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Salting-Out Assisted Liquid-Liquid Extraction for Quantification of Ammonia Compounds in Food Samples

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The extraction of ammonia compounds from food samples is a challenging topic due to the hydrophilic property of ammonia. A simple and rapid extraction method was developed for the determination of ammonia in a type of local bread (Ahari bread) and milk samples using a salting-out assisted liquid-liquid extraction and spectrophotometry. Acetonitrile was used to clean up, and potassium chloride salt solution was added to separate the acetonitrile and aqueous phase by creating two phases. The ammonia concentration in the aqueous phase was determined based on a colorimetric method. Various parameters affecting absorption intensity were optimized using the response surface methodology. Then, the optimized parameters were applied to establish a valid analytical method. The developed method was used to quantify ammonia compounds in food samples, *i.e.* Ahari bread and milk, by the standard addition method. The linearity of the method was from 2.5 to 40 μg ml with a coefficient of determination (R^2) of 0.994, and numerical values of a limit of detection, and limit of quantification was found to be 0.39 and 1.29 μg ml^{-1}, respectively. The accuracy (%error) and precision (%relative standard deviation) were in the acceptable range. The established extraction technique and standard addition method could detect ammonia in real food samples. These data showed that the established analysis approach is a simple and sensitive technique for extraction and determination of ammonia concentration in foodstuff.

Keywords: Ammonia, Bread, Liquid-liquid extraction, Milk, Salting out, Standard addition

INTRODUCTION

With the emergence of new technologies and increasing human attention to the health and safety of food and drinking water, the need for further and faster methods is felt to control food safety. On the one hand, due to population growth and lack of access to water resources and safe food, could be seen increased fraud in the food and beverage industry. The presence of ammonia in salt form in drinking water can be due to the contamination of river water with industrial wastewater or the activity of bacteria in the polluted underground water bed. They are also used in the food industry to increase the cooking speed of biscuits and create a crispy texture in them and bulk up fantasy bread, whose effects on human health have not yet been entirely determined. These compounds *e.g.*, ammonium bicarbonate, are used as a leavening agent in yeast when preparing various types of baked goods. This material creates a puffy dough that is suitable for organizing all kinds of bread, crackers, and some cookies. Ammonium bicarbonate can also be used as a foaming agent in beverages and is generally used in food, *i.e.* crackers, pasta, ice cream, biscuits, pigments and colors, and

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Fig. 1. A picture of Ahari bread, the local sweet bread of Iran.

bakery materials [1,2].

Ahari bread (Fig. 1) is one of the local sweet bread of Iran. This bread has a unique aroma and taste. The texture of this bread is not hollow and resembles a cookie. Traditionally, yogurt and vinegar were used to create the right texture in this bread. However, in the industrial baking of this bread, ammonia powder is used so that the dough increases in volume and is economically more affordable.

Milk is named a nearly perfect food and forms an important part of the human diet worldwide and across all age groups owing to its nutritional and health benefits. Milk due to its adulteration with harmful chemicals such as urea, sodium hydroxide, and ammonium sulfate, could detrimentally affect human health [3,4]. One of the easy access and low cost which can be quickly done in milk is adding ammonia to raw milk to increase total protein.

Because of impurity and knowing the food sample components, the quantification of ammonia is a critical issue in food sciences [1,5]. The Kjeldahl method is an old method for the quantification of ammonia compounds. It is considered a reference method in protein measurement. This method is used to analyze the nitrogen content in proteins. First, the proteins are digested under the influence of the acidity of the environment, and the nitrogen in them is released in the form of ammonium ions. Then, the concentration of the released ammonium ion is determined by methods such as titration and neutralization. This method includes the stages of digestion, distillation, and titration and is not used much because of its time-consuming [1]. For the quantification of ammonia compounds in water samples, an ultraviolet-visible spectrophotometric method was developed. The blue indophenol method (Berthelot reaction), Nessler method, and salicylate-chlorine reaction are standard colorimetric methods for the analysis of ammonia. The salicylate-chlorine reaction is a method based on the salicylate-chlorine reaction with two reagents (sodium dichloroisocyanurate and sodium nitroprusside) in the field of water quality control. When ammonia reacts with an alkaline mixture of sodium salicylate and sodium dichloroisocyanurate, it produces an emerald-green color that can be measured by absorption spectrophotometry. Salicylate-chlorine reaction with ammonia is sensitive, specific, stable, and reproducible [6].

