Technology Section

Pakistan J. Sci. Ind. Res., Vol. 30, No. 12, December 1987

AN ENZYMIC METHOD FOR DETERMINATION OF ADDED WATER IN MILK

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(Received December 23, 1986; revised December 28, 1987)

Tryptic digestion of milk proteins in various adulterated samples from buffalow was carried out for 60 minutes, free amino acids and smaller peptides thus released were separated from undigested proteins by precipitation with trichloro acetic acid (TCA) followed by centrifugation. The decrease in absorbence of supernatants at 280 nm of different water adulterated samples was found to be correlated with degree of dilution. Thus a simplified method of determining the percentage of added water in milk is suggested which does not involve the use of any specific dye or highly sophisticated equipments. The results are reproduceable and comparable to the extent of adulteration obtained by Cryoscope, a highly reliable instrument. Thus the technique may be adopted as common routine method in dairy industries with simple procedure based on determinations of fat and specific gravity of milk.

Key words: Enzyme, adulteration, milk.

INTRODUCTION

Adulteration of milk is a common probelm in Pakistan faced by public as well as bakery, confectionary and dairy industries. A number of methods have been proposed and used for determining the degree of adulteration in milk. The methodology is mainly based on the estimation of a component or components which are almost unaffected during seasonal, environmental or physiological changes. Protein, carbohydrate and ash are regarded as comparatively invariable constituents of milk (Markland, 1963) shown in Table 1 and 2. The ratio of lactose, protein and ash has been found constant as 13:9:2 Hatful, [7].

In most of the laboratories associated with dairy industries fat (F) is estimated by the routine Gerber or Babcock's method and density (D) is determined by using an ordinary lactometer. The total solids (T) are calculated by Richmonds formula T=0.25D + 1.22F + 0.72, which indirectly indicates the quantity of added water.

The specific gravity and refraction of copper-serum of milk Woodman, [22] is used for calculating the added water as refraction decreases by one division for each 5% addition of water.

The use of ash contents of milk in determining added water is not very useful because of added minerals in exogenous water and variation in chloride contents in milk Davies, [3] and Sanders, [19].

Freezing point determination of milk McDonald, [14] Nielson [16] is regarded as the most accurate method as all the components of milk are taken into consideration, with certain disadvantages as it requires a highly specialized apparatus i.e. Cryoscope. Secondly the depression in freezing point may be faked if chlorinated water has been used for adulteration, because chloride and lactose contents are the main factors affecting the freezing point Henningson [8].

Slavica [20] has proposed the use of lactose content as a criterion for detecting 5-30% exogenous water in milk. Recently Woolard [23] has reviewed the traditional and novel methods for estimating milk carbohydrates. Polarimetric method for routine testing is inconvenient which involves prior protein separation. Enzymic method of B galactosidase for measuring lactose in milk has been suggested by Kleyn and Trout [11] Reimerdes and Reisewitz [18] have simplified the enzymic method to semi automatic.

The present paper is based on the enzymic digestion method for estimation of proteins and their relationship to added water. Proteases found in traces in fresh milk but more profoundly in colostrum Keirmeir and Semper [12] are not supposed to affect the present methodology as the procedure is based on the total free amino acids whether present initially or released after proteolytic digestion will hardly matter.

The method is simple, economical and accurate with a variation of ± 2%. Moreover other methods of proteins estimation Hossain *et al.*, [9], Nakai, [15] used in dairy laboratory involve highly sophisticated equipments as HPIC, Humphar and Newsome, [10]. Protomat, Milkoscean

Table 1. Components of milk, divided by their average values

	H_2O	H_2O/A	Fat %	F/A	Protein p%	P/A	Lactose %	L/A	Ash %	Ash/A	SNF %	SNF/A %	Total solid	T.S./A
Cow	87.20	1.018	3.70	0.724	3.50	0.988	4.90	1.004	0.70	0.897	9.10	0.986	12.80	0.891
Goat	87.00	1.015	4.25	0.831	3.51	0.991	4.27	0.875	0.86	1.102	8.75	0.948	13.00	0.906
Buffalo	82.76	0.966	7.38	1.444	3.60	1.017	5.48	1.123	9.78	1.00	9.86	1.068	17.24	1.201
Average (A)	85.65	–	5.11	–	3.54	–	4.88	–	0.78	–	9.23	–	14.35	–
EVMM		0.052		0.72		0.029		0.248		0.205		0.12		0.31

EVMM = Extent of variation or differnce between maximum and minimum values of constitutents/their average values

