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A RAPID METHOD FOR DETERMINING THE CASEIN CONTENT OF MILK

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A method is presented for the rapid determination of the casein content of milk by Direct Injection Enthalpimetry (DIE).

A significant heat pulse of precipitation of casein is produced by adjusting the pH of the solution to the pH of the isoelectric point of casein. The method is calibrated against a real sample whose casein content has been determined by a standard method incorporating the time consuming Kjeldahl method, but once calibrated the proposed method can be operated routinely by non-skilled personnel. The method is sensitive and gives results as acceptable as those obtained by standard methods. The method can be applied to most liquid samples without the need for prior preparation. the main advantages of the method are those of time and costs of analysis and the potential of the enthalpimetric method for automation.

With ever increasing legislation covering the control of food products and steadily rising raw material costs, food processing industries must be more cost effective to remain competitive. Major savings can be made in analytical services if analysis times can be decreased in any methods which monitor changes in composition affecting product specifications. If these methods do not require highly skilled laboratory personnel or high cost laboratories and use versatile equipment which can easily be amended to meet changes in production specification then these are to be recommended.

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A knowledge of the protein content of milk is important of several reasons. It is one of the parameters that determines the price of the milk, its nutritional value and the yield of milk products.

Milk proteins may be divided into two groups by reference to their solubilities at pH 4.6: caseins which are insoluble at this pH and lactoserum proteins which are soluble at pH 4.6 [1]. The caseins or proteins which are insoluble at pH 4.6 represent about 80 % of the total proteins of cows milk. Although there are 4 types of caseins, synthesized in the mammary gland, these may be classified as phosphoproteins (casein s1, casein s2 and casein b) and a phosphoglycoprotein (casein k). This heterogeneity is due to the different binding of the phosphorus in the protein molecules. For many purposes, however, both in the food and other industries, in addition to knowledge of the protein content, it is useful to know the total casein content. This is especially the case in the manufacture of dairy products and for instance in the manufacture of glues and adhesives for wood and similar commercial products, where casein is widely used. Guillou *et al.* have reviewed methods for the determination of milk proteins [2].

The most widely used method of determining the casein content of milk is still that of Rowland [3] which involves precipitation of the casein at pH 4.6 (at 20°) and measurement of Kjeldahl nitrogen in milk and in whey and calculation of casein as the difference between total and whey proteins. An alternative method determines the whole protein using the Kjeldahl method and an empirical factor to calculate the casein content from the value of the whole protein.

Several attempts have been described to find a simple and rapid method for the determination of the casein content of milk. A dye binding method, utilising amido-black, has been proposed by McGann *et al.* [4] and improved by Kwai Hang and Hayes [5].

Infra red absorption methods have been proposed by several workers [6-10], and the application of IR reflectance methods to milk and milk products has been reviewed by Kennedy and co-workers [11]. Sjaunja *et al.* [6] reported a method with a relative error of 1.9%; Harris [7], is among several who have reported the use of the Milkoscan Infrared Milk Analyser, which measures the protein at 6.5 μm, utilising the wavelength of absorption of the peptide linkage between the amino acids of the protein molecules. She used it for the determination of the crude protein in ewes milk and used a B. S. I. method (B. S. I. 174, (1963)) involving a Kjeldahl method for checking her results. Robert *et al.* [8] have applied multivariate analysis to the near infrared spectra of milks. They report two major difficulties; the water absorption is very large in comparison with fat, protein and lactose

absorptions, and moreover, the fat globules of milk are scattering particles which produce spectral distortion. For protein analysis they assigned wavelengths of 2050 and 2180 nm. Barbano and Dellaville [9] determined the total protein in a portion of the milk with an IR analyser. The pH of a second portion was adjusted to 4.6 with phosphoric acid and the casein precipitated removed by filtration. The non-casein protein in the filtrate was determined by IR and the casein content was calculated by a difference method. They report that the method is slightly faster than the official methods and the results obtained compare well.

The literature indicates various times for each assay using infrared methods. Guillou *et al.* [2] state that 100-300 measurements of total protein can be made per hour. Karman and co-workers [10] determined the total protein in milk and in rennet whey and after applying two mathematical corrections, calculated the casein content. They report that the method takes approximately 1 hour to complete and the coefficient of variation to be about 1.5%. The accuracy is equivalent of that of the Kjeldahl method.

Casein has been determined by UV absorbance methods; Carpenter and Brown [12] reported the separation of the casein micelles for the rapid determination of casein concentration. The micelles, formed by the addition of calcium ions were separated from the whey protein in milk by size exclusion chromatography and then determined by UV absorption methods. The method was compared with precipitation of the casein by the addition of acid followed by determination of the protein in the precipitate by a Kjeldahl method. They report a coefficient of correlation of 0.92. They offer no explanation for this low correlation.

Quantitative analysis of casein in cows milk by fast protein LC has also been reported [13].

It is, however, noted that the present British Standard method uses the Kjeldahl method [14].

Most of the above non-reference methods require high cost apparatus and the use of skilled personnel to obtain acceptable reproducibility of results. The cost per analysis on a routine basis thus becomes a significant cost factor. As stated earlier, increasing legislation and steadily rising raw material costs, require food processing industries to be more cost effective. Thus methods which do not require highly skilled laboratory personnel or high cost laboratories, and use versatile equipment which can easily be amended to meet changes in production specifications are to be recommended.

The use of thermometric and enthalpimetric methods of analysis provides an ideal technique to satisfy the above criteria. When precipitation

occurs in a reaction system, there is often a significant change in the entropy of the system, manifested as a relatively high enthalpy change. This enthalpy change is observable as a temperature change, which may be used to assay the reaction, [15] since it has a linear relationship with the number of moles of product formed in the reaction.

The precipitation of the casein was considered to offer a useable reaction heat for its determination. Since several methods may be used to precipitate casein from whole milk e. g. by adjusting the pH of the solution to that of the isoelectric point (pH 4.6); saturation of the solution with a neutral salt and by combination with a large number of "alkaloidal reagents" and by bulky anions such as those from phosphotungstic acid, trichloracetic acid or by addition of complexing cations such as Cu(II), Cd(II), Co(III), Al(III); these were investigated in preliminary studies. However, using the DIE technique [16] except when using pH adjustment with the buffer or trichloracetic acid, significant heat pulses could not be obtained. Although the heat pulses with trichloracetic acid were the highest, it was not possible to obtain acceptable reproducibility with this reagent. Good reproducibility was obtained by using the first method of precipitation, thus the focus of the present investigation is on this method.

Experimental

The basic circuit of the electrical bridge system and the reaction vessels have been reported previously [17, 18].

To obtain maximum reproducibility, the disposable reaction vessel (an expanded polystyrene "food" beaker with a nominal capacity of 200 ml) is contained in a small Dewar flask fitted with a loosely fitting lid through which passes the temperature sensor (a glass coated thermistor bead) and the shaft of a constant speed stirrer. The reagent is dispensed (in 1 or 2 ml aliquots) from a semi-automatic dispenser pipette having a thermostatted 1 litre reservoir. The reaction system (Dewar flask and reagent reservoir) is contained in a water filled thermostat regulated to $25^{\circ} \pm 1^{\circ}$.

Buffer reagent

An aliquot (2 ml) of equal volumes of aqueous sodium acetate solution (1M) and aqueous acetic acid (10% v/v) is used to adjust the pH of 50 ml of the sample to that of the isoelectric point.

Comparison method

Reagents were prepared and used in accordance with the procedure for the Kjeldahl method of the British Standard Method [14].

Comparison of the Enthalpimetric method and the Kjeldahl methods for the determination of the total casein content of liquid milk samples

Prepare a series of milk samples by dilution of homogenised milk (2-10 ml diluted to 50 ml with distilled water).

(a) Determination of the total casein content by precipitation and separation of the casein.

To an aliquot (50 ml) of the diluted homogenised sample contained in the reaction vessel, add an aliquot of the buffer reagent. Record the signal produced by the heat of precipitation. Results are given in Table 1.

(b) Determination of the casein content by precipitation and determination of the nitrogen content of the precipitate by the standard Kjeldahl method.

To an aliquot (5 ml) of the homogenised sample, add sodium acetate/acetic acid (1 ml of a 1:1 mixture). Filter, wash the precipitate (with water until the washings are at pH 6-7). Quantitatively transfer the precipitate to a Kjeldahl apparatus and determine its nitrogen content. Calculate the amount of casein in the precipitate from the nitrogen content using an agreed normalisation factor. The results are given in Table 1.

(c) Determination of the casein content of milk by the Kjeldahl method via the determination of the total protein.

Table 1 Experimental results

Vol. of milk (ml) diluted to 50 ml	2.0	4.0	6.0	8.0	10.0
DIE method					
Heat pulse normalised (actual - blank), mm	8	16	24	33	41
Determination of casein after precipitation					
Amount of casein in the milk before dilution mg	50.2	102.3	149.6	200.1	256.0
Determination of casein via protein determ.					
Amount of protein in the milk before dilution, mg	66.8	140.0	189.7	262.7	326.3
Amount of casein calculated[(d)], mg	53.5	107.2	151.8	210.2	261.0

The total proteins in aliquots (5 ml) of the diluted and homogenised milk are determined using the standard method (14). The casein content was calculated using the normally accepted conversion factor (0.80) [19].

The results are given in Table 1.

Proposed method

Homogenise the milk sample. Pipette an aliquot (5 or 10 ml) into the reaction vessel. Dilute to 50 ml with distilled water. Fit the lid, thermistor and stirrer into the top of the Dewar flask.

Switch on the stirrer, electronic bridge and recorder. When temperature equilibrium has been attained (recorder reading is steady) inject an aliquot (2 ml) of the thermostatted acetate buffer. Note the heat pulse recorded. Using the previously prepared calibration graph, calculate the casein content of the original milk.

Statistical parameters of the proposed method

In separate experiments, two series of 10 aliquots of two different concentrations of fresh cows milk were assayed by the proposed method and by the Kjeldahl method for the total protein content.

The relevant statistics derived from the results are given in Table 2.

Table 2 Experimental results

Dilution of milk sample	DIE		Kjeldahl method	
	SD	RSD	SD	RSD
1:10	0.44 mm / 50 ml	1.87%	4.42 mg / 50 ml	4.35%
1: 5	0.63 mm / 50 ml	1.48%	5.08 mg / 50 ml	2.70%

Discussion

Since different methods used routinely for the determination of the protein content of milks or in control laboratories in the milk industry, produce results which are not directly comparable because they are obtained by different techniques, it is necessary to have a comparison method to evaluate and calibrate any proposed method. The comparison of results obtained by different methods or in different laboratories must be based on

a commonly accepted reference method. In the food industry, the reference method widely adopted is the Kjeldahl method.

Calibration

In Direct Injection Enthalpimetry of industrial materials it is usual to calibrate the system by the method of standard addition of the reacting analyte so that the results can be compensated for the effect of the matrix. In theory the analyte added should have the same chemical and physical composition as that being determined. The precipitate cannot be redissolved and used as the analyte since whilst it may be regarded as casein, it is not the reacting species, which is the mixture of phosphoproteins and phosphoglycoproteins described previously.

Even if it were possible to obtain a similar chemical composition it is not possible to obtain the same chemical and physical environment for the analyte. It is well established that in milk the caseins are present in the form of high molecular weight aggregates, the micelles. These are spherical particles with an average diameter in the region of 100 nm, constituting themselves from spherical subunits (submicelles) of average diameter 15-20 nm in which are the four types of casein bound by the calcium ions present. These submicelles are "cemented" together by a "colloidal phosphate" which is a phosphate-citrate complex of calcium and of amorphous magnesium [20]. Any attempt to separate these from the water, fats etc., which constitute the milk sample, will result in their decomposition, either wholly of partially, rendering them unfit for use as a substance for standard addition.

It is therefore necessary to calibrate the DIE method in a similar manner to most other methods that is via the titrimetric determination utilising the Kjeldahl procedure.

Figure 1 shows that there is a linear relationship between the dilution of the milk, the amount of casein calculated from the nitrogen values obtained by Kjeldahl analysis of (a) the precipitated casein and of (b) the total protein and (c) the signal obtained from the enthalpimetric method. Since there exists a strictly linear relationship Fig. 2 between the normalised heat pulse and the amount of casein in the sample (over the concentration range investigated), it becomes necessary only to determine one sample from a particular type of milk (fresh cows milk, powdered cows milk, goats milk) in order to establish the calibration curve. For all the commercial samples of fresh cows milk investigated a dilution of between 1:5 and 1:10 was used.

It is recognized that if frequent recalibration is necessary then it would to some extent detract from the advantage of the proposed method. In order

to ascertain the frequency of calibration required, over a period of two working weeks, a series of approximately 200 samples was determined by the proposed method and every 10th sample was also determined by the Kjeldahl method. The results indicate that it is not necessary to recalibrate the DIE method more than once per week or whenever the type of sample is altered. For example, when a sample of goat's milk was analysed it was necessary to use a different calibration curve. This is to be expected since the matrix of goat's milk is different from that of cow's.

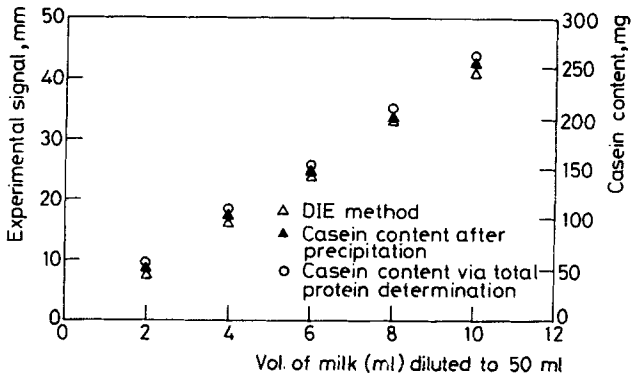

Fig. 1 Relationship between vol. of samples, heat pulse and casein content

It is emphasised that any change in the enthalpimetric system such as reaction temperature, thermal sensor, sensitivity of the bridge or the recorder will necessitate re-calibration. As indicated the method has been applied to the determination of the casein contents of various types of samples, fresh milk, homogenised (UHT treated) milk and milk prepared from a variety of commercially available dried milk powders and granules. With all samples it is essential to ensure homogeneity, with dried milk samples this was generally obtained by "blending" (using a commercial food blender) for 5 minutes prior to sampling for assay.

The statistical results outlined in Table 2 indicate that the enthalpimetric method is at least as acceptable as is the Kjeldahl and a comparison of the values with those reported obtained using infrared methods [6, 10] indicates that it is as good as those. Karman and co-workers reported that the IR method takes approximately 1 hour to complete.

The proposed enthalpimetric method takes less than 15 minutes per sample and offers additional advantages when considered for routine use in quality control or quality assurance laboratories carrying out casein assays. One of the major costs of routine assay is that of labour. Thus any method which allows the use of less skilled labour and less operational time, will overall be less costly. In Q.C. or Q.A. situations, rapid low cost analysis lowers the overall industrial costs.

By using existing standard methods for the routine assay of casein in samples, it is possible to prepare concentrations of titration solutions such that the volumes of titrants used in the standardised procedure would be able to be used directly for the determination of the casein content. Similarly standardised conditions can be established such that one set of automatic/semi-automatic digestion equipment can process several (usually 5-6) samples simultaneously for digestion and fairly rapidly in sequential titrations. Thus after preparation of standardised solutions, the values can be related directly to the concentrations of casein. By using a semi-automatic digester, capable of handling 6 samples simultaneously, calculating the time spent on titrations and accepting that all the glass ware requires washing to an analytically accepted standard of cleanliness, it can be estimated that approximately 10-12 samples can be assayed in duplicate by a skilled technician during a working day, with a delay of at least 150 minutes between the receipt of the milk sample and the calculation of the result. Using the proposed procedure, with disposable polystyrene reaction vessels, having samples determined in duplicate with a blank determination every 10

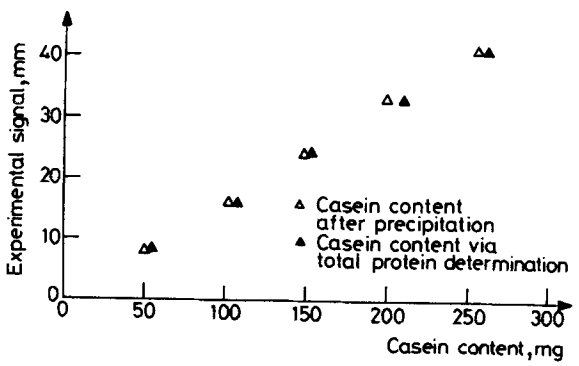

Fig. 2 Relationship between heat pulse and casein content

samples and standardisation once per working week it is possible to determine 30-35 samples per working day. The time between receiving the sample and the reporting of the results is approximately 15 minutes. Most of this time is required to allow the diluted sample to attain the temperature of the thermostat. The time per determination when the sample is at the required temperature is less than 5 minutes, since no elaborate washing of the apparatus is required, the reaction vessel is discarded, the temperature sensor probe and stirrer are washed with distilled water kept in the thermostat.

Standardisation of the titration solutions used in the Kjeldahl procedure is required daily and the solutions must be prepared to classical analytical standards. Such precision is not required in the DIE procedure, the method has the inherent advantage that the concentration of reagents used for injection need normally only be within 5% of the quoted value [15]. Since the procedure ensures that there is always an excess of unreacted injection material after precipitation of the casein, small variations in the excess of the reagent have no significant effects on the pH value of the solution and on the heat pulse measured. Thus there is no need to have precisely standardised reagent solutions and time and money are thereby saved. The consumable costs of the reference method and the DIE procedure are also significantly different. The DIE method uses 4 ml (2.2 ml) of a 1:1 mixture of 10% v/v aqueous acetic acid: 1 m sodium acetate per sample assayed in duplicate. The Kjeldahl method uses 40 ml of concentrated sulphuric acid, 4 tablets (approx 10 g) of copper sulphate catalyst, 50 ml of 0.1 M hydrochloric acid and approximately 40 ml of 0.1 M sodium hydroxide solution per sample (assayed in duplicate).

The initial outlay on apparatus is estimated to be lower for the DIE procedure, but not to any great significant extent when calculated per sample of milk analysed. Thus the overall cost of assaying each sample using the proposed method will be less than 40 % of the cost incurred when the Kjeldahl method is used.

The DIE process can readily be made semi-automatic. The potentiometric recorder can be replaced with a digital voltmeter having a sensitivity (shunt) control and simple electronic "backoff" to compensate for the blank. The reading for the commercial standard can be adjusted to 100 arbitary units and subsequent determinations will automatically be indicated as a percentage of the standard value.

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Zusammenfassung — Es wird ein Verfahren zur Schnellbestimmung des Kaseingehaltes von Milch mittels Direct Injection Enthalpimetry (DIE) beschrieben.

Durch das Einstellen des pH-Wertes der Lösung auf den pH-Wert des isoelektrischen Punktes von Kasein kann infolge des Ausfällens von Kasein ein eindeutiger Wärmeimpuls erzeugt werden. Das Verfahren wurde anhand einer wirklichen Probe geeicht, deren Kaseingehalt mittels dem zeitaufwändigen Kjeldahl-Verfahren bestimmt wurde. Einmal kalibriert kann das neue Verfahren routinehaft auch von Laien durchgeführt werden. Die Empfindlichkeit der Methode ist gut, die erhaltenen Ergebnisse können genauso akzeptiert werden, wie die in Standardverfahren erhaltenen Ergebnisse. