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Factors Affecting the Measurement of Undenatured Whey Protein Nitrogen in Dried Whey by a Modified Harland-Ashworth Test

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FACTORS AFFECTING THE MEASUREMENT OF UNDENATURED WHEY PROTEIN NITROGEN IN DRIED WHEY BY A MODIFIED HARLAND-ASHWORTH TEST

by

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This work I dedicate to my parents, Mahmoud and Hafssa Aboumahmoud for their tremendous encouragement in pursuing a graduate education.

Reyad Aboumahmoud

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ABSTRACT

Factors Affecting the Measurement of Undenatured Whey Protein Nitrogen in Dried Whey by a Modified Harland-Ashworth Test

by

Reyad Aboumahmoud, Doctor of Philosophy

Utah State University, 1981

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The Harland-Ashworth test for measuring undenatured whey protein nitrogen in nonfat dry milk was modified to measure undenatured whey protein nitrogen in dried whey. The new test was designed to measure absorbance in Harland-Ashworth filtrates at 280 nm to eliminate difficulties associated with turbidimetric measurements. One gram dried whey was reconstituted in 25 ml pH 6.7 phosphate buffer (.1M) in a 25 x 150 mm test tube. Ten grams sodium chloride were added. The test tubes were incubated for 30 min in a water bath at 37C and shaken 8-10 times in the first 15 min. The tubes were removed from the water bath and cooled to room temperature. The contents of the tubes were filtered through S & S 602 filter paper. One milliliter of filtrate was diluted with 10 ml H_2O and the total absorbance was measured against a blank of 1 ml phosphate buffer saturated with sodium chloride, then diluted with 10 ml H_2O. One milliliter of filtrate was mixed with 10 ml 13.2% trichloroacetic acid and filtered through Whatman 42 filter paper. The nonprotein absorbance was measured against

a blank containing 1 ml phosphate buffer saturated with sodium chloride, then mixed with 10 ml 13.2% trichloracetic acid and filtered through Whatman 42 filter paper. Undenatured whey protein absorbance was the difference between total absorbance and nonprotein absorbance. Undenatured whey protein nitrogen per gram dried whey equaled the undenatured whey protein nitrogen absorbance multipled by 50.5462 (R^2 = 0.9743).

The test produced highly reproducible results with nonfat dry milk as well as dried whey. The relationship between absorbance and undenatured whey protein nitrogen was highly correlated and not influenced by season, heat treatment, residual milk clotting enzymes, solids concentration during heating or pH of the whey during heating.

(87 pages)

INTRODUCTION

Whey continues to be an underutilized byproduct of the cheese industry and, in many instances, constitutes a serious disposal problem. In spite of this it contains potentially valuable food ingredients that could be used to enhance the economic position of the dairy industry and provide additional food for consumers.

The high cost of cheese whey disposal and the substantial economic and nutritional potential of whey solids justifies a considerable effort to improve its utilization (100). Whey utilization will continue to increase only if more familiarity with and understanding of cheese whey products and their properties are gained.

Holmes (65) listed the basic whey products as liquid whey, liquid whey concentrate and dried whey. Processed whey for human consumption is used as follows: 50% in the baking industry, 35% in baby foods and pharmaceuticals, 10-12% in confectionery and dairy outlets, with fermentations and chemicals occupying the balance (8). About 53% of the 34,446 million pounds of whey produced in the United States in 1978 (19) was processed. The rest was wasted or fed to animals as liquid whey.

Like nonfat dry milk (NDM), the functional properties of dried whey in different food products depend on the heat treatment applied to the whey during processing (54). NDM has been classified as low heat, medium heat or high heat powder on the basis of the extent of denaturation of the whey proteins during processing. High heat NDM contains not more than 1.5 mg undenatured whey protein nitrogen (UWPN) per gram of powder, low heat NDM contains not less than 6.0 mg UWPN per gram and medium heat NDM contains from 1.5 to 5.99 mg

per gram (5). Such standards have not been developed for dried whey, nor has an acceptable testing method been devised to measure undenatured protein nitrogen in whey.

A test for the determination of undenatured whey protein nitrogen in NDM was developed by Harland and Ashworth (53) and later modified by Kuramoto et al. (79). The test was originally developed to determine whether the heat applied to skim milk prior to drying was sufficient to make the product suitable for use in bread making. It relied on the removal of casein and denatured whey protein from reconstituted NDM by filtration after saturation with sodium chloride. The clear filtrate containing the undenatured whey protein was acidified to produce a turbidity which was measured in a spectrophotometer.

Turbidimetric methods are subject to a number of errors and show considerable variation in the degree of turbidity developed for constant levels of undenatured whey protein nitrogen (120). Beta-lactoglobulin and alpha-lactalbumin develop different levels of turbidity at equal concentrations. Reyes (111) developed a modified method for the determination of UWPN in whey powder by measuring the light absorbance of the Harland-Ashworth filtrate at 280 nm. However, the test was not evaluated against various factors which could change the composition and the properties of whey under a variety of conditions.

The objectives of the present study were to evaluate and modify where necessary the test proposed by Reyes (111) and to determine the effect of factors such as residual milk clotting enzymes, whey solids concentration, pH and seasonal changes in protein content of whey on the heat denaturation of whey proteins during processing and on the performance

of the proposed test. The modified test also will be applied to NDM and its reproducibility compared to that of the conventional Harland-Ashworth test.

REVIEW OF LITERATURE

Whey Classification and Use

Whey is a secondary byproduct of cheese making. It is a yellowish-green liquid separated from coagulated milk, cream or skim milk. It constitutes 85 to 90% of the milk volume and retains approximately 50% of its nutrients including most of the lactose, 20 to 25% of the protein and most of the vitamins and minerals. Additionally it contains variable amounts of lactic acid and soluble nitrogen (68, 76, 77, 104). Whey is classified into two groups: sweet whey is derived from products requiring rennet or other enzymes for coagulation of the milk and has a minimum pH of 5.6. Acid whey is derived from products coagulated by acidification and has a maximum pH of 5.1 (68).

Historically much of the unused whey from cheese plants was dumped into rivers or streams. Whey has a very high biological oxygen demand and if not utilized causes serious environmental problems in rivers and lakes, not to mention the loss of a nutritional by-product (47). The former was the predominant driving force behind improvements in whey utilization (134). Liquid whey production in the USA in 1979 was nearly 16 million tons. The per capita consumption of whey has increased seven-fold during the last decade despite the fact that whey processing worldwide is a young industry. However, only about half of the available whey is now being used for food and feed (131).

Whey Composition

Fluid sweet whey is about 93.7% moisture, 0.5% fat, 0.6-0.8% protein, 4.5-4.85% lactose, 0.5% ash and 0.05% lactic acid (42, 47, 77).

Dried sweet whey contains 3.5% moisture, 0.8% fat, 13.1% protein, 72-75% lactose, 7.3% ash and 0.2-1.7% lactic acid (42, 77). Fresh liquid whey contains three nitrogenous substances: casein (.01-.02%), whey protein which is soluble at pH 4.6 but precipitates in 12% trichloroacetic acid (TCA) and nonprotein nitrogen which is soluble in 12% TCA. Despite their heterogeneity, whey proteins constitute a characteristic group of soluble proteins in whey (104).

The most abundant of the whey proteins is β-lactoglobulin (β-lg). It represents 43.7% of the whey proteins and contains sulfydryl groups in the form of cysteine residues (two per dimer) as well as free disulfide groups. α-lactalbumin (α-la) represents 19.7% of the whey proteins and unlike β-lg plays an important biological role in lactose synthesis. Associated with whey proteins is a group of proteins loosely termed proteose peptones consisting mainly of glycoproteins and phosphoproteins which represent 18.9% of the whey proteins. Bovine serum albumin (BSA) is not strictly a milk protein as it is not synthesized in the mammary gland. It comes from blood and represents 4.7% of the whey proteins. The immunoglobulins are a heterogeneous group which include some molecules with antibody activity and represent 13% of whey protein (47, 104). Whey always contains a small amount of casein which represents a nearly constant level of 2% of the whey proteins (132). Whey protein is of nutritional significance when added to human diets (38). Its protein efficiency ratio is 3.2 versus 2.5 for casein (64).

Forms of Whey

Several bulk whey concentrates or derivatives are produced

industrially and enjoy commercial acceptance (76). The one of greatest importance is dried sweet whey (76, 134). It is characterized as having good flowing properties, a fine white color, and a slightly sweet taste (104). The second in importance is acid whey powder. It is primarily made from cottage cheese whey and is characterized by having a pH lower than 5.1.

The Whey Products Institute reported that production of whole dried whey for human consumption in the USA amounted to 294.3 million pounds in 1975, 373 million pounds in 1976 and over 383 million pounds in 1977 (19). Other whey products include liquid whey, liquid whey concentrate, denatured lactalbumin which is produced by pH adjustment and high temperature precipitation, and modified wheys which include partially delactosed whey, partially demineralized whey, partially delactosed/demineralized whey and whey protein concentrate (76).

Whey Utilization

Whey utilization as food and feed has been extensively reviewed in the literature (49, 65, 67, 76, 80, 84, 104). Whey utilization in human food is on the increase. This is due in part to economic advantages, better functionality and nutrition provided by the whey components. Whey imparts desirable functional properties to foods at an affordable price (19). Price increases of NDM and the reduction in its availability (89) have encouraged the use of whey as a sound functional and economical substitute in products where high levels of lactose and milk salts are acceptable. Whey protein may also be substituted for egg proteins in baked goods where eggs account for up to 50% or more of the ingredient costs (50, 104). The use of whey as

a solids-not-fat (SNF) substitute is common in ice cream (1, 6, 9, 65, 76, 117) provided that it complies with the Food and Drug Administration (FDA) regulations which specify that whey can substitute for up to 25% of the SNF (51). At present only sweet whey is permitted by the FDA as a source of whey solids in ice cream, frozen custard, ice milk and sherbet (51).

Partial substitution of NDM with Cottage or neutralized Cottage cheese whey was recommended for Swiss and Sundae style yogurts with additions of up to 5.5% whey solids provided the total SNF content is 9.5% (55). Human milk is lower in protein and higher in lactose than bovine milk which makes whey a potential ingredient in the production of humanized (122) and simulated bovine milk (6) for infant foods.

Cheese makers are always looking for ways and means to increase cheese yield without producing negative effects on the quality and acceptability of the final product. Higher cheese yields have been obtained as a result of incorporating whey protein during cheese making (1, 63, 123, 127, 134). Another economical and feasible use of cheese whey is in the production of phage inhibitory medium (PIM) for lactic bulk cultures (112). The quality of the cheese compared favorably with cheese produced from conventional media. Moreover, the cost savings with whey based culture medium were substantial. Fain et al. (34) used Cottage cheese whey derivatives in the production of Cottage cheese dressing and obtained a final product with a quality comparable to one prepared with conventional dressing. The most favorable dressing mixes were produced from either vacuum evaporated, untreated whey or ultrafiltered, vacuum evaporated whey containing a protein:lactose ratio of 3:2.

The use of whey as a partial or complete substitute for NDM in baked products is increasing (2). Approximately 50% of all the whey utilized is used by the baking industry in the USA (65), most of it being added as whole dried whey. It imparts a desirable flavor and texture as well as shorter dough time and it improves keeping quality (9, 104). For the production of a desirable loaf volume in bread making, partial denaturation of whey proteins, low lactose concentration and low ionic strength are recommended (49, 133).

Denaturation of Whey Protein

Protein denaturation is any reaction which results in changes in the secondary and tertiary structure (47). Denaturation involves the unfolding of protein molecules, sometimes followed by an extensive protein-protein aggregation which produces turbidity. The intensity of turbidity is a function of the temperature, pH and ionic strength of the system (95). Whey proteins which have been heat denatured may aggregate at appropriate pH values through disulfide groups, hydrogen bonds and hydrophobic reactions. This produces intermediate size particles which may then aggregate to form sedimentable size particles (47, 99). Denaturation of whey proteins influences the functionality and behavior of whey in food systems (54).

Factors affecting denaturation of proteins in whey were measured by sedimentation after pH adjustment to 4.6 and were listed by Nielson et al. (103) in order of decreasing importance. For Cottage cheese whey the order of importance was temperature, total solids, heating time and pH; for Colby cheese whey the order was temperature, total solids, pH and heating time. Maximum protein denaturation

occurred between pH 6.0 and 7.0 for all the total solids and heating times. An inverse relationship was observed between total denaturation and total solid concentrations. Reyes (111) found that whey protein denaturation increased when the pH of whey was adjusted from 5.75 to 6.7 prior to heating. The change in pH exhibited a larger effect on denaturation at temperatures above 70C. Grieg (47) observed that denaturation temperatures were influenced by pH. At pH 5.0 denaturation temperature was 81.5C but it was 66.5C at 9.0. Denaturation was measured by degree of precipitation after pH adjustment to 4.6.

Care is required during all steps in whey processing such as heating, agitation and pH adjustment to avoid whey protein denaturation and the concomitant loss of solubility (95). Morr (97) found that heating below the isoelectric pH range (4.5-5.0) permitted whey protein denaturation without adversely affecting protein solubility, whereas heat denaturation of cheese whey at or above the isoelectric pH produced insolubility, grittiness and inferior functional properties (94). Harwalkar (56) heated wheys at 90C for 30 min after the pH was adjusted to 2.5, 4.5 or 6.5. Whey at pH 2.5 remained in solution, whereas most of the protein was precipitated at pH 4.5 or 6.5. Hidalgo and Gamper (59) found that in the absence of calcium maximum insolubility occurred in the isoelectric range and in the presence of calcium insolubility occurred over a wide pH range (2 to 12).

Individual whey proteins vary in their sensitivity to denaturation, and also vary in their denaturation mechanisms. β-lg is characterized by the presence of disulfide bonds which are broken during heating. The subsequent free sulfhydryl groups are thought to be involved in coagulation during heat denaturation and the formation of κ-casein/

β-lg complexes in heated milk (39). Its denaturation follows a two step sequence: first, complex (MW $\leq$ 200,000 daltons) particles not sedimentable at 1000 x g are formed through disulfide linkages, then sedimentable particles at 1000 x g are formed through the involvement of calcium ions. Whey protein denaturation kinetics in liquid whey follows a course similar to pure β-lg denaturation, which can be interpreted as either a first or second order reaction (61). β-lg variants A and B are sensitive to UHT treatment (93) in whey protein concentrate and their denaturation rate decreases with increasing total solids concentration and a decrease in pH below its isoelectric range. Thermal denaturation of β-lg at 60C and pH 2.5 was irreversible, but no precipitation was evident until adjusted to neutral pH (47, 57), whereas complete denaturation and precipitation was achieved at 90C for 30 min at either pH 4.5 or 6.5 (58). Below 95C thermal denaturation of β-lg was faster in skim milk than in liquid whey due to interaction with κ-casein (39).

α-lactalbumin (α-la) consists of two variants (A and B). Variant A is the most heat stable of all whey proteins and it has been postulated that thiol groups of β-lg produced upon heat denaturation are responsible for reduction of the more stable disulphide bonds of α-la (47). Heat treatment of whey induced a complex formation between α-la and β-lg (93). Denaturation rate of α-la at pH 4.0 resembled that of β-lg; however, it was slower at higher pH values. An unusual temperature dependence of α-la denaturation similar to that of β-lg was exhibited (62, 83). Denaturation of α-la was measured on polyacrylamide gels by Hiller and Lyster (61) and found to be a first order reaction. Conformational changes of α-la were

observed by differential scanning calorimetry of temperatures as low as 45C, which explains milk thermostability at that temperature in terms of renaturation and instability of milk at higher temperatures in terms of denaturation (118). Casein interactions with α-la were more evident at pH 4.5 than at pH 6.7 (101). Aggregation of α-la below its isoelectric pH parallels an intermolecular interaction which was both pH and temperature dependent (83).

