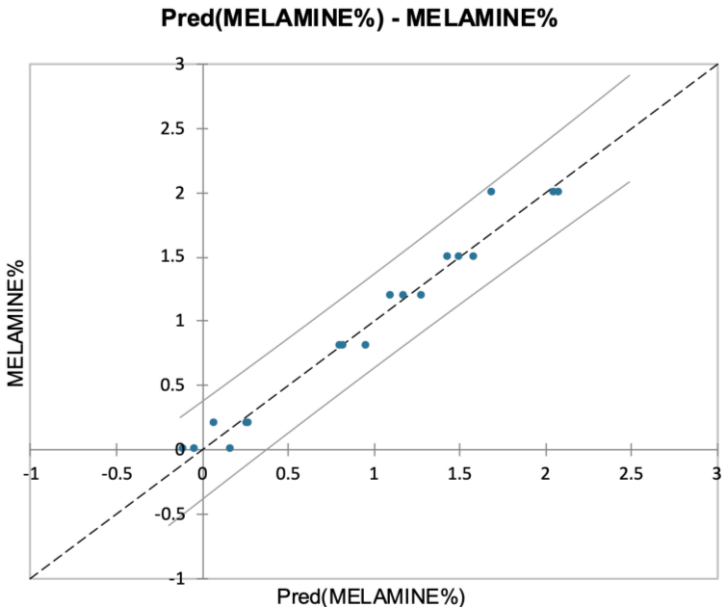

Fig. 4. Predicted level of unadulterated milk sample and melamine adulterated samples

Table 1 shows standardized coefficients of melamine obtained from MLR that highlights the variable 1560.9076 cm^{-1} was the most significant variable compared to other variables as it had the lowest Pr > |t| value compared to other variables.

Table 1
Standardize coefficients of melamine %

Source	Value	Standard error	t	Pr > |t|	Lower bound (95 %)	Upper bound (95 %)
3335.4130	0.142	0.200	0.708	0.497	-0.311	0.594
2998.5856	-0.188	0.198	-0.949	0.367	-0.637	0.260
1667.7065	-0.691	0.564	-1.226	0.251	-1.966	0.584
1659.4912	1.187	0.557	2.133	0.062	-0.072	2.447
1634.8453	0.137	0.228	0.602	0.562	-0.378	0.652
1560.9076	-0.780	0.139	-5.591	0.000	-1.095	-0.464
1429.4627	-0.462	0.144	-3.207	0.011	-0.788	-0.136
772.2385	0.454	0.129	3.505	0.007	0.161	0.746

The RMSE obtained from this MLR model was 0.158 %, which meant the MLR managed to generate an almost accurate prediction ability and the R^2 obtained was 0.975 which was good and indicated that this method can explain more than 97 % of the experimental data [20]. The MSE was also obtained to be 0.025. In addition, the cross-validation produced was 61.11 %.

3.2.2.2 MLR for formalin

The predicted levels of adulteration were compared to the actual level of adulteration and the results are as shown in Figure 5. It can be observed that although the linearity was not as good when compared to melamine data prediction, the MLR was still almost successful in predicting the actual adulteration levels of formalin in milk samples. For unadulterated milk samples, MLR predicted

formalin concentration to be -0.024 - 0.134 %, while for formalin adulterated milk samples of 0.2 %, 0.8 %, 1.2 %, 1.5 % and 2.0 %, the predicted level were 0.071 - 0.314 %, 0.720 - 1.181 %, 1.086 - 1.227 %, 1.283 - 2.004% and 1.720 - 1.797% respectively.

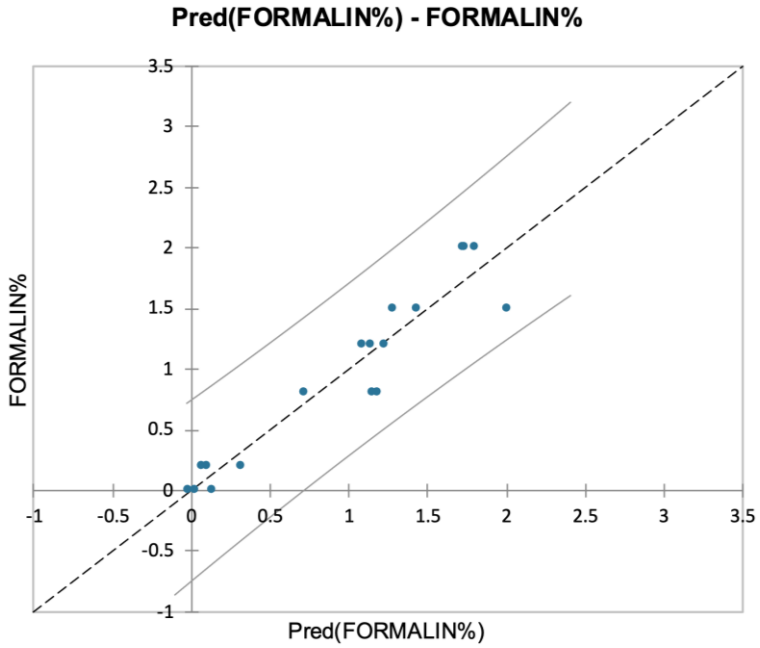

Fig. 5. Predicted level of unadulterated milk sample and formalin adulterated samples

From Table 2, the standardized coefficients of formalin obtained from the MLR model, among all the explanatory variables, variable 1536.2617 cm^{-1} was the most influential with the Pr > |t| value of 0.005.

Then, the RMSE, R^2 and the MSE values produced were 0.308 %, 0.904 and 0.095 respectively. The RMSE value of formalin prediction was bigger compared to melamine, indicating the less accurate prediction when compared to the actual value. In addition, the cross-validation obtained was 66.67 %.

Table 2
Standardize coefficients of formalin %

Source	Value	Standard error	t	Pr > |t|	Lower bound (95 %)	Upper bound (95 %)
2982.1550	0.479	0.189	2.528	0.032	0.050	0.907
1577.3382	-0.038	0.183	-0.207	0.841	-0.451	0.376
1536.2617	0.738	0.199	3.715	0.005	0.289	1.188
1519.8311	0.209	0.282	0.741	0.477	-0.429	0.848
1281.5873	0.791	0.257	3.077	0.013	0.209	1.372
1092.6353	-0.287	0.367	-0.781	0.455	-1.117	0.544
1043.3435	-0.839	0.297	-2.827	0.020	-1.510	-0.168
1010.4823	-0.662	0.231	-2.869	0.018	-1.183	-0.140

3.2.2.3 MLR for anionic detergent

From the graph in Figure 6 below, it can be seen that the prediction ability of MLR in detergent samples was not very good. This might be due to the high correlation between samples. Meanwhile, the RMSE, R^2 and MSE values obtained were 0.639 %, 0.632 and 0.408 respectively. The R^2 value of 1 refers to the perfect fit where the concentration of adulterant was fully explained and the R^2 value of 0 indicates that the model does not explain the variation of the adulterant concentration [21]. As the R^2 value obtained here was 0.632, it indicates a low percentage of samples which were explained in the results.

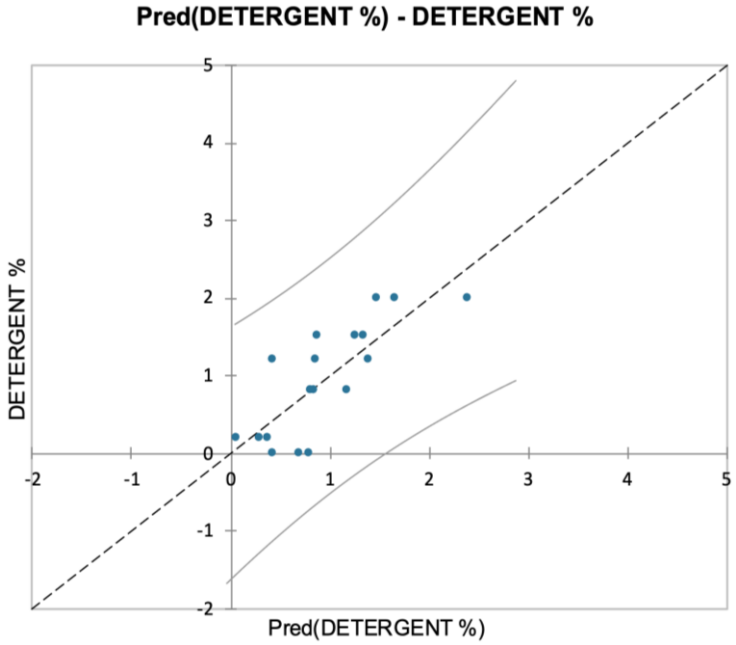

Fig. 6. Predicted level of unadulterated milk sample and anionic detergent adulterated samples

4. Conclusions

In conclusion, adulteration of milk which have proven to be detrimental to human health must be prevented. Parallel to the evolution of the world, adulteration of milk can also be carried out in a more subtle way, which makes their detection and quantification much more difficult. Thus, an advanced method that is simple yet provide high sensitivity and accuracy were needed in order to detect and quantify adulteration in milk products.

ATR-FTIR spectroscopy was used in this study, where most common adulterants (melamine, formalin and anionic detergent) applied in the milk, with concentration of 0.2 %, 0.8 %, 1.2 %, 1.5 % and 2.0 % for each adulterant. The detection of adulterants by FTIR spectroscopy was shown based on its transmittance spectra in wavenumber range of 4000 - 500 cm^{-1} and was further quantified using multivariate analysis.

PCA was proven successful in classifying melamine and formalin adulterated samples. However, in the case of detergent adulterated sample, PCA failed to classify the samples which may be due to lack of precision during sample preparation. Then, MLR was applied to quantify the adulterants detected. From all three data, data of melamine adulterated sample was the most satisfactory with

RMSE, R^2 and MSE values of 0.158 %, 0.975 and 0.025 respectively, followed by MLR performed on formalin data and detergent data. The RMSE, R^2 and MSE values produced for formalin were 0.308 %, 0.904 and 0.095 respectively while the RMSE, R^2 and MSE values obtained for detergent were 0.639 %, 0.632 and 0.408. Thus, from this study, it can be concluded that the FTIR spectroscopy coupled with multivariate analysis is a rapid, sensitive and non-destructing technique that can be used for the qualification and quantification of adulterant residues in milk products.

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