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Research Article

Comparative Protein Quantification in Ultra Heat Treated Milk Using Kjeldahl Versus Dye Binding Methods

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Abstract: The goal of this study was to compare a Dye Binding Method with the standard Kjeldahl method in the context of protein determination in Ultra-Heat Treated (UHT) milk. Protein contents were determined in (25) samples of milk collected immediately post- ultra-heath treatment of milk and (25) samples collected after six-month storage at room temperature in Kjeldahl and Amido black methods. Samples digested using concentrated sulfuric acid at high temperatures and assayed using Automated Kjeldahl (Foss, Kjeltec™ 8200). Whereas, undigested samples were mixed with Amido Black a and density of color was measured against Amido black dilution series at wavelength of 620 nm. First stage: the average concentration of protein was (2.9256±0.01193 g/100 mL) versus (2.9396±0.01338 g/100 mL) using Kjeldahl versus Amido Black A respectively, with an average difference of (0,014). The concentrations ranged between (2.890-2.940 g/100 mL) and (2.910-2.960 g/100 mL) with a Coefficient of variation is (0.41%) and (0.46%) for Kjeldahl and Amido Black, respectively. (P) Value >0.0001. Therefore, there are no variances significantly different between two methods. Second stage: First stage: the average concentration of protein was (2.9224±0.03961 g/100 mL) versus (2.932±0.007071 g/100 mL) using Kjeldahl versus Amido Black A, respectively, with an average difference of (0.0096). The concentrations ranged between (2.770-3.000 g/100 mL) and (2.920- 2.940 g/100 mL) with a Coefficient of variation is (1.36%) and (0.24) for Kjeldahl and Amido Black respectively. (P) Value<0.0001. Therefore, there are variances significantly different between two methods.

Keywords: Amido black A, Kjeldahl, milk- protein

INTRODUCTION

Milk has been the most widely consumed liquid food source for humans. Among all sources, milk produced from domesticated animals such as cow, buffalo and goat have been widely consumed by humans depending on the source of availability. Milk regarded as a safe food source for producing high value proteins that considered the most important compound for all ages (infant, children teenagers, pregnant…etc.). (Billakanti, 2009).

Proteins are found in all cells and are reflected a biological important for almost all cells. Food proteins are very complex. Many proteins have been purified and characterized. Proteins are consisted of hydrogen, carbon, nitrogen, oxygen and sulfur. Nitrogen is the most peculiar element existing in proteins. Food proteins nitrogen content ranges from 13.4 to 19.1%. One problem of the analysis of proteins is that some food components have similar physicochemical properties. Nonprotein nitrogen could come from free amino acids, small peptides, nucleic acids, phospholipids, amino sugars, porphyrin and some vitamins, alkaloids, uric acid, urea and ammonium ions. Therefore, the total organic nitrogen in foods would represent nitrogen primarily from proteins and to a lesser extent from all organic nitrogen-containing Nonprotein substances. Depending upon Methodology, other major food components, including lipids and carbohydrates may interfere physically with analysis of food proteins. Numerous methods have been developed to measure protein content. The basic principles of these methods include the determinations of nitrogen, peptide, bonds, aromatic amino acids, dye-binding capacity, ultraviolet absorptivity of proteins and light scattering properties. In addition to factors such as sensitivity, accuracy, precision, speed and cost of analysis, what is actually being measured must be considered in the selection of an appropriate method for a particular application (Nielsen, 2010).

The Kjeldahl method is a main technique for protein quantities and used as a reference in comparisons against all other methods. The Kjeldahl method confirms high precision and good reproducibility. During a routine assay, the mean error does not exceed 1%. The major difference between the very numerous Kjeldahl nitrogen assays lies in the catalyst used for mineralization and in the technique of ammonia distillation and titration (Semih, 2005). Although the accuracy of Kjeldahl method, the use

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of hazardous and potentially toxic chemicals is creating concern (Owasu-Apenten, 2002; Frankel-Conrat and Cooper, 1944; Udy, 1956). The other method is dye-binding method. In Dye binding method proteins react with certain organic dyes to produce insoluble complexes. The quantity of dye bound is proportional to:

- The Dye-Binding Capacity (DBC)
- The protein concentration

A farm-gate price for milk (in Australia, Denmark, France, Netherlands, New Zealand and United States) is partly determined by its protein content. Dye-binding assays are widely used for milk protein determination. Amido Black 10B (C.I. 20470), Acid Orange 12 (C.I. 15970) and Orange-G (C.I. 16230) are the three main dyes used (Owasu-Apenten, 2002).