Aurand et al. (13) studied the resistance of various whey proteins to heat denaturation and found that the order of increasing resistance in whey was immunoglobulins, α-la, BSA and β-lg. When fresh liquid whey (pH 4.5) was heated to 90C for 20 min, cooled to 25C and then centrifuged, the denatured proteins (42% of the total protein) precipitated and the supernatant contained undenatured proteins. BSA was essentially all precipitated while some α-la remained in solution (3). Gradual coagulation of whey proteins was observed above 60C. Immunoglobulins were the most sensitive and were completely denatured at 70C. The most heat stable protein was α-la with BSA and β-lg being intermediate (47, 81). Reyes (111) found that α-la, BSA and transferrin were more resistant to denaturation at pH 5.75-6.2 than at pH 6.5 and 6.67. On the other hand, the polyacrylamide gel electrophoretic patterns showed β-lg and immunoglobulins to be unaffected when pH changed in the above range. Harper and Raman (54) observed a comparable relationship for denaturation of total whey protein and BSA. The relationship was not necessarily the same on a percentage basis, the largest difference was noted at a level of 40-60% total whey protein denaturation.

Functional Properties of Whey

Some of the important functional properties of whey protein are whippability, emulsifying capacity, gelling ability, solubility and water holding capacity (47). Moderate protein and high reducing sugar concentrations make whey a prime substrate for the Maillard reaction (9, 88). Denaturation of proteins during whey preparation is the major factor in its uses as a food ingredient (25, 47). A relationship between functional characteristics of whey in model food systems and the degree of protein denaturation was particularly obvious in samples with less than 20% and over 80% denaturation of the total whey protein (49, 54). Denatured whey protein has limited applications in food because of poor solubility (15, 46, 49, 69, 104, 114, 116, 118). The presence of high denatured whey protein concentrations produces poor whipability, poor foaming stability and low overrun. On the other hand, the removal of denatured whey protein greatly improves whippability and foaming properties of whey (70, 118).

Solubility is an important functional property of protein in many food applications (75, 86). The major functional advantages of undenatured whey protein (UWP) include its solubility throughout the entire pH range, gelation when subjected to heat treatments under the proper conditions, emulsification, foam stabilization and excellent nutritional quality (96, 98, 104). Relative concentration of the individual proteins in whey was thought to influence its functionality (27).

Insolubility of whey protein can be brought about by several treatments: denaturation through high heat plus pH adjustment to isolectric pH range and high ionic concentration (104). Mild protein

denaturation appeared to enhance functional properties of whey such as foaming stability, whippability and water holding capacity (50, (114). The desirable degree of denaturation depends largely on both pH and the desired functionality (50, 94, 114). Undenatured whey protein concentrates can be produced by electrodialysis, ion exchange, ultrafiltration and gel filtration (7, 104). Dalan (20) and other workers (4, 59, 72) suggested that precipitation of denatured whey protein can be achieved by the aid of partial whey proteolysis, polyacrylic acid modification (124)or succinic anhydride modification (125).

Milk Clotting Enzymes

The coagulant traditionally used for cheese making is calf rennet extract. However, there exists a worldwide shortage of calf rennet due to the continued increase in cheese production and consumption accompanied by a decline in veal calf slaughter. Therefore, a great deal of interest in finding rennet substitutes has been generated. Only a few animal proteases such as pepsin (porcine and bovine) and microbial protease such as Mucor miehei (MMP), Mucor pusillus var Lindt (MPP) and Endothia parasitica (EPP) are being used in cheese making (32, 35, 45). Interest in other rennet substitutes is considerable, including plant sources such as Withania coagulans, Carcia papaya, Ficus carica, Cynara cardunculus, Cucurbita pepo and Ricinus communis and microbial sources such as Bacillus subtilis, Bacillus brevis, Bacillus fusiformis, Bacillus mesentericus, Bacillus cereus, Streptococcus liquifaciens, Asperigillus candidus and Fomitopsis pinicola (32, 45, 78).

The key criterion determining the usefulness of an enzyme for

coagulation of milk in cheese making was cited by Green (45): ..."the ratio of the enzyme's milk clotting activity to its proteolytic activity should be high, as is the case for rennin." Plant proteases are capable of clotting milk; however, the majority are unsuitable for cheese production because of their extensive proteolytic activity which results in digestion of the curd. A satisfactory cheese was claimed to be produced using extracts of Ficus carica and the Palestinian Ricinus communis for milk clotting (135). Krishnamurthy et al. (78) used enzymic extracts from Bacillus subtilis and Bacillus cereus in clotting milk and a good quality cheese was produced.

Distribution of milk clotting enzymes during cheese making between curd and whey was influenced by both pH and the milk clotting enzyme used. In freshly coagulated milk that has not been subjected to cooking the distribution of rennin between curd and whey was highly pH dependent (66). At pH 6.6 about 31% of the enzyme activity remained in the curd and 69% in the whey. When coagulated at pH 5.2 about 86% of the enzyme remained in the curd and only 14% was lost in the whey. On the other hand, the distribution of Mucor enzymes between curd and whey was not pH dependent. About 90% of the activity went into the whey (66). During Cheddar cheese making the whey at draining contained about 58% of the original rennet activity. On the other hand Cheddar cheese whey from milk coagulated with Mucor enzymes contained about 94% of the original milk clotting activity (66). Distribution of residual milk clotting enzymes in whey was investigated by Thunell et al. (126). Animal and microbial enzymes were used in Cheddar cheese making. Whey was collected and its pH was adjusted from 5.2 to 7.0 prior to heating at 68.3 and 73.9C for

.25, .5, 1, 5 and 10 min. Enzymic activity was measured before and after heating. MMP was the most heat stable at all pH values followed by MPP, rennet, bovine pepsin, EPP and Porcine pepsin (PP). The heat stability of all enzymes increased with decreasing pH except EPP which was most stable at pH 7.0.

The effect of rennet on proteolysis of whey proteins was studied (19). At pH 4.5 drastic differences among whey proteins were produced. BSA was the most easily degraded protein, immunoglobulins exhibited limited proteolysis while little or no effect was observed on β-lg and α-la. At pH 5.5 and 6.5 rennin, MMP and papain hydrolysed β-lg, κ-casein and κ-casein/β-lg complex (22). Casein hydrolysis was affected by the heat induced κ-casein/β-lg complex and was dependent on the specificity of the enzymes used (23). Rennet wheys exhibited a negligible endopeptidase activity which indicated the absence of both neutral and alkaline proteases (73). Hydrolysis of whey proteins by milk clotting enzymes occurred in the following order of activity: MPP>PP>rennet and the hydrolytic activity was higher at pH 5.5 than 6.5; however, heat treatment progressively reduced proteolytic susceptibility (24).

Variations in Whey Proteins

A number of factors affect the relative proportion of individual proteins in whey. The most studied of these factors is a seasonal variation. A study over a three year period during which weekly samples were collected and analyzed for protein, indicated mean values of 12.12, 12.13 and 12.12% protein on dry basis for the three consecutive years (41). The lowest protein concentration occurred

(30, 31, 48, 109). Emmons et al. (31) found that nitrogen losses in whey during cheese making influence the yield at a rate of 2.62% per 0.1% increase in whey protein. Compared with calf rennet, reduction in cheese yield was 0.16% for bovine pepsin, 0.1%for 1:1 calf rennet plus porcine pepsin, 0.5-0.6% for Mucor enzymes, 1.2% for EPP and 4.9% for Bacillus polymyxa protease.

Determination of Undenatured Whey Protein

The literature is full of reports dealing with denaturation studies on individual as well as collective whey proteins. It is difficult to relate and interpret these results unless consideration is given to the methods by which denaturation was measured. Some workers (53, 58, 59, 60, 61, 62, 74, 79, 99) have used precipitation as a measure of denaturation. This is not strictly correct because denatured whey proteins may remain in solution at appropriate pH values and protein concentrations (132). For the purpose of this review denatured whey protein was defined in terms of the Harland-Ashworth test and its modifications. However, it is recognized that it may not be a true measure of denaturation in the strict sense. This method is chosen because it is a test which can be used by industry as a measure of heat treatment applied to skim milk or whey during processing.

Dry whey analysis is generally carried out by the same procedures used for NDM. However, some difficulties arise with the determination of undenatured whey protein nitrogen (UWPN) (132). Measurements of UWPN require removal of both casein and denatured whey protein from whey samples. Precipitation of casein and denatured whey protein is

the most widely used technique to remove these materials. Adjustment to pH 4.6 (58) or saturation with NaCl (79) are the most common preparation methods. Values for UWPN obtained by sodium chloride saturation were considerably lower than those obtained by acid precipitation (132).

UWPN values in NDM (53) were used to predict the quality of baked goods when NDM was used as a major ingredient. The best treatment applied during manufacture of NDM had a great effect on the properties of baked goods in which it is used. It is necessary to heat skim milk to high temperatures prior to drying in order to control water absorption by dough and to produce maximum loaf volume. On the other hand, low heat NDM is preferred for fortification of skim milk for Cottage cheese and in ice cream making. The generally accepted classification of NDM as adapted by the American Dry Milk Institute (5) is:

Classification	mg UWPN/gm NDM
Low Heat	>6.0
Medium Heat	1.51-5.99
High Heat	<1.5

Estimation of UWPN by the Harland-Ashworth procedure for NDM has little value for whey powder because of its different composition and likelihood of pH variation (132). Therefore, it was proposed that NDM (89) and whey powder (132) be heat classified according to the percentage of UWPN/total whey protein nitrogen. Wyeth (132) proposed a heat treatment classification of whey powder as follows:

Classification	Percent UWPN in whey powder
Low Heat	>75
Medium Heat	20-75
High Heat	<20

Total nitrogen minus nonprotein nitrogen was used as a measure of whey protein nitrogen. UWPN was estimated by Kjeldahl analysis of Harland-Ashworth filtrates from whey powder (132).

The procedure for estimating UWPN in NDM was developed by Harland and Ashworth (53) and later modified by Kuramoto et al. (79). It relied on the saturated sodium chloride precipitation of casein and denatured whey protein in reconstituted NDM. It was then filtered to collect the Harland-Ashworth filtrate which contained the undenatured whey protein (UWP). Subsequent precipitation of UWP from Harland-Ashworth filtrate under acidic conditions produced turbidity which was measured by spectrophotometry. Turbidimetry is read as percent transmittance which is converted to mg UWPN/gm NDM with the aid of standard curves. The standard curve is constructed from different dilutions and combinations of Harland-Ashworth filtrates collected from high and low heat NDM. The transmittance is plotted on the Y axis and the corresponding mg UWPN/g NDM (determined by kjeldahl analysis) is on the X axis.

Sanderson (120) discussed the errors and limitations associated with the use of the turbidimetric method. A considerable variation in the degree of turbidity was developed at constant UWP concentrations. β-lg and α-la develop different levels of turbidity at equal concentrations. Seasonal variations in whey protein are accompanied by variations in the relative amounts of individual whey proteins. The later

variations resulted in variations in the standard curves.

A procedure to determine UWPN in whey powder was developed by Reyes (111) in which turbidimetric difficulties were avoided. Dried whey was reconstituted to 6.5% solids. The pH was adjusted to 6.7 and the solution saturated with sodium chloride to precipitate casein and denatured whey protein. UWPN was calculated from the difference in absorbance at 280 nm of the filtrate before and after complete deproteinization with 12% TCA.

Use of sulfonic acid-azo dyes (amido black, orange G and acid orange 12) have been investigated by dairy scientists for protein estimation in milk. Fraenkel and Cooper (40) believed that sulfonate groups on these dyes form insoluble salts with positively charged groups of basic amino acids in the protein. The amount of dye bound by a measured quantity of milk indicates the protein content. Use of amido black for whey protein determiantion in dry whey and NDM was investigated (11, 17, 28, 89, 90, 91, 110). UWPN was measured on either Harland-Ashworth filtrate or supernatant of centrifuged solution after pH adjustment to 4.6. The results correlated highly with Kjeldahl methods. Orange G was used to a lesser extent (12, 28, 121).

Sample preparation for estimating UWP was suggested by Harwalkar (58). The precipitate was removed by centrifugation. The pH of the supernatant was then adjusted to 4.5 and recentrifuged. The UWP in the supernatant can then be determined by gel electrophoresis, optical rotation, sedimentation velocity, gel permeation or differential scanning calorimetry (57, 58, 118). Kearney and McGann (74) developed a method for determining protein denaturation in NDM using cellulose acetate strips. A co-precipitated κ-casein β-lg band resulted from

heat denaturation of whey proteins and was quantified by densitometry.

Many of the early determinations of specific denatured whey proteins used zonal electrophoresis and immunoelectrophoresis (47). Quantitative estimation of a mixture of whey proteins was determined by spectrophotometric scanning of stained protein bands following polyacrylamide gel electrophoresis. β-lg A was usually incorporated as an internal standard (25, 36, 60, 100, 103). Agarose plates contained antiserum to α-la and β-lg, these antisera being used for quantitative determination of the two proteins by measuring and comparing migration distance for the samples and controls (29).

Richert et al. (115) studied denaturation of whey protein by determining the increase in viscosity with a Brookfield LVF viscometer. A method for determining the extent of denaturation was developed by Manning (85) in which heat induced redistribution of SH and SS groups was measured in the protein precipitated at pH 4.6 and in the supernatant.

METHODS AND MATERIALS

Modified Harland-Ashworth Test for Nonfat Dry Milk

The Harland-Ashworth test (49) as modified by Kuramoto et al. (79) was combined with the recommendation of Reyes (111) and applied to NDM.

Two grams of NDM were reconstituted in 20 ml distilled water in a 25 x 150 mm test tube. Eight grams of NaCl were added and the test tube was stoppered and placed in a water bath at 37C for 30 min. The contents were shaken 8-10 times during the first 15 min of the incubation period to insure complete saturation of the sample with NaCl.

After cooling to 22C the mixture was filtered through S & S No. 602, 9 cm filter paper, and 5 ml of filtrate was collected (Harland-Ashworth filtrate). The mixture was refiltered through the same filter paper when the first portion of the filtrate was cloudy.

Total absorbance. One millilter of filtrate was diluted with 10 ml distilled water in a 25 x 150 mm test tube, stoppered and mixed by inverting three times before measuring total absorbance at 280 mn against a blank prepared by diluting 1 ml saturated sodium chloride solution with 10 ml distilled water.

Nonprotein absorbance. One millilter filtrate was added to 10 ml 13.2% (w/v) trichloroacetic acid (TCA) in a 25 x 150 mm test tube, stoppered and mixed by inverting three times, incubated at 37C for 5-15 min then filtered through Whatman No. 42 filter paper. The nonprotein was measured at 280 nm. A blank prepared by the addition of 1 ml saturated sodium chloride phosphate buffer to 10 ml 13.2% TCA (w/v). Undenatured

whey protein nitrogen (UWPN) absorbance was calculated in total absorbance - nonprotein absorbance.

The spectrophotometer used was a Beckman DB-GT (Beckman Instruments, Inc., Instruments Division, Fullerton, CA).