The salicylate-chlorine reaction is a cheap, simple, and useful method for the analysis of ammonia in water samples. However, applying these methods to complex matrix *i.e.*, biological and food sample, is impossible without applying extraction methods. Various ways have been reported for the analysis of food samples. However, most of the methods e.g., liquid-liquid extraction, is appropriate for hydrophobic compounds. The use of extraction methods with the help of salts can be used to concentrate and clean samples containing hydrophilic compounds such as ammonia, considering the solubility of ammonia compounds in water. Previous studies on the use of salt solutions, such as potassium chloride to extract ammonia from different samples such as plant samples, the use of salt-based extraction methods along with alcoholic solvents (substitute solvent halogenated) can be a suitable method for extracting ammonia and its derivative forms such as indophenol [2,7].

Therefore, salting-out assisted liquid-liquid extraction (SALLE) could be applied for the extraction of ammonia from the complex matrices. This method can separate a wide range of drugs and metabolites, including many hydrophilic compounds. It also provides cleaner extracts due to proper phase separation. This method is faster, environmentally friendly, and more economical. It is used to extract organic compounds from different samples with an organic solvent that can be mixed with water, such as acetonitrile or ethanol. A high concentration of electrolyte salt in an aqueous solution reduces the solubility of the analyte or solvent with

low polarity. It causes the analyte to precipitate from the solution, or the solvent forms a separate phase [8,9].

The main objective of this study was to establish a rapid simple, and environmentally friendly analytical method for ammonia extraction and measurement in food samples. This extraction method could be used for the clean-up of studied food samples and extraction of ammonia as a hydrophilic compound and analyzed based on the salicylate-chlorine reaction. For resolving the matrix effect, we used the standard addition method for the analysis of each sample.

MATERIALS AND METHODS

Chemical and Apparatus

Sodium carbonate (Na_2CO_3), potassium chloride (KCl), sodium hydroxide (NaOH) sodium chloride (NaCl), salicylic acid, sodium nitroprusside, ammonium hydroxide (25%), acetonitrile, hydrochloric acid (HCl) (37%) were purchased from Merck chemical company (Germany). Sodium hypochlorite (6%) was provided from Tage Company (Iran). Lab-made double-distilled water was used to prepare the solutions. Spectrophotometer-SPEKOL 1500 (Germany) was used for the analysis of samples at 640 nm. Hettich centrifuge (Germany) was obtained at 3000 rpm for 3 min to separate the precipitate from the solution. KERN Laboratory digital scale (Germany) was used for weighing samples.

Analysis of Ammonia Using a Colorimetric Method

For the analysis of ammonia, a colorimetric method based on the salicylate-chlorine reaction was applied [10,11]. Ammonia converts to monochloroamine with the addition of chlorine. The monochloroamine then reacts with salicylate to form 5-aminosalicylate. 5-aminosalicylate is oxidized by nitroferricyanide and forms indosalicylate which is a colorful compound.

For this purpose, two solutions as follow were prepared:

Solution 1: Hypochlorite solution- It was prepared by adding 1 ml of sodium hypochlorite and 0.5 g of NaOH was added into a 100 ml volumetric flask and filled with 70 ml of distilled water. It was mixed until dissolved and filled flask to the 100 ml mark.

Solution 2: Salicylate solution: Placed 5 g of salicylic acid into a 50 ml volumetric flask and fill it with 35 ml water until dissolved then added 0.02 g of sodium nitroprusside and mix it until dissolved then added 2.5 g NaOH to adjust the pH to the 12.0 range and fill the flask to the 100 ml mark. Then the solution was transferred into a dark, airtight glass bottle for longevity.

Optimization of SALLE Extraction Method and Validation Procedures

Optimization of parameters is an essential aspect of an analytical method. Multivariate experimental design strategies are becoming increasingly favored in the optimization of analytical methods against univariate procedures because of taking lower time, attempt, work, and resources. The goals of these methods are to make a regression model to check the effects of two or more factors with their interactions on the response function [12-14]. For optimization, different values will be selected for each parameter (pH, type of salt, the concentration of a saline solution, *etc*.), and the proficiency of the method will be evaluated for each parameter while keeping other analytical conditions constant.

In this study, to achieve optimal conditions, various parameters such as pH (the effect of three media, HCl (0.1 M), water, NaOH (0.1 M)), and the type of salt, volume, and concentration of salt was optimized.

The method used to extract ammonia based on optimized conditions includes 900 µl of acetonitrile containing an appropriate amount of ammonia being added to a microtube. Then, 200 µl of KCl 2 M dissolved in HCl 1 M was added to acetonitrile, and two phases were formed. The lower phase was separated with a syringe, and the acidic pH of the solution was neutralized by adding 100 µl NaOH 1 M. Finally, 250 µl of solution 1 and 250 µl of solution 2 were added to the resulting mixture, and it was necessary to wait for a fixed time (1 h) for color change at 40 °C.

The calibration curve was also drawn for different ammonia concentrations against absorption. The method validation and sensitivity parameters included accuracy, linearity, and precision, LOD and LOQ were evaluated according to the international guidelines.

Application of the Developed Method on Real Samples

The amount of ammonia in real samples *i.e*., milk (pasteurized and raw milk), and Ahari bread (five samples

provided from local confectionary or bakery from Tabriz, Iran) was measured by the desired method and under optimized conditions. Different types of the studied real samples *i.e.* Ahari bread and milk are available in the market. Therefore, A standard addition calibration curve for bypass of the matrix effect was also applied to find the amount of ammonia in the analyzed sample [15].

Analysis of Ammonia in the Milk Sample

Pasteurized milk was purchased from a local manufacturer (Tabriz, Iran). Then, various concentrations of ammonia were added to the milk sample. Then, 100 µl was taken from the mixture of milk, and ammonia, and 900 µl of acetonitrile was added to it and centrifuged for 3 min at 3000 rpm. Then, 900 µl of the supernatant was taken, ammonia extraction by SALLE was performed according to the optimal conditions, and absorption was measured with a spectrophotometer at a wavelength of 640 nm.

The calibration curve for different concentrations of ammonia in milk was drawn based on optimal conditions, and the accuracy and repeatability of the method were also investigated. This method was also performed under optimal conditions for the raw milk sample.

Analysis of Ammonia in Ahari Bread Samples

Ammonia quantification in Ahari bread samples was done according to standard increments and optimal conditions. In this method, first, 0.5 g of Ahari bread sample was weighed then 3 ml of acetonitrile was added to the desired sample. It was centrifuged for 3 min at 3000 rpm. Then, 900 µl of the supernatant was collected and transferred to a microtube. The rest of the steps were performed by SALLE according to the optimal conditions to calculate the ammonia concentration based on absorption in spectrophotometry.

The calibration curves for different samples of Ahari bread were drawn based standard addition method, and the accuracy and repeatability of the method were also evaluated based on optimal conditions.

RESULTS AND DISCUSSION

Optimization of Extraction Parameters

Response surface methodology (Box Behnken design) was used to optimize the method. It determines the main and interactive effects of the factors and gives a possibility to attain the most information according to the minimum number of experiments. Box Behnken is a useful method for a few factors and avoids the extreme conditions of the experiment and is suitable when all design points fall within a safe operating zone unlike the central composite design [16,17].

In this study, a fractional factorial design was employed to determine optimal conditions. It was accomplished with MINITAB software (Minitab Inc., release 17). Therefore, the effect of the main parameters affecting the extraction improvement, including salt type, salt volume, salt concentration, and pH was selected as independent variables. The absorption values were considered to choose the best conditions for ammonia extraction. The experimental matrix and obtained absorbance have been reported in Table 1. To attain proper phase separation and optimal extraction efficiency, three types of salts, NaCl, KCl, and Na_2CO_3, were studied. The analysis of results showed the type of salt for extraction has a considerable effect. Compared to other salts, KCl caused sufficient phase separation between the organic and aqueous phases. It is an excellent desalting agent for sample processing and improves phase separation and ammonia extraction.

Analysis of quantitative parameters stepwise indicate that pH, volume, and concentration and some of the quadratic parameters and binary interactions have a statistically significant effect ($p < 0.05$), and the following equation is obtained:

$$\begin{aligned}\text{Absorbance} = {} & 3.090 - 0.1207 \times \text{pH} - 0.00297 \times \text{Volume} \\ & - 0.135 \times \text{Concentration} + 0.00351\ \text{pH} \times \\ & \text{pH} - 0.000002\ \text{Volume} \times \text{Volume} \\ & + 0.089\ \text{Concentration} \times \text{Concentration} \\ & + 0.000091\ \text{pH} \times \text{Volume} - 0.0070\ \text{pH} \times \\ & \text{Concentration} + 0.000753\ \text{Volume} \times \\ & \text{Concentration}\end{aligned}$$

Correlation coefficient (R^2), adjusted-R^2, predicted R^2 values were 0.94, 0.90, and 0.82. These data confirm a good correlation between absorbance and the studied parameters. In addition, adjusted-R^2 predicted R^2 values confirm the accuracy and adequacy of the proposed model by Box-Behnken design for optimization of parameters.

Table 1. Optimization of Salt Type (Na_2CO_3, NaCl, and KCl), pH, and Salts Concentration for Extraction of Ammonia (40 µg ml^{-1}) by SALLE and the Effect of these Values on UV Absorption

No.	pH	Volume (µl)	Concentration (M)	Salt	Absorbance
1	7	600	1.0	Na_2CO_3	1.80
2	7	400	1.5	NaCl	0.60
3	7	400	1.5	NaCl	0.56
4	7	200	1.0	NaCl	0.55
5	7	600	1.0	KCl	0.69
6	13	600	1.5	NaCl	0.42
7	1	200	1.5	KCl	2.51
8	7	600	2.0	Na_2CO_3	1.78
9	7	600	2.0	NaCl	0.78
10	7	200	2.0	NaCl	0.70
11	7	600	1.0	NaCl	0.38
12	13	400	1.0	KCl	1.25
13	1	400	1.0	KCl	1.86
14	7	600	2.0	KCl	1.40
15	7	400	1.5	Na_2CO_3	1.46
16	1	400	2.0	Na_2CO_3	1.95
17	13	400	2.0	Na_2CO_3	2.07
18	1	200	1.5	Na_2CO_3	1.89
19	13	400	2.0	KCl	1.58
20	13	400	1.0	Na_2CO_3	2.17
21	1	400	1.0	NaCl	0.45
22	7	400	1.5	Na_2CO_3	1.46
23	7	200	2.0	Na_2CO_3	1.29
24	1	400	2.0	KCl	2.34
25	7	200	2.0	KCl	2.13
26	13	200	1.5	NaCl	0.51
27	7	400	1.5	KCl	1.71
28	1	400	2.0	NaCl	0.60
29	7	400	1.5	Na_2CO_3	1.50
30	1	600	1.5	Na_2CO_3	1.92
31	7	200	1.0	Na_2CO_3	1.29
32	7	400	1.5	NaCl	0.81
33	13	200	1.5	KCl	1.59
34	7	400	1.5	KCl	1.75
35	1	600	1.5	NaCl	0.44
36	1	400	1.0	Na_2CO_3	1.85
37	7	400	1.5	KCl	1.67
38	13	400	2.0	NaCl	0.64
39	13	400	1.0	NaCl	0.39
40	1	600	1.5	KCl	1.11
41	7	200	1.0	KCl	2.10
42	13	200	1.5	Na_2CO_3	1.84
43	1	200	1.5	NaCl	0.62
44	13	600	1.5	KCl	1.20
45	13	600	1.5	Na_2CO_3	2.09

Surface plots for the pH, concentration, and volume were illustrated in Fig. 2. Different concentrations of three types of salts with values of 1, 1.5, and 2 M were selected, and absorption was measured. The concentration of salt has a minimum effect on extraction recovery (Fig. 2). However, the maximum values were observed in KCl 2 M. Finally, the volume of salt solution for separating and cleaning ammonia was 200 µl of KCl 2 M. The lowest volume of the salt solution decreases the volume of an aqueous phase, and the maximum amount of ammonia is extracted in this phase. The effect of pH on ammonia extraction was determined by dissolving KCl in hydrochloric acid 1 M (pH = 1), sodium hydroxide 1 M (pH = 13), and water, so acidic media was chosen as the suitable environment for preparing the KCl solution. The ionization of ammonia to ammonium form in acidic media is appropriate for its affinity to aqueous media and distribution from the acetonitrile phase to the water phase. The generated data were used to justify the optimal volume for better ammonia recovery from the samples.