Table 2. Composition of milk from different breeds of cow and average values of components

Bread	Fat %	Fat/A	Protein %	Prot./A.	Lactose %	L/A
Holestein	3.40	0.796	3.32	0.919	4.87	0.993
Shorthorn	3.94	0.922	3.32	0.919	4.99	1.018
Aryshire	4.00	0.936	3.58	0.991	4.67	0.953
Brown swiss	4.01	0.939	3.61	1.000	5.04	1.028
Guernsey	4.95	1.160	3.91	1.083	4.93	1.006
Jersey	5.37	1.257	3.92	1.086	4.93	1.006
Average (A)	4.27		3.61		4.90	
EVMM = Extent of variation between max. & min. values			0.461		0.167	0.135

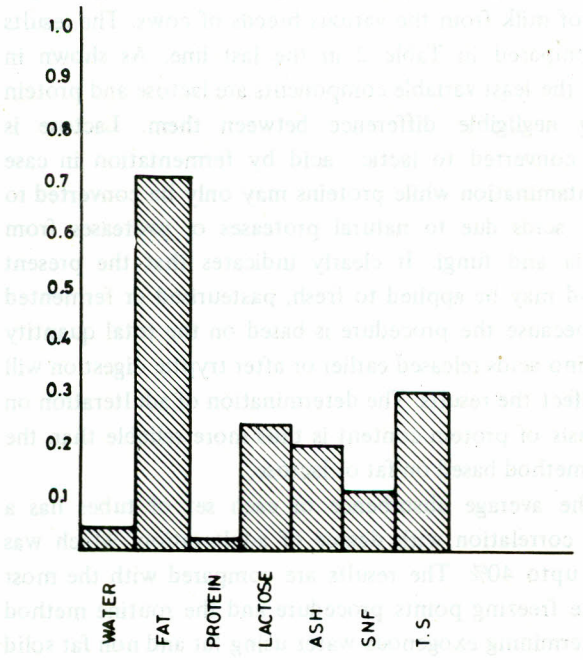

Fig. 1 Comparison of variation in components of milk from various animals.

Table 3. Comparison of the results of the three methods of determination of adulteration

S. No.	Adulteration %	Absorbence (280 nm)	L.R. %	Fat %	SNF %	T.S. %	F.P. °C
1	0	1.44	28	3.5	7.84	11.34	–0.462
2	10	1.31	25	3.15	7.02	10.17	–0.409
3	20	1.20	22	2.8	6.2	9.0	–0.366
4	30	1.11	20	2.45	5.63	8.08	–0.318
5	40	0.96	16	2.1	4.56	6.66	–0.271
6	(27%) unknown	1.14	21	1.8	5.91	7.71	–0.337

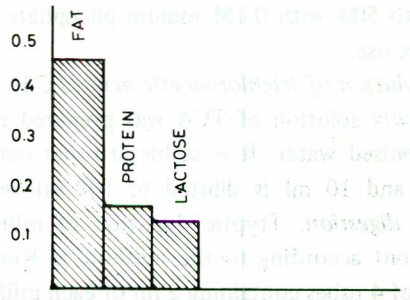

Fig. 2 Comparison of variation of milk components of different breed of cows.

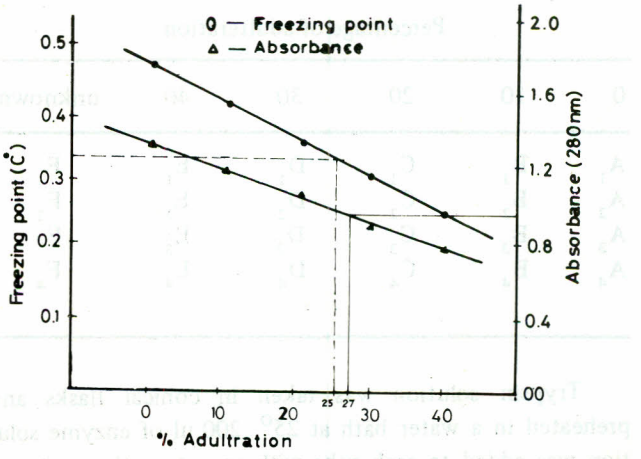

Fig. 3 Graph showing the correlation of values of absorbence and degree of adulteration.

or the usual titration, Kjeldahl and dye binding methods Dolby, [4], and Weik *et al.*, [21] which are time consuming.

MATERIALS AND METHODS

The chemicals used were from E. Merck (analytical grade) and solvents were redistilled before use. Trypsin of fine chemical grade from Sigma Chemical Co. was obtained and stored in refrigerator before use.

Preparation of milk samples. Approximately 100 milk samples of different quality and origin, raw as well as processed were analysed separately. Milk smaple (1) was taken and pH was adjusted to 7.4 with a drop of two of 10% NaOH solution and it was heated in a boiling water bath for 15 minutes to denature the proteins Dunn, [5] present. It was transferred into six different flasks and water (double distilled deionised) was added to flasks B, C, D and E so that the quantity of added water was 10, 20, 30 and 40%. Flask A has 0% adulteration and an unknown quantity of water was added to flask F.

Preparation of trypsin stock solution. Hundred ml of trypsin solution was prepared in 0.1M sodium phosphate buffer of pH 7.4 having concentration of 100 ug/ml. The solution was stored in refrigerator at 10-15^{o} for a week and diluted to 50% with 0.1M sodium phosphate buffer of pH 7.4 before use.

Stock solution of trichloroacetic acid (TCA). Hundred ml of 50% w/v solution of TCA was prepared in double distilled deionised water. It is stable at room temperature for a week and 10 ml is diluted to 100 ml before use.

Tryptic digestion. Tryptic digestion of milk protein was carried out according to the method of Kunitz [13] and six sets of 4 tubes containing 2 ml of each milk samples A to E were placed in water bath at 25^{o} for 10 minutes. The sets of tubes were numbered, as given below.

Percentage of adulteration

0	10	20	30	40	unknown
A_1	B_1	C_1	D_1	E_1	F_1
A_2	B_2	C_2	D_2	E_2	F_2
A_3	B_3	C_3	D_3	E_3	F_3
A_4	B_4	C_4	D_4	E_4	F_4

Trypsin solution was taken in conical flasks and preheated in a water bath at 25^{o}. 200 ul of enzyme solution was added to each tube with an automatic pipette at an interval of 30 seconds. The tubes were incubated for 90 minutes to provide reasonable digestion time. Two ml of 5% TCA solution was added to each tube wtih automatic pipette in the same order as before with an interval of 30 seconds. The tubes were shaken thoroughly in a test tube shaker for 5 minutes for complete precipitation of undigested proteins by TCA. The tubes were centrifuged or filtered through (S&S) filter paper of 1 mm thickness. The optical density of the supernatent or filterate was measured at 280 nm against the blank without the trypsin solution.

Cryoscopic method. The freezing points of all six samples were determined by using the automatic Cryoscope model Cryostar II Funke Gerber as described by Elsdon and Stubbs [6].

RESULTS AND DISCUSSION

The milk consumed by human beings and commonly supplied in the market in Pakistan is a mixture of milk from mainly three animals i.e. cow, goat and buffalow. The composition of milk from these animals Corbin and Whittier, [2] is compared in Table 1 where in the preceding columns, each component is divided by its average (A). The lowest and the highest figures obtained by dividing each of the component by its average is given in the Table 1 in the last line.

It looks obvious that the least invariable, factor in the milk components is protein (Fig. 1). Protein percentage is also a comparatively constant parameter among components of milk from the various breeds of cows. The results are compared in Table 2 in the last line. As shown in Fig. 2 the least variable components are lactose and protein having negligible difference between them. Lactose is easily converted to lactic acid by fermentation in case of contamination while proteins may only be converted to amino acids due to natural proteases or proteases from bacteria and fungi. It clearly indicates that the present method may be applied to fresh, pasteurised or fermented milk because the procedure is based on the total quantity of amino acids released earlier or after tryptic digestion will not affect the results. The determination of adulteration on the basis of protein content is thus more reliable than the usual method based on fat or lactose.

The average absorbence of each set of tubes has a direct correlation with degree of adulteration, which was linear upto 40%. The results are compared with the most reliable freezing points procedure and the routine method of determining exogenous water using fat and non fat solid (SNF) as given in Table 3. The difference in values of SNF is more irregular than variation in values of absorbence,

which coincide more with freezing points. The graph (Fig. 3) shows that if the absorbence of the unknown sample is determined, its degree of adulteration can be easily calculated and it coincides more with values obtained by freezing points as a highly recognised method for calculating added water in milk. Thus a new simple and accurate method to determine the exogenous water in milk is introduced, which may be of practical value for routine analysis of added water in milk.

Acknowledgement. We are grateful to Chiniot Beradri Trust for the financial assistance to one of the authors (A. Hasnain).

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