When reacting, under specific conditions, with dyes containing acid sulfonic groups (-SO3H), the functional groups of proteins, particularly the basic groups in the side chains of arginine, lysine and histidine, induce a color reduction in the dye solution, in proportion to the protein content of food. Organic dyes bind with proteins by ionic or electrostatic interactions, although van der Waals forces participate as well. The reaction is optimal in a strongly acidic medium and produces soluble or insoluble complexes. Thus, the concentration of protein in a sample could be determined by measuring the decreasing of absorbance of the dye solution. Dye-binding methods are very simple, rapid, sensitive, inexpensive and useful for routine analysis. There are many advantages of the dye-binding method, there is also no need for skillful manipulation and use of corrosive reagents of the Kjeldahl procedure and it is not a time consuming. Color usually progresses in few minutes (usually <5 min) and remains stable for at least 0.5 to 1 h. (Semih, 2005; Kolakowski, 1974; Anon, 1979). In this study, we compared a Dye Binding Method with the standard Kjeldahl method in the context of protein determination in Ultra-Heat Treated (UHT) milk.

METHODS

Samples: Our analysis is category in two groups: Milk samples immediately post- ultra-heat treatment and samples after six-month storage at room temperature. 25 samples of milk immediately post-ultra-heat treatment are taken in July 1st 2013. After that, 25 samples of storage ultra-heat treatment are taken in December 12th 2013. All samples are taken from farms and supermarkets in Damascus and countryside. Each sample is analyzed three times by each method (Kjeldahl and Amido black) and then we took the average for each method.

MATERIALS

Kjeldahl method (Horwitz and Jr. Latimer, 2002): Sulfuric acid H_2SO_4 (95-98%), Nitrogen free (Panreac, Spain). Copper catalyst solution $CuSO_4.5H_2O$, Nitrogen free, we prepared solution (0.05 g/mL H2O), (Scharlau chemie, Spain). Sodium hydroxide solution NaOH 50% (w/w), nitrate-free (Tekkim, Turkey 99.0% extra pure). Boiling chips, mesh size 10, high purity, amphoteric alundum granules, plain. Methyl red/bromocresol green indicator solution; we dissolved 0.2 g methyl red and diluted to 100 mL in 95% ethanol. Then we dissolved 1.0 g bromocresol green and diluted to 500 mL in 95% ethanol. Finally, mixed 1 part methyl red solution with (5) parts bromocresol green solution (Hemedia laboratories, India). Boric acid solution 4% (Hemedia laboratories, India 99.5%) with indicator. We dissolved 40 g H_3BO_3, diluted to 1 L in water and added 3 mL methyl red/bromocresol green indicator solution. Solution will be light orange color. Hydrochloric acid standard solution 0.1000 N (Tekkim, Turkey). Ammonium sulfate $(NH_4)_2SO_4$ 99.9%. Potassium sulfate K_2SO_4, nitrogen free.

Amido black method (Horwitz and Jr. Latimer, 2002): Amido Black 10B (Carl Roth, Germany). Citric Acid 99,5% (Tekkim, turkey). Disodium hydrogen phosphate $Na_2HPO_4.2H_2O$ (AVON chem, United Kingdom). Thymol blue (Surechem, England).

Apparatus:

- Digestion flask, Kjeldahl, hard, moderately thick. Total capacity is ca 500 or 800 mL. Distillation flasks. Same as Kjeldahl flask, fitted with rubber stopper through which passes lower end of efficient connecting bulb or trap to prevent mechanical carryover of NaOH during distillation. Digestion/distillation system (Kjeltec™ 8100/8200, Foss, Sweden). Titration buret 50 mL.
- Spectrophotometer (Hitachi U-1800).

RESULTS AND DISCUSSION

First stage: Milk samples immediately post-ultra-heat treatment: Table 1 shows the results that we have gotten from the determination of milk protein content in both methods Kjeldahl and Amido black. The average in Kjeldahl method is (2.9256 g/100 mL) and in Amido black is (2.9396 g/100 mL). The average of difference between two methods is (0.014). We compare results in Table 2. In Kjeldahl method, the results ranged between (2.890-2.940 g/100 mL). The standard deviation (STDV) is (0.01193) and the Coefficient of variation is (0.41%). In Amido black method, the results ranged between (2.910-2.960 g/100 mL). The STDV is (0.01338) and the Coefficient of variation is (0.46%). The results are undergone t test analysis, the p-value>0.0001. Therefore, there are no variances significantly different between two methods. Figure 1

Table 1: Compare results of protein milk concentration by Kjeldahl method vs. Amido black method (ultra-heat treatment milk)

Samples	Concentration g/100 mL		Differences between Kjeldahl and Amido black method
	Kjeldahl	Amido black	
1	2.91	2.94	0.03
2	2.89	2.92	0.03
3	2.94	2.93	-0.01
4	2.93	2.92	-0.01
5	2.93	2.94	0.01
6	2.93	2.95	0.02
7	2.92	2.93	0.01
8	2.94	2.95	0.01
9	2.92	2.95	0.03
10	2.92	2.94	0.02
11	2.93	2.94	0.01
12	2.94	2.91	-0.03
13	2.93	2.94	0.01
14	2.91	2.96	0.05
15	2.91	2.93	0.02
16	2.94	2.94	0
17	2.93	2.94	0.01
18	2.93	2.96	0.03
19	2.93	2.96	0.03
20	2.93	2.92	-0.01
21	2.93	2.95	0.02
22	2.92	2.93	0.01
23	2.94	2.95	0.01
24	2.92	2.95	0.03
25	2.92	2.94	0.02
Average	2.9256	2.9396	0.014

Table 2: Compare results of t test analysis between Kjeldahl method vs. Amido black method (ultra-heat treatment milk)

Unpaired t test	Kjeldahl	Amido black
Number of values	25	25
Minimum	2.890	2.910
Maximum	2.940	2.960
Average	2.9256	2.9396
Sum	73.14	73.49
Std. Deviation	0.01193	0.01338
Coefficient of variation	0.41%	0.46%
Difference between means	0.01400 ± 0.003585	
p-value	0.5789 Variances not significantly different	

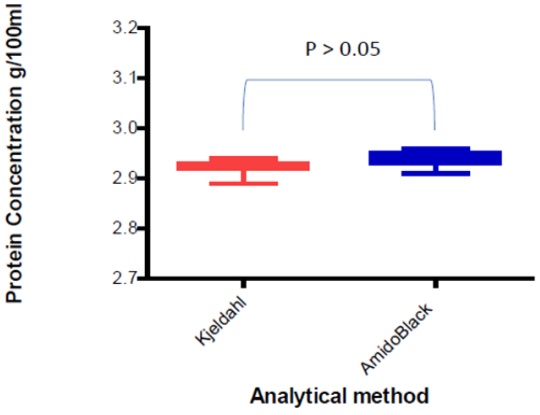

Fig. 1: t-test of Kjeldahl method and Amido black method (ultra-heat treatment milk)

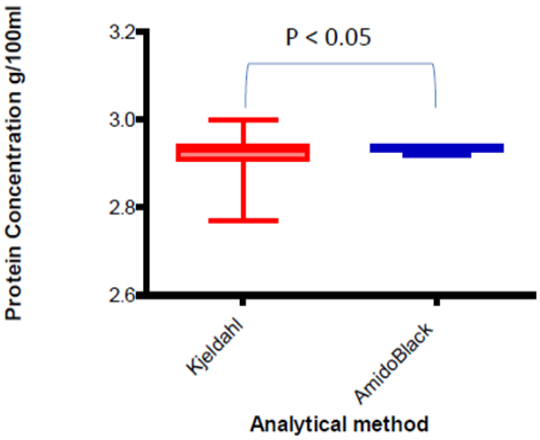

Fig. 2: t-test of Kjeldahl method and Amido black method (ultra-heat treatment milk after six months storage)

gives the t test analysis graph and p-value of Kjeldahl and Amido black methods.