Validation of the Analytical Method

The SALLE method based on the best condition in the previous section (200 µl KCl 2 M in HCl 1 M) was optimized and the analytical characteristics of the method were determined under the above experimental conditions.

Calibration Curve

Under optimal conditions, the calibration graph linear relationship was observed between the concentrations of ammonia and intensity in the range of (0, 2.5, 5, 10, 20, and 40 µg ml^{-1}) with a determination coefficient (R^2) of 0.994. The linear equation is described by the regression equation as follows: Absorbance = 0.0468 × concentration (mg l^{-1}) + 0.0102 (R^2 = 0.994).

The LOD and LOQ were computed as follows: LOD = 3 × standard deviation of blank/slope of the calibration curve and LOQ = 10 × standard deviation of blank/slope of the calibration curve. Numerical values of LOD and LOQ were found to be 0.038 and 1.29 µg ml^{-1}, respectively.

Accuracy and Precision

The precision and accuracy (inter-day) concentration of ammonia were determined by evaluating three replicates at three concentrations (7.5, 15, and 30 µg ml^{-1}). The results are

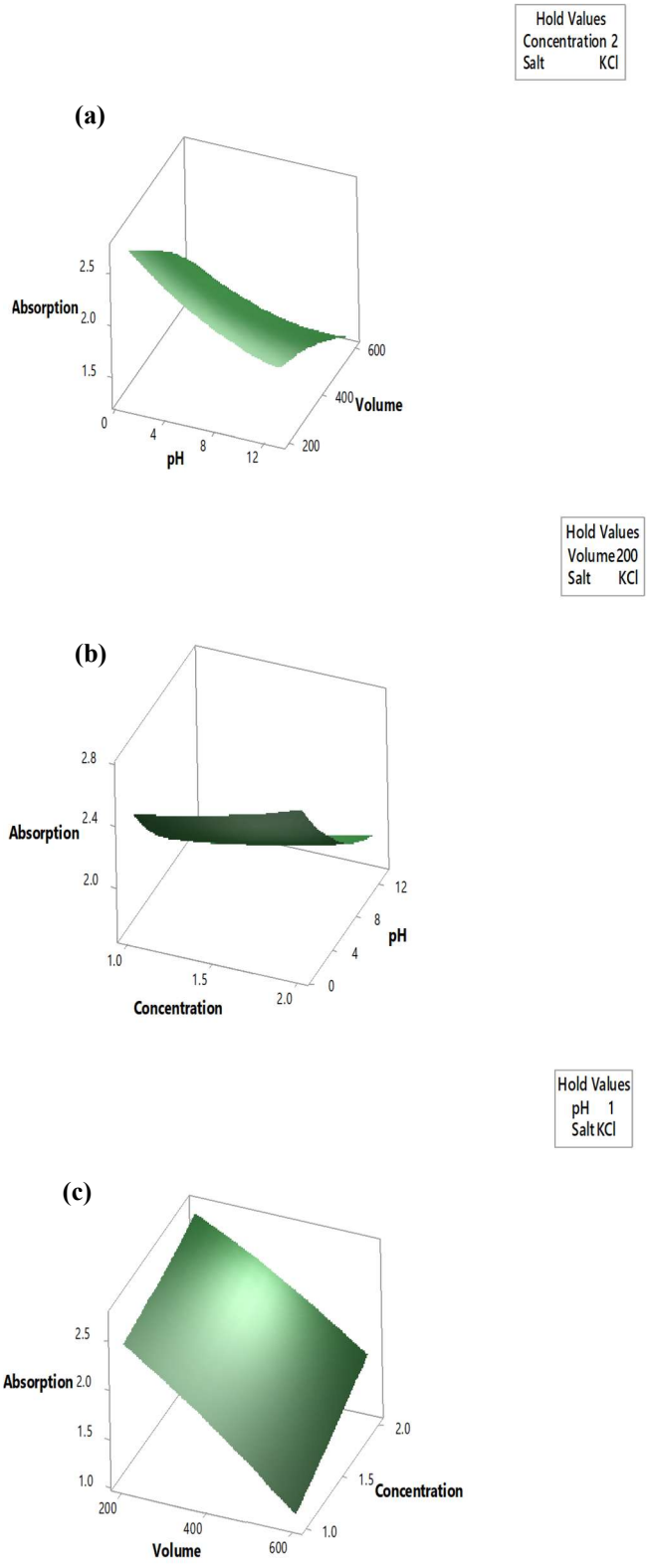

Fig. 2. Surface plot of absorption of ammonia (a) pH *vs*. volume of KCl, (b) pH *vs*. concentration of KCl after SALLE.

Table 2. Precision (%RSD), and Accuracy (%Relative Recovery) of the Developed Method

Nominal concentration (µg ml^{-1})	%RSD	Founded concentration (µg ml^{-1})	%Relative recovery[a]
7.50	1.0	6.8	90.7
15.0	6.5	13.9	92.7
30.0	11.4	25.0	83.3

[a]%Relative recovery (accuracy) = (Founded concentration/Nominal concentration) × 100%.

listed in Table 2. The mean observed concentrations are within < ± 17% of the nominal levels. The acquired results for precision (%RSD) are less than 12%, at all concentrations acceptable. Therefore, it indicated that the technique was satisfactory in accuracy and precision.

Application of the Method to Real Samples

To evaluate the applicability of the established method and ensure the accuracy of the procedure for the quantification of ammonia, the standard addition method [18] was used on milk and Ahari bread samples. In this method, it is assumed that the complex matrix of food such as milk and Ahari bread does not affect the overall results. Because it is not possible to find a sample of a food matrix, especially for Ahari bread. Therefore, analysis of real samples was performed by this method. In this method, equal volumes of food samples were taken, except for one, spiked with known and different amounts of ammonia. Then all of them were diluted to the same volume, and the next steps were performed according to the optimal conditions. Finally, the amount of absorption for the samples was measured, and the standard addition curve was drawn. In this curve, X-axis is the concentration of ammonia added to the samples, and the Y-axis is the absorption rate drawn. It minimizes the matrix (*i.e.*, food samples) effects that interfere with analyte measurement signals.

Milk samples. For analysis of ammonia levels in milk samples, the calibration curve was constructed using five spiking levels of ammonia to commercial pasteurized milk samples and raw milk in concentrations. Then, the ammonia concentration was determined by the SALLE method after the precipitation of matrix components by acetonitrile is described in detail in the method section. The concentration of ammonia was determined by the absolute value of the X-intercept (Fig. 3). According to the obtained results from the calibration curve in pasteurized and raw milk samples, the concentration of ammonia in both samples was less than the LOD of the method.

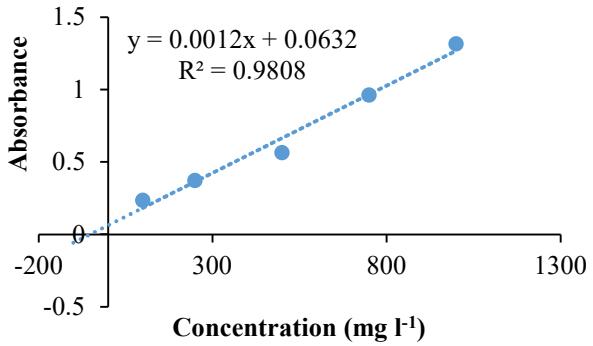

Fig. 3. A calibration curve (standard addition method) for quantification of ammonia in milk samples.

Ahari bread samples. The standard addition method was used to calculate the concentration of ammonia in five different samples of Ahari bread. To measure the concentration of ammonia, different concentrations of ammonia were spiked in Ahari bread samples. After extraction by the SALLE method, the absorption of each sample was also measured. For each sample, a separate calibration curve based on the standard addition method was drawn. In some cases, like to studied milk samples, the concentration of ammonia in both samples was less than the LOD of the method (Fig. 4a), while in some samples, a significant amount of ammonia based on the value of the x-intercept was observed (Fig. 4b).