Modified Harland-Ashworth Test for Whey Powder

One gram of whey powder was reconstituted in 25 ml of 0.1 M phosphate buffer (0.1 M of NaH_2PO_4 was titrated with 0.1 M Na_2HPO_4 to pH 6.7), in a 25 x 150 mm test tube. Ten grams of NaCl were added. The tube was stoppered and placed in a water bath at 37C for 30 min. The contents were shaken 8-10 times during the first 15 min of incubation to insure complete saturation of the sample with NaCl.

After cooling to 22C the mixture was filtered through S & S No. 602, 9 cm filter paper, and 5 ml filtrate was collected. The mixture was refiltered through the same filter paper when the first portion of the filtrate was cloudy.

Total absorbance. One milliliter filtrate was diluted with 10 ml distilled water in a 25 x 150 mm test tube, stoppered and mixed by inverting three times before measuring the total absorbance at 280 nm against a blank prepared by diluting 1 ml phosphate buffer saturated with sodium chloride with 10 ml distilled water.

Nonprotein absorbance. One milliliter filtrate was added to 10 ml 13.2% TCA (w/v) in a 25 x 150 mm test tube, stoppered and mixed by inverting three times. The nonprotein absorbance was measured at 280 nm against a blank prepared by the addition of 1 ml phosphate buffer saturated with sodium chloride to 10 ml 13.2% TCA (w/v) in a 25 x 150 mm test tube. The sample and blank tubes were incubated at 37C for 5-15 min

then filtered through Whatman No. 42 filter paper. UWPN absorbance was calculated as total absorbance - nonprotein absorbance.

Standard curve for the determination of mg undenatured whey protein nitrogen. Four grams of whey powder (no heat treatment) were reconstituted in 50 ml of 0.1 M phosphate buffer, pH 6.7 in a 25 x 150 mm test tube. Twenty grams of NaCl were added. The tube was stoppered and placed in a water bath at 37C for 30 min. The contents were shaken 8-10 times during the first 15 min of the incubation period to insure complete saturation of the sample with NaCl.

After cooling to 22C the mixture was filtered through S & S 602 filter paper, and 20 ml of filtrate was collected. The mixture was refiltered through the same filter paper if the first portion of the filtrate was cloudy. A blank was prepared by addition of 10 g NaCl to 25 ml 0.1 M phosphate buffer in a 25 x 150 mm test tube, stoppered, incubated and shaken and then filtered through S & S 602, 9 cm filter paper in the same manner described for the sample. Portions of the blank and sample filtrates were pipetted into 25 x 150 mm test tubes as follow:

Tube No.	Sample Filtrate (ml)	Blank Filtrate (ml)
1	5.0	0.0
2	4.0	1.0
3	3.0	2.0
4	2.0	3.0
5	1.0	4.0

Total absorbance, nonprotein absorbance and UWPN absorbance were measured as previously described. A curve was plotted with UWPN concentration on the X axis and absorbance on the Y axis.

Determination of Nitrogen

All nitrogen analyses were made by a semi-micro kjeldahl procedure (10).

Total nitrogen in whey powder. One gram of whey was reconstituted in 25 ml distilled water and tested for nitrogen.

Nonprotein nitrogen in whey powder. Two milliliters of reconstituted whey (1 g in 25 ml distilled water) were mixed with 2 ml 24% TCA (w/v) and filtered through Whatman No. 42 filter paper. Two milliliters of the filtrate were tested for nitrogen.

Nonprotein nitrogen in NaCl saturated filtrates. Two milliliters of NaCl saturated filtrate was mixed with 2 ml distilled water. Four milliliters of 24% TCA (w/v) were added to the sample and filtered through Whatman No. 42 filter paper prior to nitrogen analysis. All tests were run in duplicate against the appropriate blank.

Milk Clotting Enzymes

The milk clotting enzymes included, single strength rennet (New Zealand Cooperative Rennet Co., Ltd., Eltham, New Zealand), adult bovine gastric extract (BGE) (Chr. Hansen's Laboratory, Inc., Milwaukee, Wisconsin), porcine pepsin (PP) (Pfizer Inc., Chemicals Division, New York, N.Y.) Mucor meihei protease (MMP) (Marschall Division, Miles Laboratories Inc., Madison, Wisconsin). Mucor pusillus Var. Lindt protease (MPP) (Dairyland Food Laboratories, Inc., Waukesha, Wisconsin) and Endothia parasitica protease (EPP) (Pfizer Inc., Chemicals Division, Milwaukee, Wisconsin).

Enzyme Activity

Enzymatic activities were measured in rennin units (RU) by

comparing the milk clotting ability of the enzyme solution with that of single strength rennet. The procedure of Ernstrom (32) was modified by diluting the standard enzyme 1:200 instead of 1:250 to produce a substrate clotting in 100 ± 5 revolutions.

Monthly Whey Samples

Monthly samples of raw skim milk from a single herd were obtained from the Utah State University Dairy Products Laboratory. The skim milk was clotted at 37C for 30 min with single strength rennet (18.4 RU/ml of milk). The clot was broken and the resulting slurry filtered through glass wool to separate the curd and whey. The whey was freeze-dried at a pressure of 100 millitorr (FTS System, Inc., Stone Ridge, N.Y.) and stored at room temperature until needed. Whey samples were divided into three portions and reconstituted in distilled water. The pH was then adjusted to 6.4, 6.0 or 5.6 with 0.1 N HCl. Each portion was adjusted with distilled water to produce a final whey concentration of 6, 12 or 18% solids. Each of the concentrations were subdivided into 3 portions which were placed 25 x 150 mm test tubes and subjected to heat treatments at 63, 77 or 91C for 30 min. Tubes were immersed in a water bath at 94C and agitated vigorously until the desired temperature was reached. They were then placed in a second water bath at the same temperature. After 30 min the tubes were removed to an ice bath, freeze-dried and stored in plastic bags. Whey sample preparation and treatments are shown in figure 1.

Whey samples from different milk clotting enzymes. A sample of raw skim milk was subdivided into six portions. Each was treated at 37C for 30 min with one of the previously described enzymes. The clot

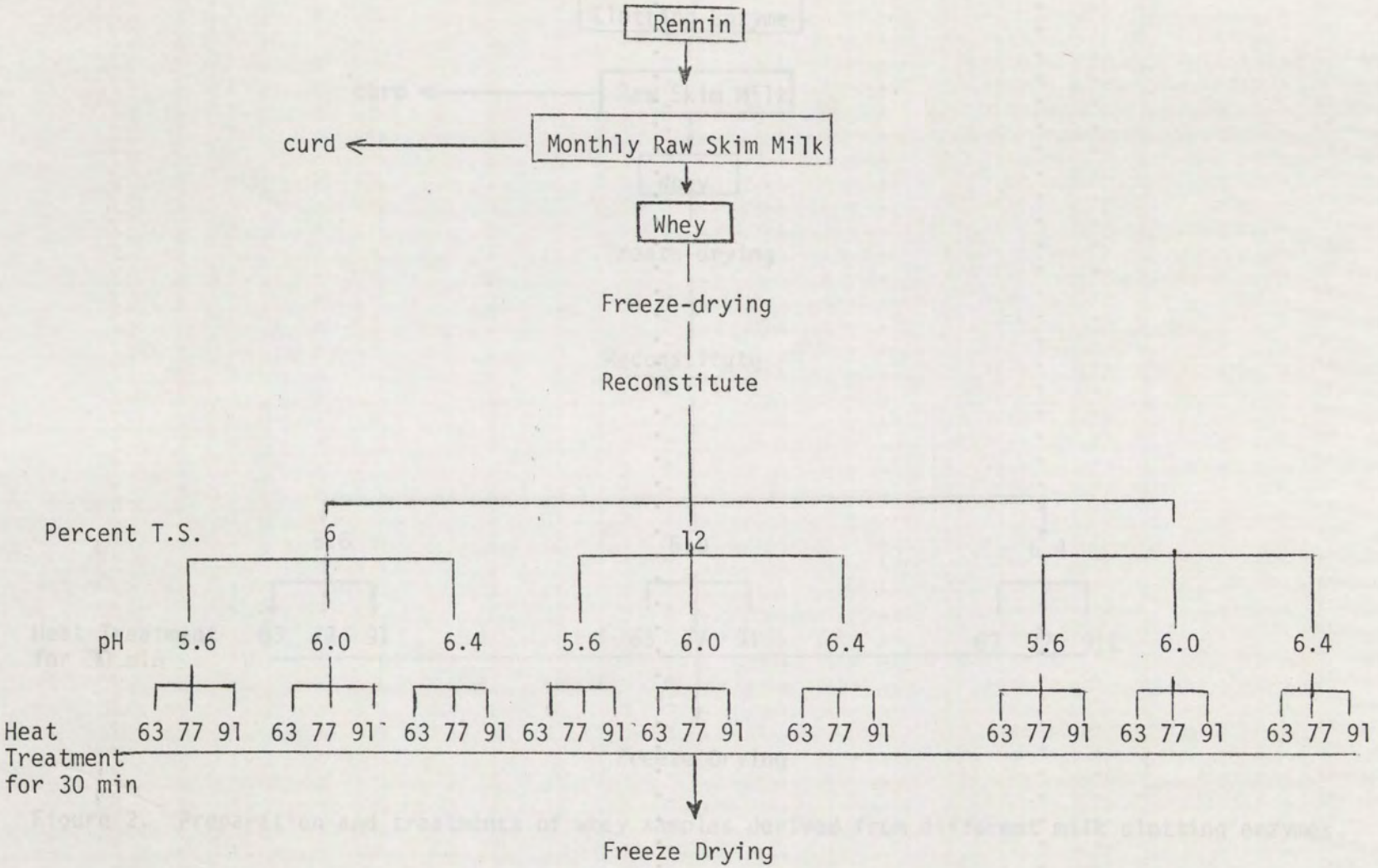

Figure 1. Preparation and treatments of monthly whey samples.

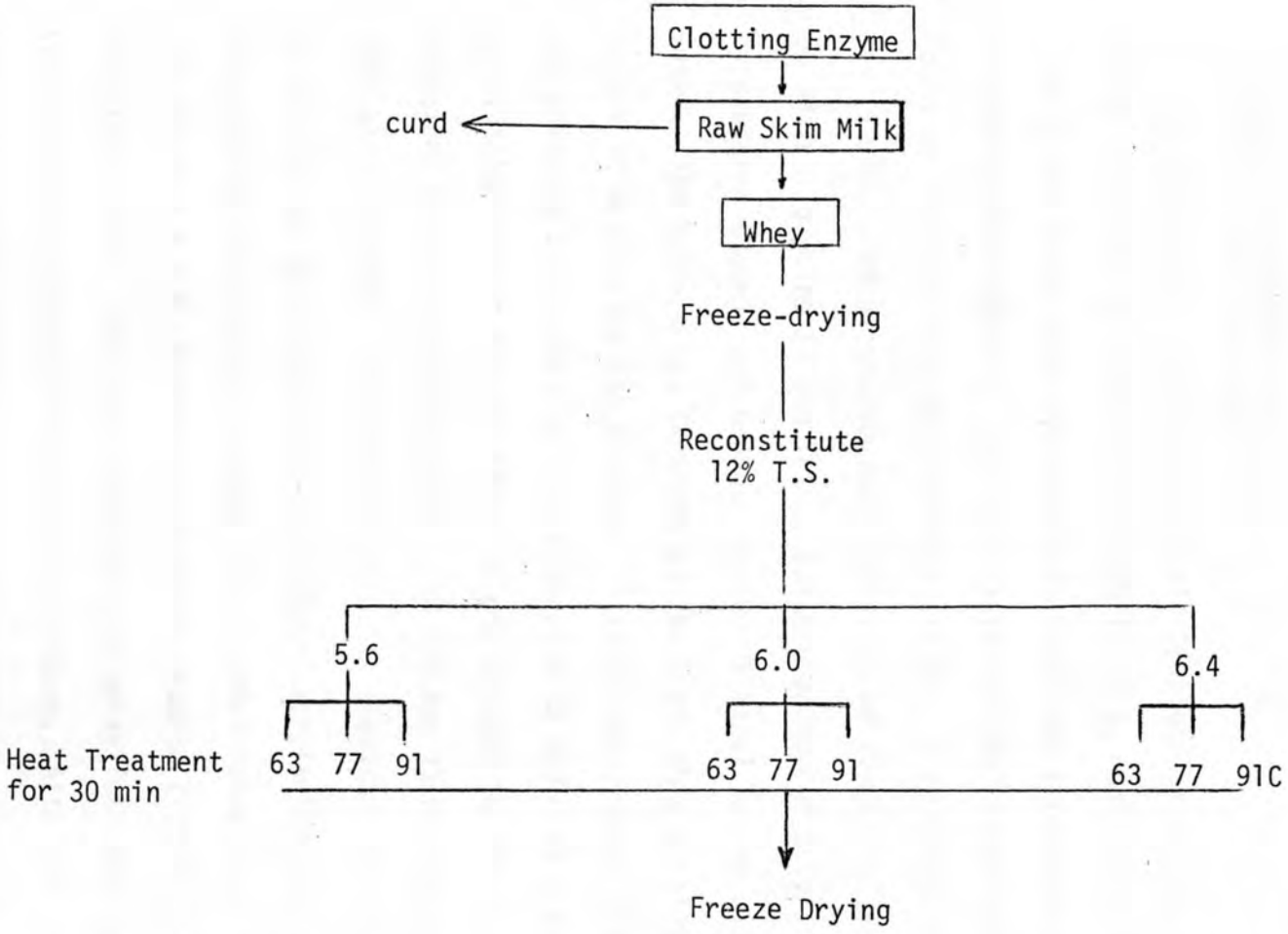

Figure 2. Preparation and treatments of whey samples derived from different milk clotting enzymes.

was broken and the slurry filtered through glass wool. The whey fraction was collected and freeze-dried. The whey powder was reconstituted to 12% solids and the pH adjusted to 5.6, 6.0 or 6.4. Each sample was heated to 63, 77 or 91C for 30 min then freeze-dried. Sample preparation and treatments are shown in figure 2.

SDS Gel Electrophoresis of the Proteins of Raw Dried Whey

Sodium dodecyl sulfate (SDS) electrophoresis was carried out using the method of Weber and Osborn (127) as modified (105). One half gram of raw dried whey (no heat treatment) was reconsitituted in 25 ml chamber buffer (pH 8.8). One milliliter of the reconstituted whey was added to 1 ml tracking dye solution (1% SDS, 5 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT), 20 mM glycerol, .02% pyronin Y, 50 mM Tris/glycine (0.5 m Tris: 1.5 M glycine, pH 8.8, from a previously prepared stock), and brought to 100 ml final volume with distilled water. The mixture was then heated for 7 min in a boiling water bath to aid in dissolving the protein. A stock solution of 25% acrylamide was prepared by dissolving 25.0 g acrylamide and 0.25 g bis acrylamide in distilled water and the final volume brought to 100 ml. Stock solutions of 2.0 M Tris/glycine, 2.5% SDS and 1% ammonium persulfate were also prepared. The casting solution consisted of stock solutions in the following proportions: 10 volumes acrylamide-bis, 5 volumes Tris/glycine buffer, 2.5 volumes 50% glycerol solution, 1 volume SDS, .01 volume N,N,N,N, tetramethylethylene diamine (TEMED), 5.75 volumes distilled water. One volume of ammonium persulfate was added immediately before casting to initiate polymerization. After the gel was loaded to within 5 mm of the top of the tube (8 cm x 5 mm ID tube), a

solution of electrode buffer was layered on top of the crylamide solution to form a smooth interface. After polymerization was completed (20 min), the layering solution was removed.

Electrophoresis was conducted in a Bio-Rad unit. Each chamber filled with 500 ml of electrode buffer consisting of 200 mM Tris/glycine (pH 8.8) and 0.1% SDS. A Heathkit IP - 17 constant voltage power supply of 0- 400 V, 100 mA capacity was employed. Twenty microliter of whey-tracking dye mixture was loaded on the gel.

Electrophoresis was initiated at 0.5 mA per gel. After the dye had completely entered the gel (about 30 min), the current was raised to 1.0 mA per tube (12 mA at for 12 gels). Electrophoresis was continued until the dye front was within 5 mm of the bottom of the gel. The power was shut off, the gels were removed from the tubes and the dye front marked by insertion of a fine wire. The proteins in the gel was fixed overnight in a solution consisting of 50% (V/V) methanol and 7.5% (V/V) glacial acetic acid. The gels were scanned at 280 nm in a DU-8 Beckman spectrophotometer (slit width was 5, gel slit .2 mm, read average 1, gel speed 1 cm/min and chart speed was 1 cm/min).

Reagents and Chemicals

Crystalline bovine serum albumin was obtained from the Schwarz-Man Chemical Company, Orangeburg, New York. Alpha - lactalbumin was obtained from Sigma Chemical Company, St. Louis, Missouri and beta - lactoglobulin from United State Biochemical Corporation, Cleveland, Ohio.

All reagents used in electrophoresis were Bio - Rad Chemicals and were purchased directly from them. Reagent grade sodium chloride, mono and dibasic sodium phosphate and trichloroacetic acid were used.

RESULTS

Seasonal Variation in Nitrogen Fractions of Whey

Whey samples were collected monthly from raw skim milk coagulated with rennet. The whey fractions were freeze-dried. Total, protein and nonprotein nitrogen (milligram N per gram dried whey) were determined by a semi-micro kjeldahl procedure (10). The results are presented in Figure 3. The modified test was carried out and the Harland-Ashworth filtrate was analyzed for total, undenatured protein and nonprotein nirgogen were determined (Figure 4).

A parallel seasonal flucuation in total and protein nitrogen was observed. On the other hand there was very little seasonal flucuation in nonprotein nitrogen in dried whey (Figure 3). Total and protein nitrogen varied from a maximum of (25.984 mg/g dried whey and 21.01 mg/g dried whey) in the month of October to a minimum of (22.33 mg/g dried whey and 17.559 mg/g dried whey) in the month of August. This represents 13.9% seasonal variation in total nitrogen and 16.4% in protein nitrogen. Similarly seasonal flucuations in total and undenatured protein nitrogen of raw (no heat treatments) dried whey were essentially parallel (Figure 4). However, seasonal flucuation in nonprotein nitrogen in the Harland-Ashworth filtrates was more pronounced than in dried whey.

Nitrogen Fractions in Whey From Milk Clotted with Different Milk Clotting Enzymes

Raw skim milk was coagulated with rennet, BGE, PP, EPP, MMP and MPP at concentrations usually used for cheese making. The whey was collected and freeze-dried. Nitrogen fractions in the raw dried whey were

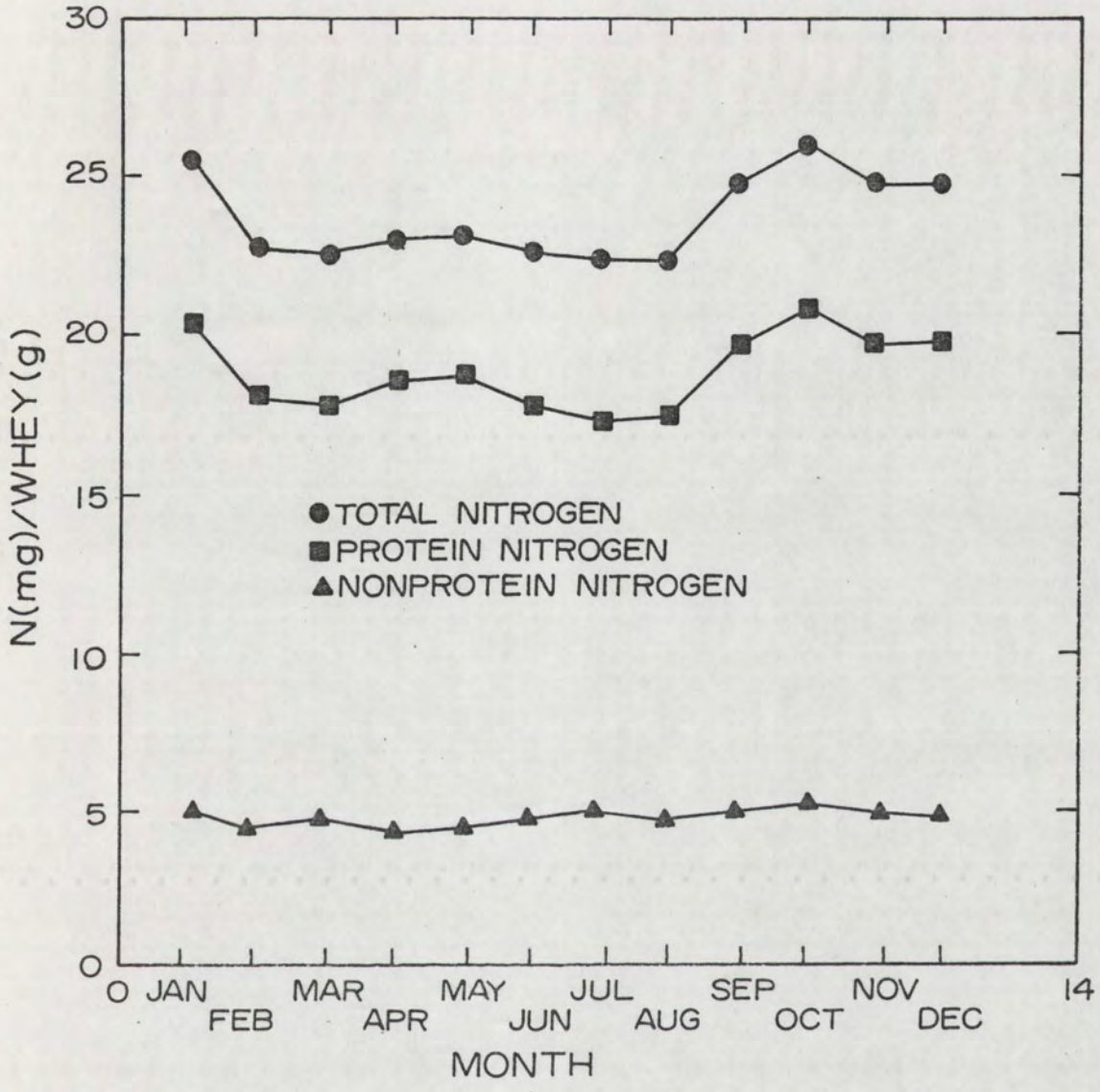

Figure 3. Total, protein and nonprotein nitrogen in raw dried whey collected monthly over one year.

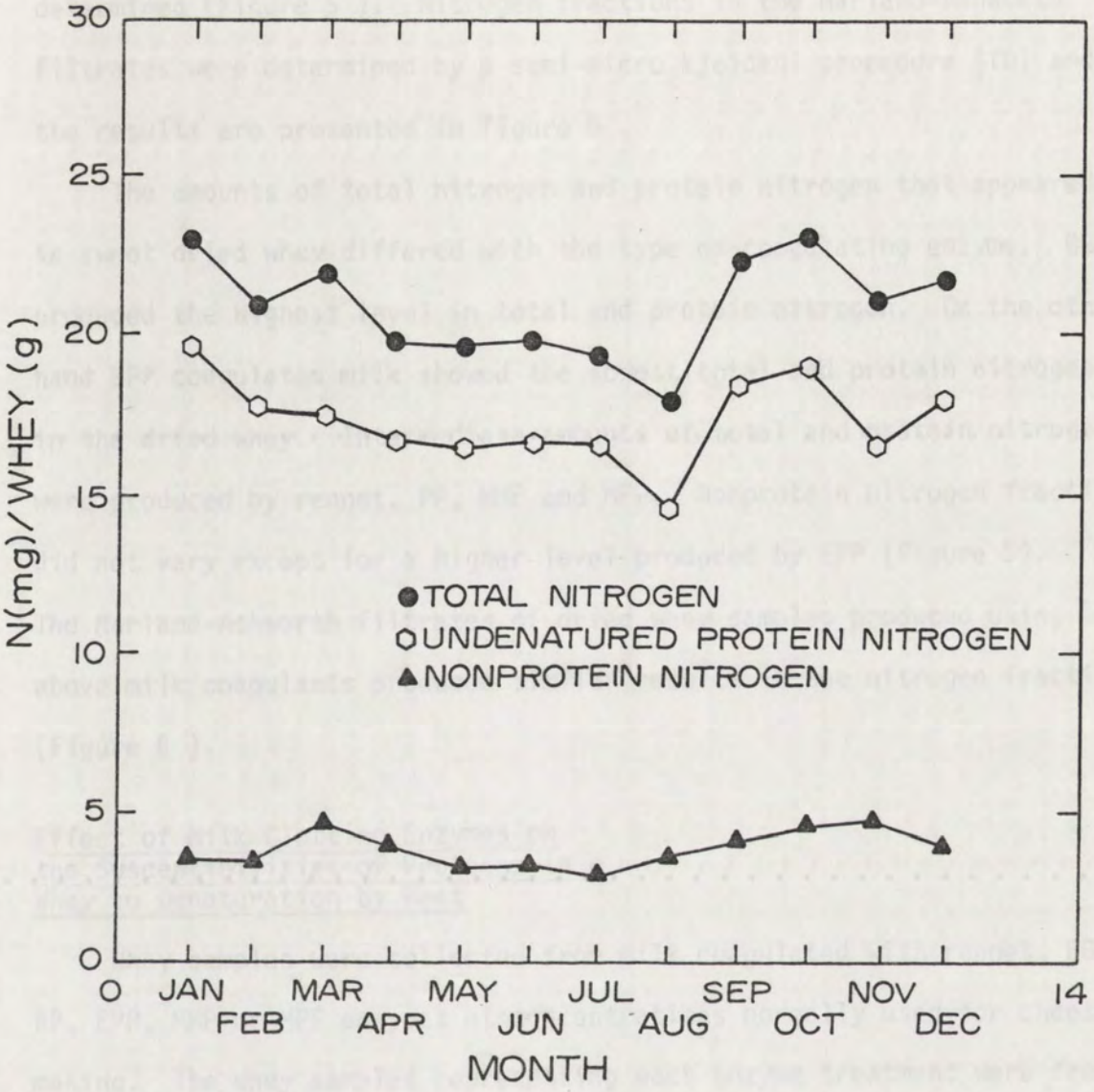

Figure 4. Total, undenatured protein and nonprotein nitrogen in Harland-Ashworth filtrates from raw dried whey collected monthly over one year.

determined (Figure 5). Nitrogen fractions in the Harland-Ashworth filtrates were determined by a semi-micro kjeldahl procedure (10) and the results are presented in figure 6 .

The amounts of total nitrogen and protein nitrogen that appeared in sweet dried whey differed with the type of coagulating enzyme. BGE produced the highest level in total and protein nitrogen. On the other hand EPP coagulated milk showed the lowest total and protein nitrogen in the dried whey. Intermediate amounts of total and protein nitrogen were produced by rennet, PP, MMP and MPP. Nonprotein nitrogen fractions did not vary except for a higher level produced by EPP (Figure 5). The Harland-Ashworth filtrates of dried whey samples produced using the above milk coagulants produced similar results in the nitrogen fractions (Figure 6).

Effect of Milk Clotting Enzymes on the Susceptibilities of Proteins in Whey to Denaturation by Heat

Whey samples were collected from milk coagulated with rennet, BGE, PP, EPP, MMP or MPP enzymes at concentrations normally used for cheese making. The whey samples representing each enzyme treatment were freeze-dried, divided into three portions, reconstituted to12% solids and then heated at 63, 77, 91C for 30 min. The heated samples were freeze-dried and the undenatured whey protein nitrogen in the Harland-Ashworth filtrates was determined by a Kjeldahl procedure (10). A raw whey sample from each enzyme (no heat treatment) served as a control. The results are presented in figure 7. Heating at 63C for 30 min produced a maximum denaturation of 17.3% of the total undenatured whey protein in dried whey samples from milk coagulated with BGE and a minimum denaturation of

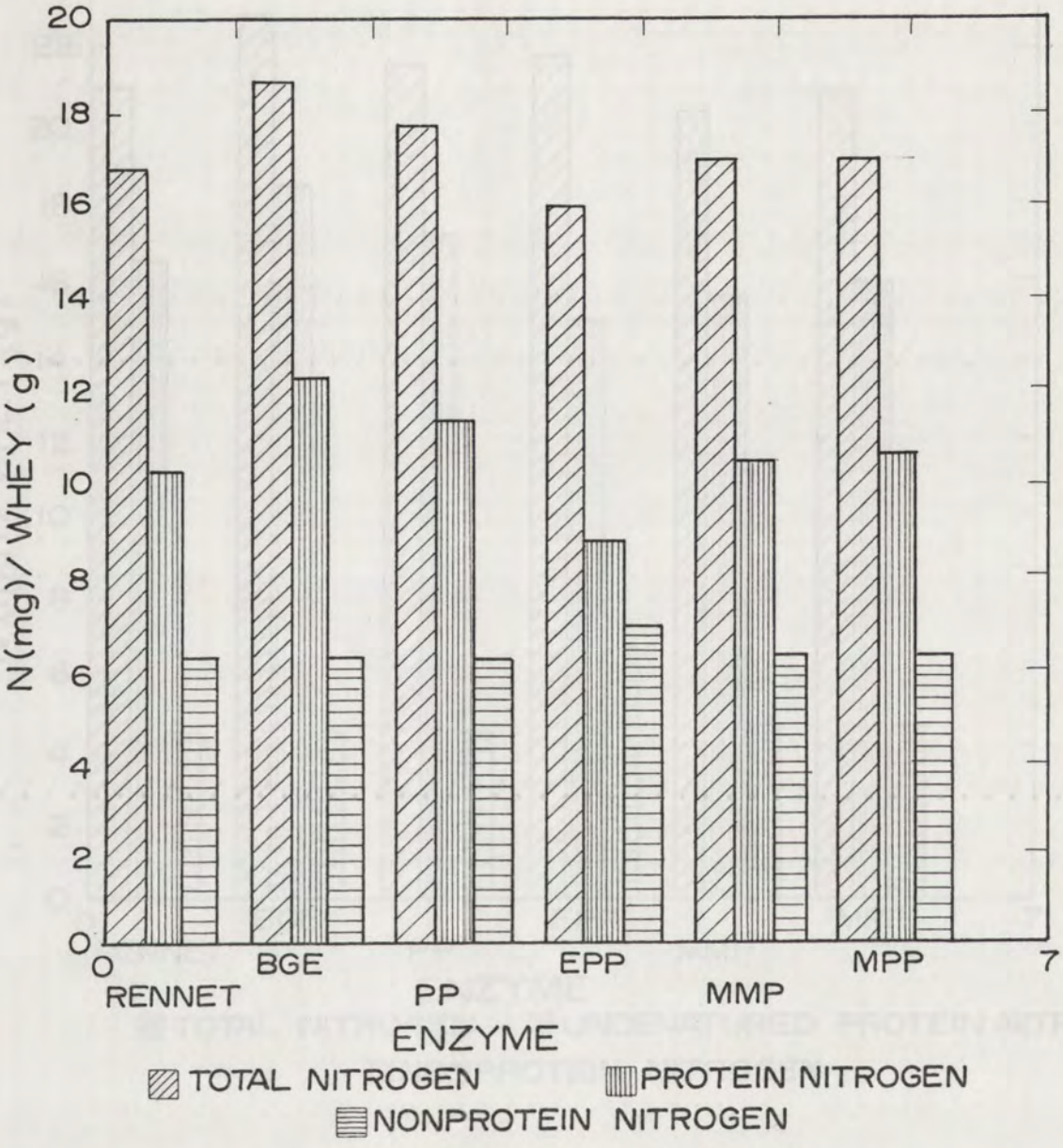

Figure 5. Total, protein and nonprotein nitrogen in raw dried whey from the same milk coagulated with six different milk clotting enzymes.

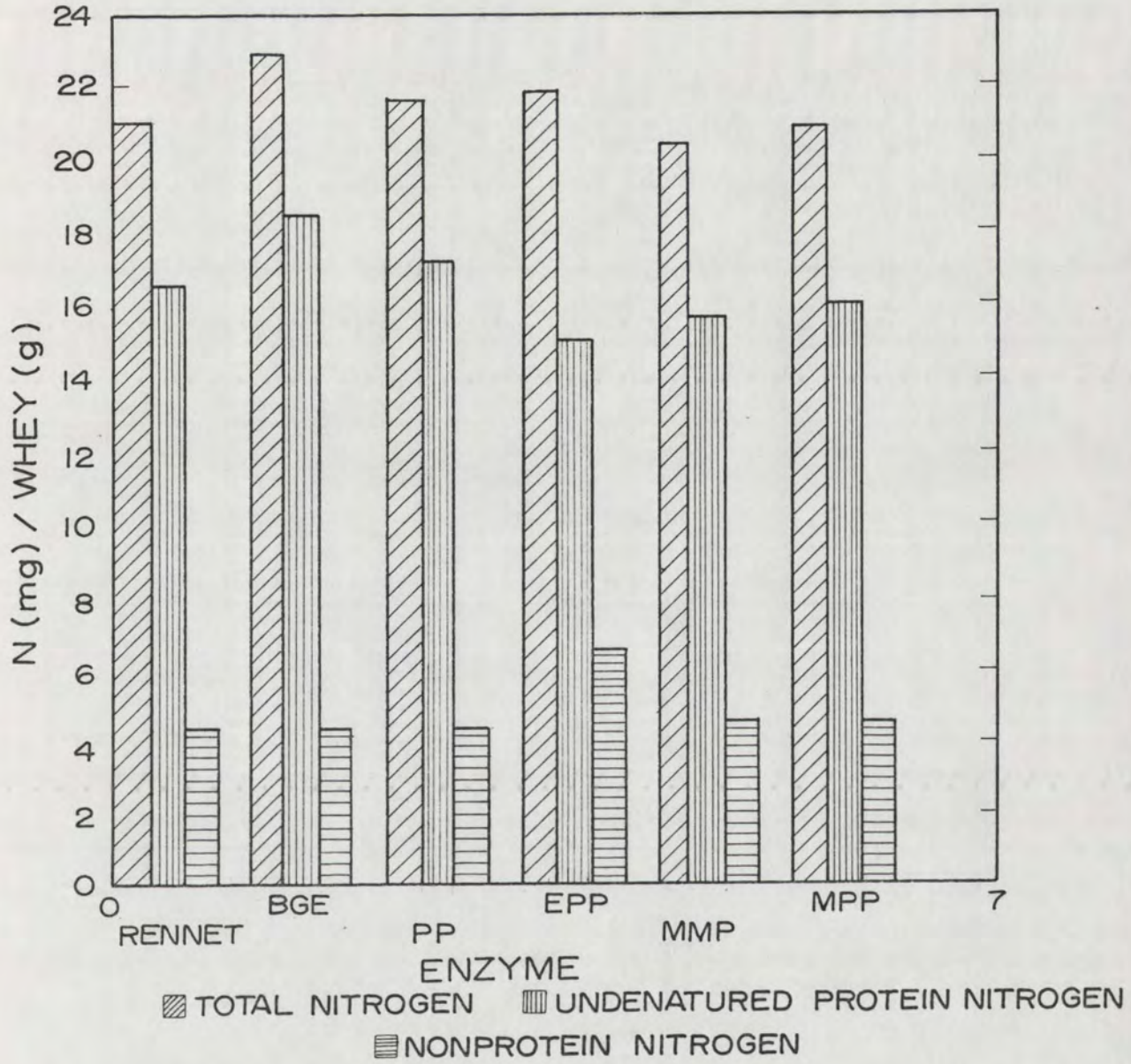

Figure 6. Total, undenatured protein and nonprotein nitrogen in Harland-Ashworth filtrates in raw dried whey from the same milk coagulated with six different enzymes.

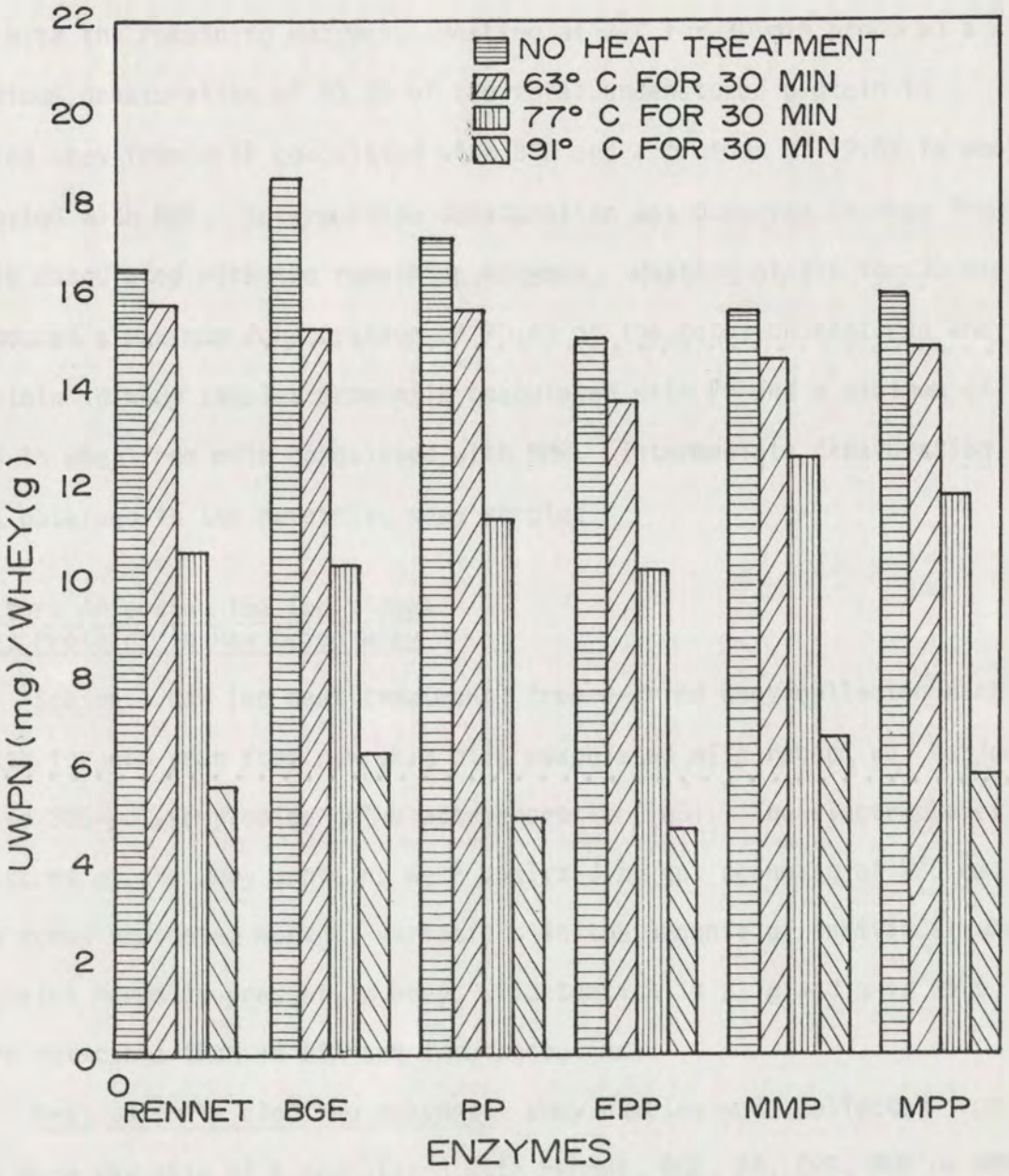

Figure 7. Effect of milk clotting enzymes on the susceptibilities of whey protein to denaturation by heat.

4.7% of the whey proteins in whey from milk clotted with rennet. Intermediate denaturation was observed in whey samples from milk coagulated with the remaining enzymes. Heating at 77C for 30 min produced a maximum denaturation of 43.9% of the total undenatured protein in dried whey from milk coagulated with BGE and a minimum of 19.6% in whey clotted with MMP. Intermediate denaturation was observed in whey from milk coagulated with the remaining enzymes. Heating at 91C for 30 min produced a maximum denaturation of 70.4% of the total undenatured whey protein in whey samples from milk coagulated with PP and a minimum of 57% in whey from milk coagulated with MMP. Intermediate denaturation was observed in the remaining whey samples.

Factors Affecting the Individual Whey Proteins in Raw Dried Whey

Season. Raw (no heat treatment) freeze-dried whey collected each month for one year from raw skim milk coagulated with rennet was subjected to SDS-polyacrylamide gel electrophoresis (105). The electrophoretic patterns of the whey proteins were analyzed by gel scanning at 280 nm. The scans indicated monthly variations in the amounts of individual whey proteins normally present in whey. Fluctuation in β-lg and α-la were more noticable than in BSA and immunogobulins.

Residual milk clotting enzymes. Whey samples were collected from the same raw skim milk coagulated with rennet, BGE, PP, EPP, MMP or MPP in concentrations usually used for Cheddar cheese making. The whey samples were divided into four portions. One was left at its normal pH (about 6.7) while the other portions were adjusted to pH 6.4, 6.0 or 5.6 with 0.1 N HCl. Each portion was further subdivided into three portions and the twelve whey samples were incubated to 2, 4 or 6 hr at 37C. After

incubation the whey samples were freeze-dried and analyzed by SDS-polyacrylamide gel electrophoresis.

The scans showed differences in the protease peptone fraction of wheys from milk coagulated with different milk clotting enzymes. However, the immunoglobulins and BSA did not show detectable proteolytic changes at any pH value or incubation time. On the other hand the effect of milk clotting enzymes on α-la and β-lg were not conclusive and further studies are needed to clarify their effect on these proteins.

Evaluation of the Modified Harland-Ashworth Test for Measuring Undenatured Whey Protein Nitrogen in Nonfat Dry Milk

The reproducibility of the modified and the conventional Harland-Ashwroth test (79) were compared. The results are presented in Table 1. The modified Harland-Ashworth test was significantly more reproducible than the conventional test. Therefore, it should be considered as an alternative method for measurement of undenatured whey protein nitrogen in NDM.

Table 1. Reproducibility of absorbance values of Harland-Ashworth filtrates prepared by the modified and conventional Harland-Ashworth test on twenty replicates of nonfat dry milk sample.

	Modified test (A_{280})	Conventional test (A_{420})
Mean	0.253	0.269
Minimum	0.250	0.250
Maximum	0.255	0.300
Standard Deviation	0.002	0.015

Evaluation of the Modified Harland-Ashworth Test for Measuring Undenatured Whey Protein Nitrogen in Whey Powder

The conventional Harland-Ashworth test for NDM (79) was modified by replacing 2g of NDM with 1g dried whey and measuring absorbance of the turbid filtrate at 420 nm instead of percentage transmittance at 420 nm. The reproducibility of the two tests is presented in Table 2. The results indicate that the modified test was significantly more reproducible than the conventional test. It is therefore, recommended as a suitable method for measuring UWPN in dried whey.

Table 2. Reproducibilities of absorbance values of Harland-Ashworth filtrates prepared by the modified and conventional Harland-Ashworth tests on twenty replicates of whey powder sample.

	Modified test (A_{280})	Conventional test (A_{420})
Mean	0.465	0.798
Minimum	0.450	0.710
Maximum	0.485	0.900
Standard Deviation	0.009	0.083

Factors Affecting the Correlation Between Absorbance and Undenatured Whey Protein Nitrogen

The suggested modified Harland-Ashworth test for the determination of milligrams undenatured whey protein nitrogen per gram of dried whey (UWPN) was carried out on dried whey samples subjected to different treatments and preparation procedures. Absorbance of UWPN at 280 nm (A_{280}) was measured by a spectrophotometer whereas the corresponding value of UWPN was determined by a semi-micro Kjeldahl procedure (10).

The application of the test on a routine basis would be to predict the UWPN from A_{280} in the Harland-Ashworth filtrate without carrying out Kjeldahl analysis. Linear regression analysis of the data was conducted (12) and several regression equations were produced.

Effect of season on standard curves for the modified test. Raw dried whey samples were collected each month for one year, and used to establish standard curves correlating A_{280} with UWPN. The twelve month data were subjected to analysis of variance (12). The results of the statistical analysis showed that whey samples obtained at different months did not significantly affect the relationship between A_{280} and UWPN values or vice versa. Alpha levels for these relationships were less than 0.9984 and 0.9997 respectively for A_{280} to UWPN and UWPN to A280 as a function of season (Tables 3 and 4).

Table 3. Analysis of variance of seasonal effect on the standard curve of the modified Harland-Ashworth test of raw whey samples collected over twelve months. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Month	11	0.0701	0.0060	0.17	0.9984
Error	48	1.7735	0.0369		
Corrected Total	59	1.8435			

$R^2 = 0.0380$

Table 4. Analysis of variance of seasonal effect on the standard curve of the modified Harland-Ashworth test of raw whey samples collected over twelve months. Milligrams undenatured whey protein nitrogen per gram dried whey is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Month	11	161.9432	14.7221	0.12	0.9997
Error	48	5714.0791	119.0433		
Corrected Total	59	5876.0222			

$R^2 = 0.0276$

Effect of milk clotting enzymes on standard curves for the modified test. Untreated freeze dried whey samples (raw)from raw skim milk coagulated with rennet, BGE, PP, EPP, MMP or MPP were used to establish standard curves correlating A_{280} with UWPN values. The data were subjected to analysis of variance. Milk coagulants used did not significantly affect the relationship between absorbance and UWPN or vice versa. Alpha levels for these relationships were 0.9829 and 0.9918 for A_{280} to UWPN and UWPN to absorbance respectively as a function of milk clotting enzyme used to prepare the whey samples (Tables 5 and 6).

Table 5. Analysis of variance of the effect of milk clotting enzymes on the standard curve of the modified Harland-Ashworth test of raw whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Enzyme	5	0.0228	0.0045	0.13	0.9829
Error	24	0.8170	0.0340		
Corrected Total	29	0.8398			

$R^2 = 0.0271$

Table 6. Analysis of variance of the effect of milk clotting enzymes on the standard curve of the modified Harland-Ashworth test of raw whey samples collected from raw skim milk coagulated with different milk clotting enzymes. Milligrams of undenatured whey protein nitrogen per gram dried whey is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Enzyme	5	53.2629	10.6525	0.10	0.9918
Error	24	2645.6582	110.2248		
Corrected Total	29				

$R^2 = 0.0197$

Factors Affecting the Results of the Modified Harland-Ashworth Test

Freeze-dried whey samples were prepared each month. They were then divided into three portions and reconstituted in distilled water. The pH was adjusted to 6.4, 6.0 or 5.6 with 0.1N HCl. The whey solutions were adjusted with distilled water to produce final whey solids concentrations of 6, 12 or 18%. Each concentration was divided

and heated at 63, 77 or 91C for 30 min, cooled then freeze-dried and tested by the modified Harland-Ashworth test. Analysis of variance and Duncan's multiple range test were used for statistical analysis of the results (12).

Effect of heat treatment. Statistical analysis indicated that the heat treatments used (63, 77 or 91C for 30 min) significantly affected the absorbance and UWPN values (Tables 7 and 12). The above heat treatments produced significant α levels less than 0.0001. The above conclusion was evident from Tables 8 and 13.

Effect of concentration. Solids concentrations in whey during heating (6, 12 or 18%) did not significantly affect A_{280} or UWPN values of the subsequently dried product when subjected to the modified Harland-Ashworth test or Kjeldahl analysis. Alpha values of 0.2198 and 0.1464 are presented in Tables 7 and 12. The same implication can be deduced from Tables 9 and 14.

Effect of pH. The pH (6.4, 6.0 or 5.6) during heating significantly affects A_{280} and UWPN values (Tables 7 and 12). The changes in pH during heating produced significant α levels less than 0.001. The above conclusion was further verified from Tables 10 and 15.

Effect of season. The change in season (12 months) significantly affected UWPN in whey samples. They also were reflected in absorbance values of Harland-Ashworth filtrates. Alpha levels were below 0.0001 (Table 11) for A_{280} and below 0.0222 (Table 16) for UWPN. The seasonal effect was even more evident in Tables 11 and 16.

Table 7. Analysis of variance of factors affecting the results of the modified Harland-Ashworth test on dried whey samples collected over twelve months. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Heat Treatment	2	1.6178	0.8084	840.53	0.0001
Concentration	2	0.0029	0.0014	1.53	0.2198
pH	2	0.0322	0.0161	16.75	0.0001
Month	11	0.0884	0.0080	8.35	0.0001
Error	162	0.1559	0.0010		
Corrected Total	179	1.8972			

$R^2 = 0.9178$

Table 8. Duncan's multiple range test comparisons of the effect of heat treatment on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	Heat Treatment (Temperature C)	Mean (A_{280})	Grouping*
48	63	0.2835	A
84	77	0.1977	B
48	91	0.0300	C

*Means with the same letter are not significantly different.

Table 9. Duncan's multiple range test comparisons of the effect of whey solids concentration during heating on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	Concentration (Percent Solids)	Mean (A_{280})	Grouping*
48	6	0.1771	A
84	12	0.1779	A
48	18	0.1710	A

*Means with the same letter are not significantly different.

Table 10. Duncan's multiple range test comparisons of the effect of pH during heating on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	pH	Mean (A_{280})	Groupings*
36	5.6	0.1899	A
72	6.0	0.1615	B
72	6.4	0.1832	A

*Means with the same letter are not significantly different.

Table 11. Duncan's multiple range test comparisons of the effect of season on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	Month	Mean (A_{280})	Grouping*
15	Jan	0.1977	A
15	Feb	0.2113	A
15	Mar	0.1873	A, B
15	Apr	0.1553	C, D
15	May	0.1560	C, D
15	Jun	0.1443	D
15	Jul	0.1510	C, D
15	Aug	0.2063	A
15	Sep	0.1727	B, C
15	Oct	0.1673	B, C, D
15	Nov	0.1970	A
15	Dec	0.1640	B, C, D

*Means with the same letter are not significantly different.

Table 12. Analysis of variance of factors affecting the results of the modified Harland-Ashworth test of dried whey samples collected over twelve months. Milligrams undenatured whey protein nitrogen per gram dried whey is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Heat Treatment	2	3785.9909	1892.9954	1160.80	0.0001
Concentration	2	6.3417	3.1	1.94	0.1464
pH	2	53.5767	26.7883	16.43	0.0001
Month	11	37.8641	18.9320	2.11	0.0222
Error	162	264.1851	1.6308		
Corrected Total	179	4147.9586			

$R^2 = 0.9363$

Table 13. Duncan's multiple range test comparisons of the effect of heat treatment on undenatured whey protein nitrogen values.

Number of Observations	Heat Treatment (Temperature C)	Mean (UWPN)	Grouping*
48	63	14.8048	A
84	77	11.2798	B
48	91	2.7243	C

*Means with the same letter are not significantly different.

Table 14. Duncan's multiple range test comparisons of the effect of whey solids concentration during heating on the undenatured whey protein nitrogen values.

Number of Observations	Concentration (Percent Solids)	Mean (UWPN)	Grouping*
48	6	9.8616	A
84	12	10.0424	A
48	18	9.8331	A

*Means with the same letter are not significantly different.

Table 15. Duncan's multiple range test comparisons of the effect of pH during heating on the undenatured whey protein nitrogen values.

Number of Observations	pH	Mean (UWPN)	Grouping*
36	5.6	10.3393	A
72	6.0	9.2531	B
72	6.4	10.4231	A

*Means with the same letter are not significantly different.

Table 16. Duncan's multiple range test comparisons of the effect of season on undenatured whey protein nitrogen values.

Number of Observations	Month	Mean (UWPN)	Grouping*
15	Jan	9.8843	A
15	Feb	9.5301	A, B
15	Mar	9.9657	A
15	Apr	9.6130	A, B
15	May	9.8321	A
15	Jun	10.4302	A
15	Jul	8.7681	B
15	Aug	10.3249	A
15	Sep	9.9337	A
15	Oct	10.2701	A
15	Nov	10.4554	A
15	Dec	10.2525	A

*Mean with the same letter are not significantly different.

Effect of milk clotting enzyme. Whey samples were obtained after clotting the same raw skim milk with rennet, BGE, PP, EPP, MMP or MPP enzymes at concentrations normally used for cheese making. The whey samples were freeze-dried. Each sample was reconstituted to 9% solids, heated at 63, 77 or 91C for 30 min, then cooled and freeze-dried prior to analysis by the modified Harland-Ashworth test. Heat treatments significantly affected both UWPN and A_{280} in samples. The results are presented in tables 17, 18, 20 and 21. The milk clotting enzymes used in clotting the raw skim milk significantly affected both A_{280} and UWPN of the Harland-Ashworth filtrates (Tables 19 and 22). The significant effect of milk clotting enzymes on A_{280} was deduced from the α level of less than 0.0001 (Table 17). However, the milk clotting enzymes did not significantly affect UWPN values (Table 22).

Table 17. Analysis of variance of factors affecting the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Enzyme	5	0.0139	0.0028	10.84	0.0001
Heat Treatment	3	0.1667	0.0556	217.15	0.0001
Error	15	0.0038	0.0002		
Corrected Total	23	0.1843			

$R^2 = 0.9792$

Table 18. Duncan's multiple range test comparisons of the effect of heat treatment on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	Heat Treatment (Temperature C)	Mean (A_{280})	Grouping*
6	37	0.2742	A
6	63	0.2467	B
6	77	0.1650	C
6	91	0.0600	D

*Means with the same letter are not significantly different.

Table 19. Duncan's multiple range test comparisons of the effect of milk clotting enzymes on absorbance measurements of the modified Harland-Ashworth test.

Number of Observations	Enzyme	Mean (A_{280})	Grouping*
4	Rennet	0.2125	A
4	MPP	0.2075	A
4	MMP	0.2013	A
4	BGE	0.1888	A
4	EPP	0.1600	B
4	PP	0.1488	B

*Means with the same letter are not significantly different.

Table 20. Analysis of variance of factors affecting the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. Milligrams undenatured whey protein nitrogen per gram dried whey is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
Enzyme	5	6.8018	1.3603	1.94	0.1463
Heat Treatment	3	413.4393	137.8131	196.95	0.0001
Error	15	10.4958	0.6997		
Corrected Total	23	430.7370			

$R^2 = 0.9756$

Table 21. Duncan's multiple range test comparisons of the effect of heat treatment on undenatured whey protein nitrogen values.

Number of Observations	Heat Treatment (Temperature C)	Mean (UWPN)	Grouping*
6	37	16.5644	A
6	63	15.0610	B
6	77	11.2227	C
6	91	5.8186	D

*Means with the same letter are not significantly different.

Table 22. Duncan's multiple range test comparisons of the effect of milk clotting enzymes on undenatured whey protein nitrogen values.

Number of Observations	Enzyme	Mean (UWPN)	Grouping*
4	BGE	12.6951	A
4	MMP	12.4621	A
4	PP	12.3663	A
4	MPP	12.2225	A, B
4	Rennet	12.2224	A, B
4	EPP	11.0318	B

*Means with the same letter are not significantly different.

Factors Affecting Regression Equations for Measuring Milligrams Undenatured Whey Protein Nitrogen Per Gram Dried Whey

The results of the modified Harland-Ashworth test were subjected to linear regression (12). Several regression equations correlating absorbance of undenatured whey protein at 280 nm (A_{280}) with milligrams undenatured whey protein nitrogen per gram dried whey (UWPN) as determined by Kjeldahl analysis (10) were produced. The dried whey samples were exposed to the commonly used preparation and treatments by the dried whey industry.

Effect of season on regression equation from standard curves data. Two regression equations correlating A_{280} with UWPN in dried whey were obtained. One equation included an intercept value obtained in which R^2 of the regression model was 0.9394 (Table 23). Another regression model with an intercept at 0.0 produced an R^2 of 0.9849 (Table 24). Plots of the egression equations are presented in figure 8.

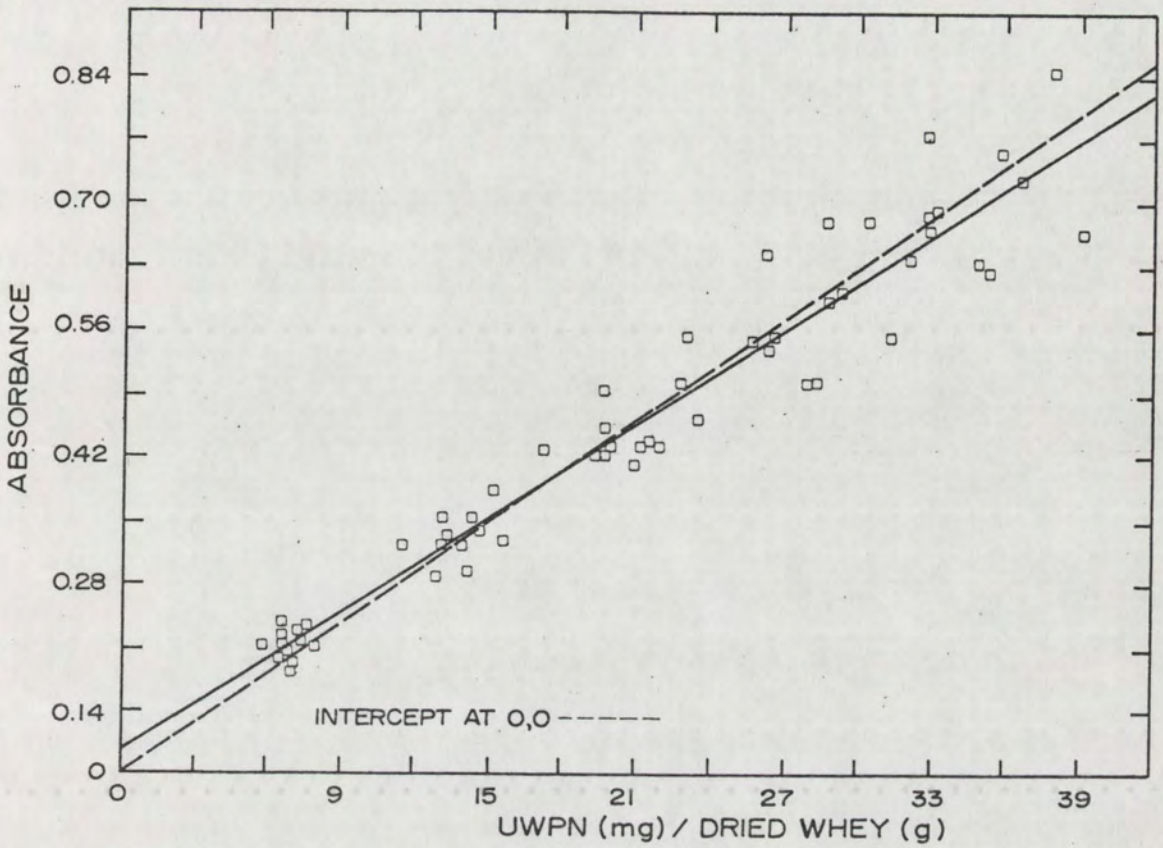

Figure 8. Relationship and regression line of milligram undenatured whey protein nitrogen per gram dried whey versus absorbance for standard curves constructed from raw dried whey collected each month over a year (60 observations).

Table 23. Regression analysis of the effect of undenatured whey protein nitrogen values on the standard curve of the modified Harland-Ashworth test of raw whey samples collected over twelve months. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	1.7319	1.7319	899.68	0.0001
Error	58	0.1116	0.0019		
Corrected Total	59	1.8435			

$R^2 = 0.9394$

$A_{280} = 0.0931 + 0.0172$ (UWPN)

Table 24. Regression analysis of the effect of undenatured whey protein nitrogen values on the standard curve of the modified Harland-Ashworth test of raw dried whey samples collected over twelve months. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	13.6455	13.6455	3852.83	0.0001
Error	59	0.2090	0.0035		
Uncorrected Total	60	13.8544			

$R^2 = 0.9849$

$A_{280} = 0.0208$ (UWPN)

Effect of milk clotting enzyme on regression equation from standard curves data. Untreated whey samples (raw) were collected from milk clotted with different milk clotting enzymes and used to establish standard curves. Two regression equations correlating A_{280} with UWPN in dried whey were produced. One of the equations included an intercept value in which R^2 of the regression model was 0.9155 (Table 25). Another regression model with the intercept at 0.0 produced an R^2 value of 0.9804 (Table 26). Plots of the data and regression equations line are presented in figure 9.