Second stage: Samples after six-month storage at room temperature: According to Table 3, the average in Kjeldahl method is (2.9224 g/100 mL) and in Amido black is (2.932 g/100 mL). The average of difference between two methods is (0.0096). We compare results in Table 4. In Kjeldahl method, the results ranged between (2.770-3.000 g/100 mL). The standard deviation (STDV) is (0.03961) and the Coefficient of variation is (1.36%). In Amido black method, the results ranged between (2.920-2.940 g/100 mL). The

Table 3: Compare results of protein milk concentration by Kjeldahl method vs. Amido black method (ultra-heat treatment milk after six months storage)

	Concentration g/100 mL		
Samples	Kjeldahl	Amido black	Differences between Kjeldahl and Amido black method
1	2.93	2.92	-0.01
2	2.94	2.93	-0.01
3	2.87	2.93	0.06
4	2.91	2.92	0.01
5	2.94	2.92	-0.02
6	2.94	2.94	0
7	2.91	2.93	0.02
8	2.93	2.94	0.01
9	2.92	2.94	0.02
10	2.93	2.93	0
11	2.94	2.94	0
12	2.77	2.93	0.16
13	2.94	2.93	-0.01
14	2.89	2.94	0.05
15	2.93	2.92	-0.01
16	2.93	2.93	0
17	3	2.93	-0.07
18	2.96	2.93	-0.03
19	2.94	2.94	0
20	2.91	2.93	0.02
21	2.94	2.94	0
22	2.91	2.93	0.02
23	2.93	2.94	0.01
24	2.92	2.94	0.02
25	2.93	2.93	0
Average	2.9224	2.932	0.0096

Table 4: Compare results of t test analysis between Kjeldahl method vs. Amido black method (ultra- heat treatment milk after six months storage)

Unpaired t test	Kjeldahl	Amido black
Number of values	25	25
Minimum	2.770	2.920
Maximum	3.000	2.940
Average	2.9224	2.932
Sum	73.06	73.30
Std. Deviation	0.03961	0.007071
Coefficient of variation	1.36%	0.24%
Difference between means	0.009600±0.008047	
p-value	<0.0001 variances significantly different	

Table 5: Linarity equation, liner range, correlation coefficient, LOD, LOQ of Amido black and Kjeldahl methods

Analytical method	Liner equation	Liner range g/mL	Correlation coefficients	LOQ g/mL	LOD g/mL
Amido black	Y = 0.237x+0.0145	0.015-0.0032	0.40%	0.21	0.046
Kjeldahl	Y = 0.1576+0.06053	0.004-0.03	0.39%	0.729	0.240

LOD: Limit of detection. LOQ: Limit of quantitative

STDV is (0.007071) and the Coefficient of variation is (0.24%). The results are undergone t test analysis, the p-value<0.0001. Therefore, there are variances significantly different between two methods. Figure 2 gives the t test analysis graph and P value of Kjeldahl and Amido black methods.

Validation: Validation of the methods was done to demonstrate the results we have gotten. Table 4 shows the confirmation results of Kjeldahl and Amido black.

In order linearity of Amido black, a series of milk solutions at the concentrations of 0.015, 0.012, 0.0098, 0.0078, 0.0006, 0.005, 0.004, 0.0032 g/mL of milk were prepared and analyzed. To determine the linearity of Kjeldahl method, a series of milk solution at the concentration of 0.004, 0.008, 0.0122, 0.016, 0.018, 0.02, 0.024, 0.026, 0.03 g/mL of milk were prepared and analyzed. The linear equations, linear ranges and correlation coefficients are shown in Table 5.

CONCLUSION

The values for standard deviations are both small and this means the precision of these determinations is high. The Amido black will rapidly (≤ 30) min determine protein. It is simple to operate. Another

major advantage is safety. We are not dealing with any harsh chemicals, high temperatures, or unusual conditions, so you can actually run this analysis in normal laboratories. It also is very green technology in that it doesn't generate any hazardous waste. Current Kjeldahl methods generate hazardous waste with sulfuric acid and metal catalysts that have to be disposed of. The acquisition cost is less expensive than typical Kjeldahl equipment. Dye binding will allow testing to be done more reliably and easily than is possible in Kjeldahl method. Therefore, we can use Amido black method in routine analysis instead off Kjeldahl method.

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