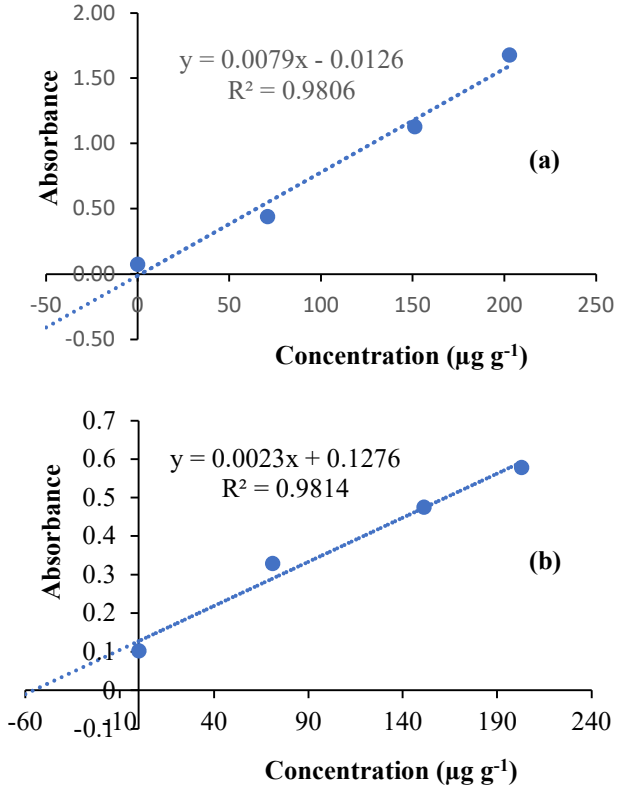

Fig. 4. A calibration curve (standard addition method) for quantification of ammonia in Ahari bread samples a) sample 1, b) sample 2.

In the studied five samples, in one of the samples, the concentration of ammonia was less than the LOD of the method, which indicates that ammonia compounds have not been added in the cooking of the bread or completely evaporated in the cooking process. However, four samples have ammonia with 43 µg g^{-1}, 21 µg g^{-1}, 55 µg g^{-1}, and 36 µg g^{-1}.

The result of using ammonium bicarbonate in a baked product will be a light, crispy, and somewhat soft texture. Today, this compound is replaced by baking powder or baking soda. However, real ammonium bicarbonate is still preferred by people who want a lighter or crisper texture. But remember that the final product should be something that essentially dries out when baked. Otherwise, some unpleasant smell will remain. Also, in Ahari bread, because there is no standard for the maximum amount of ammonia powder used for baking this bread, its excessive use causes a soapy taste. In addition, some of this ammonia can enter the bloodstream and be transported throughout the body within seconds and has adverse effects on the human digestive system. [19] Ammonium bicarbonate is widely recognized as a safe compound approved by the US FDA as GRAS (Generally Recognized as Safe) and is accepted as a safe food additive in many countries under the number E503. However, consumers are still advised to consult with experts before using foods containing large amounts of ammonium bicarbonate for long periods. On the other hand, minerals such as ammonia and its salts, which are components of food ingredients used for this purpose, are allowed to remove entirely, from food after cooking. However, research is still not fully established. It has been not done how many compounds such as ammonia remain in food after cooking and what effects it has on the health of the consumer [20]. In addition, in milk samples, there are some hazardous chemicals added such as ammonia, melamine, ammonium sulfate, urea, and nitrate to improve its physical appearance and shelf life. Moreover, the addition of milk nitrogen compounds to increase/correct protein content. Some of them are very hazardous and can lead to mortal diseases [21,22]. This function is not only injurious to the health of consumers but also affects the economy of a country. Control of potential contaminants is a problem because as laboratories improve detection, fraudulent producers introduce new alternatives that cannot be detected by established techniques [5]. Therefore, with the increase in consumer awareness, their concern about the safety and health of the food they consume has also increased.

CONCLUSION

This study developed the SALLE method to extract ammonia from food samples like Ahari bread and milk, then extracted ammonia quantified by a colorimetric method. The experimental results indicate that the presented method is proper for the determination of the concentration of ammonia in food samples, so compared to other methods, this method is simple, fast, economical, safe for the environment, and repeatable. Therefore, it could be applied for routine analysis of ammonia concentration in foodstuffs.

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