Table 25. Regression analysis of the effect of undenatured whey protein nitrogen on the standard curve of the modified Harland-Ashworth test on raw dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	0.7688	0.7688	303.21	0.0001
Error	28	0.0710	0.0025		
Corrected Total	29	0.8398			

$R^2 = 0.9155$

$A_{280} = 0.1020 + 0.0169$ (UWPN)

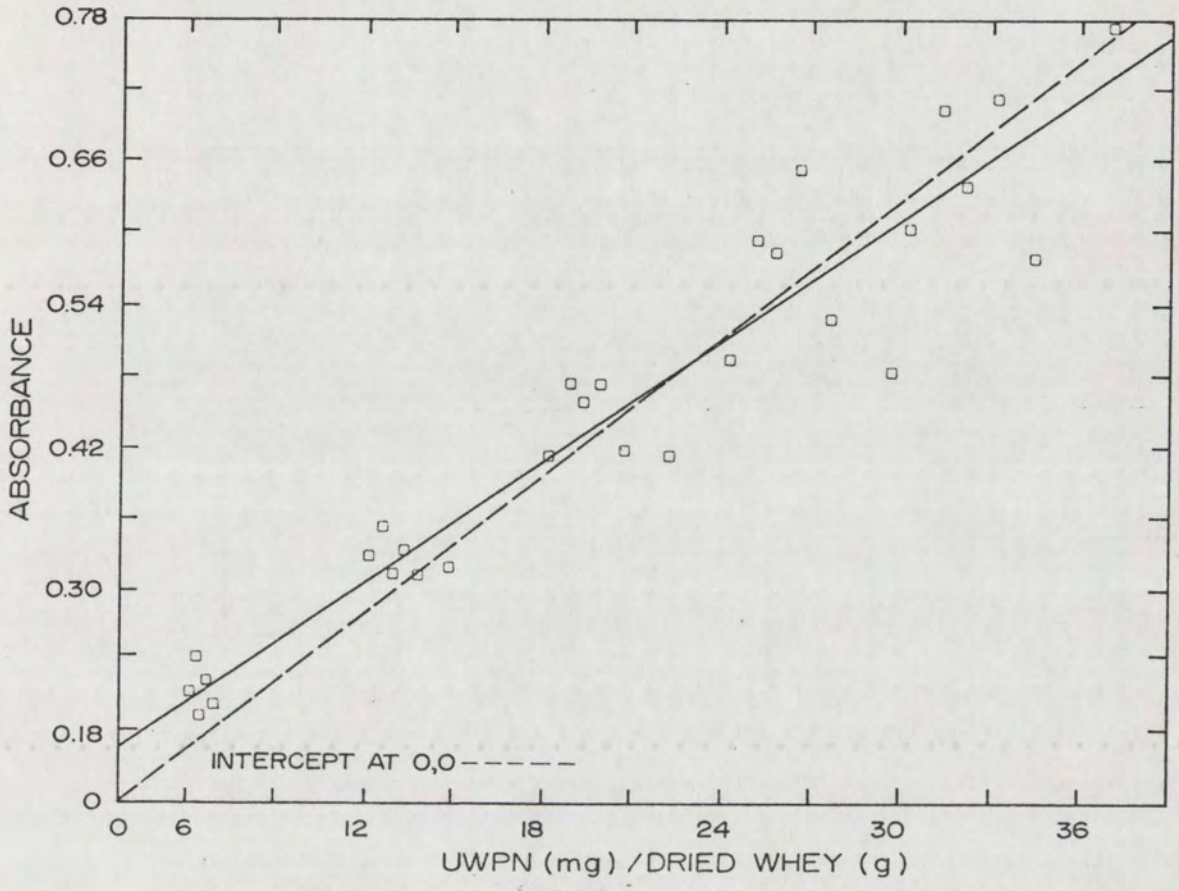

Figure 9. Relationship and regression line of milligrams undenatured whey protein nitrogen per gram dried whey versus absorbance for standard curves constructed from raw dried whey collected from milk coagulated with different milk clotting enzymes (30 observations).

Table 26. Regression analysis of the effect of undenatured whey protein nitrogen values on the standard curve of the modified Harland-Ashworth test on raw dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Suqares	Mean Square	F	α
UWPN	1	6.4531	6.4531	1452.37	0.0001
Error	29	0.1289	0.0044		
Uncorrected Total	30	6.5820			

$R^2 = 0.9804$

$A_{280} = 0.0211$ (UWPN)

Effect of temperature and pH on the relationship of absorbance and undenatured whey protein nitrogen (regression equation). Whey samples collected each month at different pH, solids concentrations and heat treatments were subjected to the modified Harland-Ashworth test. Two regression equations correlating A_{280} with UWPN in dried whey, heat treatment (temperature) of whey and pH were produced. One equation included an intercept value in which R^2 of the regression model was 0.9158 (Table 27). Another regression equation with intercept at 0.0 was developed in which the R^2 value for the regression model was 0.9774 (Table 28). The effect of heat (temperature) and pH on the equations were very small. Therefore, two more regression equations correlating A_{280} with UWPN in dried whey were obtained in which the pH and heat treatment factors were omitted. The equation with an intercept had an R^2 of 0.9158 (Table 29). The regression model with an intercept at 0.0 produced an R^2 of 0.9774 (Table 30).

Plots of data and regression lines are presented in figure 10.

Table 27. Regression analysis of factors affecting the results of the modified Harland-Ashworth test on dried whey samples collected over twelve months. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	1.7259	1.7259	1902.25	0.0001
Heat Treatment	1	0.0078	0.0078	8.62	0.0038
pH	1	0.0038	0.0038	4.21	0.0417
Error	176	0.1597	0.0009		
Corrected Total	179	1.8972			

$R^2 = 0.9158$

$A_{280} = 0.2535 + 0.0167\ (UWPN) - 0.0019\ (Temperature\ C) - 0.0158\ (pH)$

Table 28. Regression analysis of factors affecting the results of the modified Harland-Ashworth test on dried whey samples collected over twelve months. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	7.2682	7.2682	7627.17	0.0001
Heat Treatment	1	0.0271	0.0271	28.40	0.0001
pH	1	0.0002	0.0002	0.17	0.6780
Error	177	0.1687	0.0011		
Uncorrected Total	180	7.4641			

$R^2 = 0.9774$

$A_{280} = 0.0196\ (UWPN) - 0.0004\ (Temperature\ C) + 0.0022\ (pH)$

Table 29. Regression analysis of the effect of undenatured whey protein nitrogen values on the results of the modified Harland-Ashworth test on dried whey samples collected over twelve months. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	1.7259	1.7259	1793.19	0.0001
Error	178	0.1713	0.0009		
Corrected Total	179	1.8972			

$R^2 = 0.9097$

$A_{280} = -0.0269 + 0.0204$ (UWPN)

Table 30. Regression analysis of the effect of undenatured whey protein nitrogen values on the results of the modified Harland-Ashworth test on dried whey samples collected over twelve months. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	7.2682	7.2682	6641.35	0.0001
Error	179	0.1959	0.0011		
Uncorrected Total	180	7.4641			

$R^2 = 0.9737$

$A_{280} = 0.0182$ (UWPN)

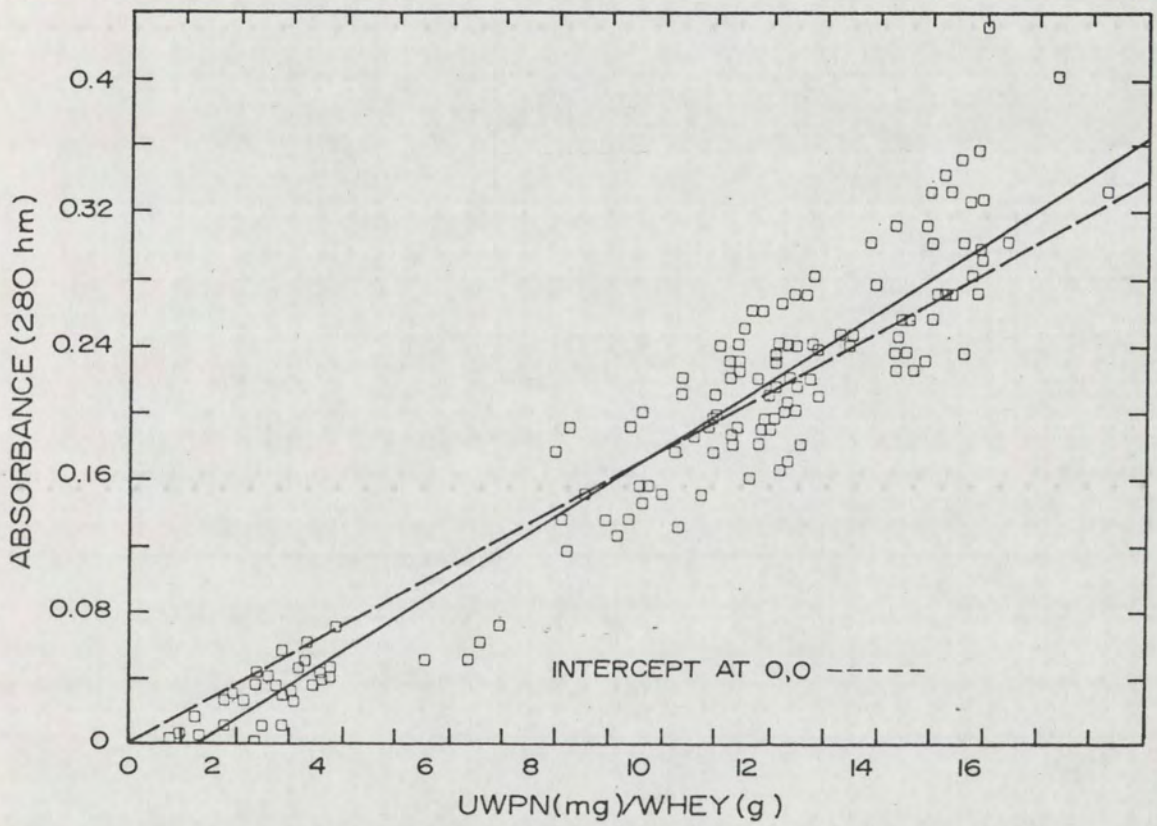

Figure 10. Relationship and regression line of milligram undenatured whey protein nitrogen per gram dried whey versus absorbance for dried wheys collected each month over a year (180 observations).

Effect of milk clotting enzyme on the relationship of absorbance and undenatured whey protein nitrogen (regression equation on heated whey samples. The whey collected by coagulating milk with different milk clotting enzymes was heat treated then subjected to the modified test. The linear regression of the resulted data produced two regression equations correlating A_{280} with UWPN in dried whey and heat treatment (Temperature) were produced. One had an intercept value and an R^2 of 0.9098 (Table 31). When the intercept was moved to 0.0 the R^2 of the regression model was 0.9837 (Table 32). Again the heat factor had so little effect on the equation therefore it was eliminated. This resulted in an equation with an R^2 of 0.9087 (Table 33). When the intercept of the equation was moved to 0.0 the R^2 became 0.9762 Table 34). Plots of data and regression lines are presented in figure 11.

Table 31. Regression analysis of factors affect the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	0.1675	0.1675	211.62	0.0001
Heat Treatment	1	0.0002	0.0002	0.26	0.6122
Error	21	0.0166	0.0008		
Corrected Total	23	0.1843			

$R^2 = 0.9098$

$A_{280} = -0.0125 + 0.0183\ (UWPN) - 0.0003\ (Temperature\ C)$

Table 32. Regression analysis of factors affect the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	0.9945	0.9945	1314.84	0.0001
Heat Treatment	1	0.0076	0.0076	10.06	0.0044
Error	22	0.0166	0.0008		
Uncorrected Total	24	1.0187			

$R^2 = 0.9837$

$A_{280} = 0.0178$ (UWPN) - 0.0004 (Temperature C)

Table 33. Regression analysis of the effect of undenatured whey protein nitrogen values on the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	0.1675	0.1675	218.94	0.0001
Error	22	0.0168	0.0008		
Corrected Total	23	0.1843			

$R^2 = 0.9087$

$A_{280} = -0.0535 + 0.0197$ (UWPN)

Table 34. Regression analysis of the effect of undenatured whey protein nitrogen values on the results of the modified Harland-Ashworth test on dried whey samples collected from raw skim milk coagulated with different milk clotting enzymes. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	0.9945	0.9945	943.30	0.0001
Error	23	0.0242	0.0011		
Uncorrected Total	24	1.0187			

$R^2 = 0.9762$

$A_{280} = 0.0158$ (UWPN)

Effect of combined data on relationship of absorbance and undenatured whey protein nitrogen (regression equation). All the above data were analyzed to produce a regression equation which correlate A_{280} with UWPN in dried whey. Regression analysis produced two equations. One which included an intercept value and an R^2 of 0.9228 (Table 35). Another with an intercept of 0.0 produced an R^2 of 0.9743 (Table 36). When the predicted absorbance value of the regression equation in Table 35 was subjected to regression analysis versus the measured absorbance values the R^2 was 0.9228 (Table 37). Plots of the combined data are presented in figure 12.

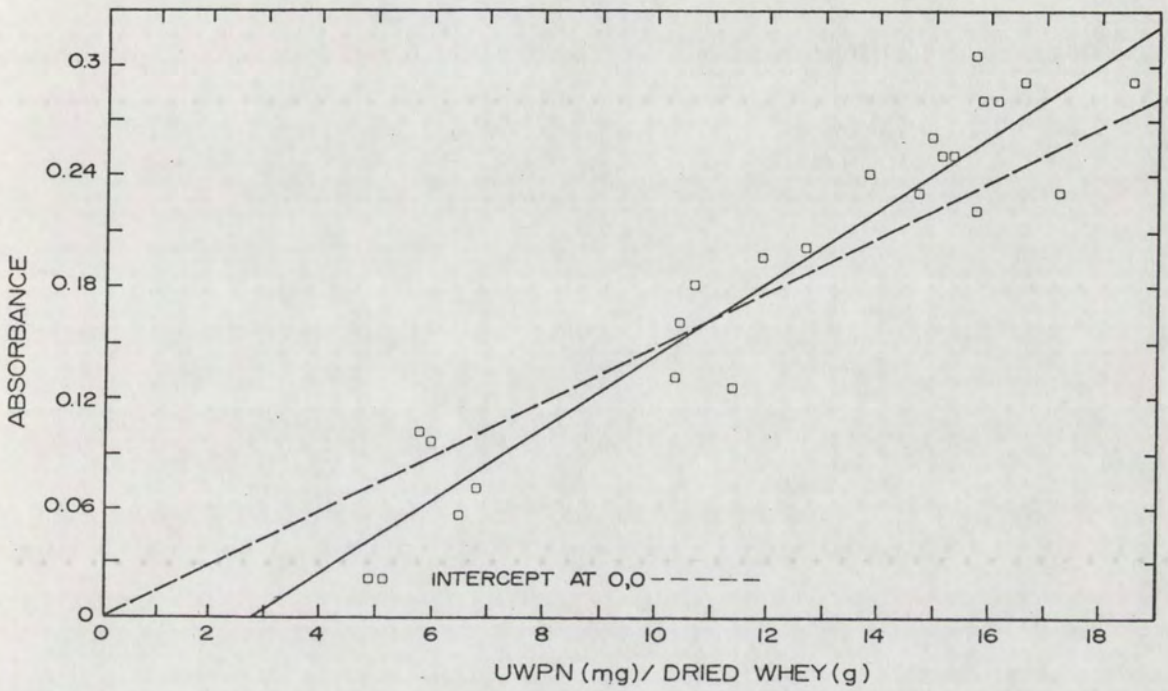

Figure 11. Relationship and regression line of milligram undenatured whey protein nitrogen per gram dried whey versus absorbance for dried wheys collected from milk coagulated with different milk clotting enzymes (24 observations).

Table 35. Regression analysis of the effect of undenatured whey protein nitrogen values on all the results of the modified Harland-Ashworth test. A_{280} is the dependent variable.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	8.4381	8.4381	3464.34	0.0001
Error	290	0.7064	0.0024		
Uncorrected Total	291	9.1445			

$R^2 = 0.9228$

$A_{280} = -0.0177 + 0.0207$ (UWPN)

Table 36. Regression analysis of undenatured whey protein nitrogen values effect on all data produced from the Harland-Ashworth filtrates. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	27.7795	27.7795	11051.05	0.0001
Error	291	0.7315	0.0025		
Uncorrected Total	292	28.5111			

$R^2 = 0.9743$

$A_{280} = 0.0198$ (UWPN)

Table 37. Regression analysis of the effect of undenatured whey protein nitrogen values on all the results of the modified Harland-Ashworth test. A_{280} is the dependent variable and intercept = 0.0.

Source	df	Sum of Squares	Mean Square	F	α
UWPN	1	27.7795	27.7795	11051.05	0.0001
Error	291	0.7315	0.0025		
Uncorrected Total	292	28.5111			

$R^2 = 0.9743$

$A_{280} = 0.0198$ (UWPN)

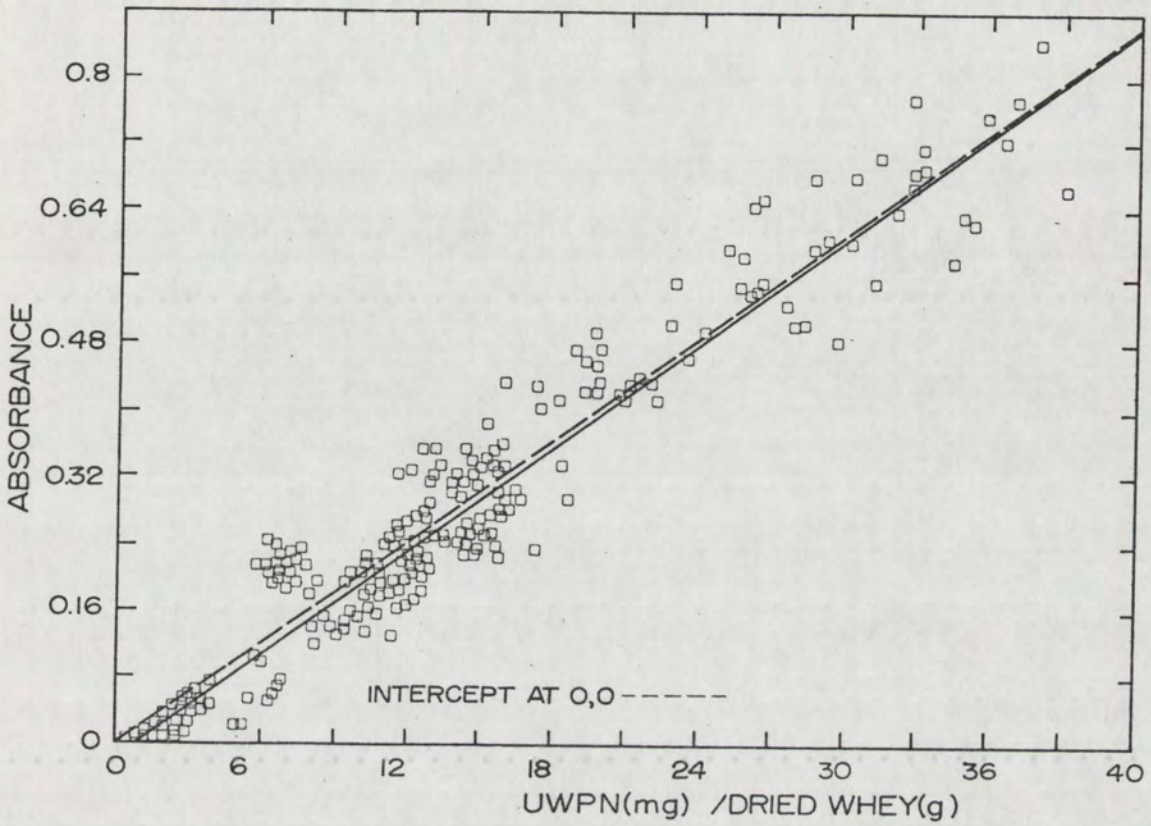

Figure 12. Relationship and regression line of milligram undenatured whey protein nitrogen per gram dried whey (29 observations).

DISCUSSION

The term denaturation in this discussion is limited in its meaning to denaturation as measured by the Harland-Ashworth test and its modifications. Wyeth (132) stated that dried whey analysis are generally carried out by the same procedures used for NDM. This creates a particular problem for the determination of UWPN in dried whey by the Harland-Ashworth test that was designed only for NDM. Whey protein constitutes about 13% of dry whey (42) as opposed to about 7-8%in NDM. Also there is more variation in pH of whey than in the pH of NDM. Sanderson (120) stated that turbidimetric methods are subject to a number of errors. The method of Harland and Ashworth (53) and its subsequent modifications (79) can show different degrees of turbidity for the same level of undenatured whey protein nitrogen. β-lg and α-la developed different levels of turbidity at equal concentrations. Wyeth (132) found that below pH 6.0 saturation with sodium chloride precipitates some native whey protein with denatured whey protein and casein. The amount of undenatured protein that precipitates increases with decreasing pH until, at pH 2.0, nearly all of the whey protein is insoluble.

A modified Harland-Ashworth test was developed which gave more reproducible results than the conventional test when it was carried out on both NDM and dried whey. The modifications were made in light of the difficulties encountered with the conventional test. Dried whey was reconstituted in a pH 6.7 buffer to simulate the pH of fresh milk and to prevent the effect of differences in the degree of protein solubility due to pH. Another modification was the measurement of UWPN in the Harland-Ashworth filtrate by UV absorbance. This eliminated all problems associated with turbidity measurement. The dried whey

sample used was 0.1 g in order to keep the absorbance within the range of most available spectrophotometers.

Preliminary analysis of raw (no heat treatment) freeze-dried whey indicated that total and protein nitrogen fluctuations were parallel throughout the year. On the other hand very little seasonal fluctuation in nonprotein nitrogen was observed. Maximum total and protein nitrogen were observed in the month of October and a minimum value was observed in August. Similarly, undenatured whey protein nitrogen in Harland-Ashworth filtrates from raw dried whey were essentially parallel to total and protein nitrogen values over a twelve month period. This is in general agreement with the findings of Giroux et al. (41), Delamonica et al. (26) observed a cyclic seasonal fluctuation of total and individual whey proteins in skim milk. Heat denaturation of whey protein varies with season (120) which reflects differences in the relative amounts of the individual whey proteins and their heat sensitivities. Changes in total whey protein content in whey could be important if the percent denaturation in dry whey is to be determined.

Total and protein nitrogen in raw sweet dried whey differed with the type of milk clotting enzyme used for coagulation of the milk. The highest level of total and protein nitrogen was produced when milk was clotted with BGE and lowest when clotted with EPP. Intermediate levels were in wheys from milk clotted by rennet, PP, MMP or MPP. The nonprotein nitrogen fraction of wheys produced from the same milk clotted by the above enzymes did not show any marked variation except for a higher level produced by EPP. Different milk

clotting enzymes (21, 43, 48, 87, 108, 113) produced differences in whey protein concentrations which were inversely related to cheese yields. Therefore, variations in nitrogen in wheys produced from milk coagulated with different enzymes probably reflected the extent of casein degradation during coagulation.

The use of different enzymes to coagulate milk had a significant effect on the ease with which the proteins in the resulting wheys were heat denatured. This could mean that partially degraded casein resulting from the action of some enzymes was included in the whey and responded to heat treatments to different extents than whey proteins. It might also mean that some milk clotting enzymes produced minor changes in whey protein structure that slightly altered their heat sensitivity. It was interesting that the effect of the different enzymes on heat susceptibility of the whey proteins varied with temperature, i.e. proteins in whey from milk clotted with BGE were more easily denatured at 63C and 77C for 30 min than in whey from milk clotted with rennet. However, at 91C for 30 min proteins in whey from milk clotted with PP were more easily denatured than those in whey from milk clotted with MMP.

All the protein in raw dried whey could be considered "undenatured". However, UWPN in all raw whey samples examined in this study was slightly higher than total protein nitrogen. Total protein nitrogen was determined by 12% TCA precipitation and it was found that the protein precipitating effect of 12% TCA was less in the presence of concentrated salt solutions obtained by diluting Harland-Ashworth filtrates than in the normal reconstituted whey.

Raw whey samples collected during one year were adjusted to pH 6.4, 6.0 or 5.6 (68), and heated to 63, 77 or 91C for 30 min. Initial concentrations of whey solids prior to heating were 6, 12 or 18%. The effect of milk clotting enzymes commonly used in cheese production was also considered. The effects of all these factors on the relationship between UWPN and absorbance as measured by the proposed modified Harland-Ashworth test were statistically analyzed.

A very high overall correlation between absorbance values and undenatured whey protein nitrogen per gram dried whey (Kjeldahl) (10) was observed, and an α level of less than 0.0001 was produced. Variations due to season or milk clotting enzyme used in whey production did not affect this relationship. Therefore, standard curves relating absorbance with UWPN can be prepared from raw whey regardless of the season of the year or the enzyme used to clot the milk. This is an advantage over the use of turbidimetric methods in which the standard curves were influenced by the season (120).

Analysis of variance of the various factors and their individual effects on the relationship between absorbance and UWPN in Harland-Ashworth filtrates were determined. Heat treatment of whey produced an insignificant effect on the relationship between UWPN and absorbance as did season, pH of the whey during heating, solids concentration during heating and kind of residual enzymes in the whey. However, heat significantly influenced the extent of protein denaturation in whey. Season, pH during heating, and kind of residual clotting enzyme in the whey also had significant effects on the extent of heat denaturation of protein in whey. Heat denaturation of whey protein was not affected by solids concentration of 6, 12 and 18%.

The results of the modified Harland-Ashworth test on dried whey were subjected to regression analysis of UWPN versus absorbance at 280 nm. The original regression equation contained factors for pH during heating and heat treatment (temperature). However, these were shown to make an insignificant contribution to the overall relationship of UWPN and absorbance and were removed from the subsequent equations. The original regression lines did not pass through the origin (0, 0) and had an R^2 value of 0.9097. However, when the regression line was shifted to pass through the origin (0, 0), the R^2 was improved to 0.9743. The final regression equation was produced from data representing all conditions and treatments used in this study.

$UWPN = A_{280}$ (50.5462) in which UWPN is expressed as milligrams of undenatured whey protein nitrogen per gram of dried whey and A_{280} is the difference between total absorbance and nonprotein absorbance.

Modified Harland-Ashworth Test for Undenatured Whey Protein Nitrogen in Raw Whey

Apparatus and Reagents

Test tubes: 25 x 150 mm lipless pyrex or Kimax or soft glass.

Funnels: Short stem, diameter 50 mm, stem length 65 mm; short stem, diameter 90 mm, stem length 65 mm.

Filter paper: S & S 602 9 cm, and 15 cm Whatman 42, 9 cm washed with 5 changes of distilled water over a period of 24 hours to remove all absorbing substances. The washed paper is then air dried and stored for use.

Curvettes: Matched quartz cuvettes for ultraviolet analysis.

Pipettes: 1, 2, 3, 4, 5 and 10 ml volumetric.

Balance: Top loading balance (± 10 mg), tared 500 gram capacity.

Water bath: Thermostatically controlled at 37 (± 1C).

Spectrophotometer: Ultraviolet, capable of measurement at 280 nm.

Phosphate buffer: 0.1 M and pH 6.7.

Trichloracetic acid: 13.2% solution in distilled water (W/V).

Standard whey samples: Raw freeze dried whey sample with known undenatured whey protein nitrogen content.

Sodium chloride: Analytical grade.

Preparation of Standard Curve

Reconstitute 4.0 g of raw whey powder in 50 ml 0.1M phosphate buffer (pH 6.7) in a 25 x 150 mm test tube. Add 20 g of NaCl and incubate in a 37C water bath for 30 min. Shake the contents 8-10 times during the first 15 min of incubation. Cool to 22C (room temperature), filter through S & S 602 15 cm filter paper and collect 20 ml filtrate. If the first portion of the filtrate is cloudy then refilter through the same filter paper. Prepare a blank filtrate by adding 10 g NaCl to 25 ml 0.1M phosphate buffer in a 25 x 150 mm test tube, stopper, incubate, shake and then filter through S & S 602 9 cm filter paper in the same manner described for the sample. Pipet portions of the blank and the sample filtrates into 25 x 150 mm test tubes as follows:

Tube No.	Sample Filtrate (ml)	Blank Filtrate (ml)
1	5.0	0.0
2	4.0	1.0
3	3.0	2.0
4	2.0	3.0
5	1.0	4.0

Total absorbance. Dilute 1 ml of each of the combined (sample and blank) filtrates with 10 ml distilled water in a 25 x 150 mm test tube. Stopper and mix by inverting three times before measuring the total absorbance at 280 nm against a blank prepared by diluting 1 ml phosphate buffer saturated with sodium chloride with 10 ml distilled water.

Nonprotein absorbance. Mix 1 ml of the combined sample and blank filtrates with 10 ml 13.2% TCA in a 25 x 150 mm test tube, stopper and mix by inverting three times, incubate at 37C for 5-10 min and then filter through Whatman 42 filter paper. Measure absorbance against a blank prepared by adding 1 ml phosphate buffer saturated with sodium chloride to 10 ml 13.2% TCA.

Undenatured whey protein nitogen absorbance. UWPN absorbance is calculated from total absorbance - nonprotein absorbance.

Procedure. Reconstitute 1 g dry whey in 25 ml 0.1M phosphate buffer (pH 6.7) in a 25 x 150 mm test tube. Add 10 g NaCl, stopper and incubate in a water bath at 37C for 30 min. Shake 8-10 times during the first 15 min, cool to 22C, filter through S & S 602, 9 cm filter paper and collect 5 ml filtrate. Filter through the same

filter paper if the first portion of the filtrate is cloudy. A blank with no whey sample is prepared in the same manner.

Total absorbance. Dilute 1 ml filtrate with 10 ml distilled water in a 25 x 150 mm test tube, stopper and mix by inverting three times before measuring total absorbance at 280 nm against a blank. Blank is prepared by diluting 1 ml of the blank filtrate with 10 ml distilled water.

Nonprotein absorbance. Mix 1 ml filtrate with 10 ml 13.2% TCA in a 25 x 150 mm test tube, stopper, incubate at 37C for 5-15 min and then filter through Whatman 42 filter paper and mix by inverting three times. Measure absorbance against a blank. Blank is prepared by addition of 1 ml phosphate buffer saturated with sodium chloride to 10 ml 13.2% TCA.

Undenatured whey protein nitrogen absorbance. UWPN absorbance is calculated from total absorbance - nonprotein absorbance.

UWPN can be determined from a standard curve (UWPN vs A_{280}) or from the regression equation UWPN = A_{280} x 50.5462.

The above test is different from that suggested by Reyes (111) who prepared a blank from the same Harland-Ashworth filtrate by deproteinizing with 12% TCA. This allowed the blank to subtract out any nonprotein absorbing substances and eliminated the problem of variation in amounts of nonprotein absorbing substances between different samples. Several commercial TCA were tested. When put into solution, all of them caused sufficient absorbance at 280 mm to seriously interfere with the test. Therefore, an additional blank was introduced to account for the UV absorbance caused by TCA. Also Reyes (111)

adjusted the pH of the reconstituted filtrate to 6.7 by the addition of either HCl or NaOH whereas in this test the pH was held at 6.7 by the use of phosphate buffer to reconstitute dried whey.

Heat Classification of Whey

A possible classification system for dried whey based on the results of this study might be:

Low heat: > 14.5 mg UWPN/g dried whey
(63C for 30 min)

Medium heat: < 14.5 > 6.0 mg UWPN/g dried whey

High heat: < 6.0 mg UWPN/g dried whey
(91C for 30 min)

However, before establishing these classification limits, it would be appropriate to evaluate a large number of whey samples from different sources throughout the country.

Now that a suitable test is available, studies should be directed toward determining a correlation (if any) between UWPN in dried whey and its functional properties in food mixes. This relationship could also have a bearing on the heat classification system.

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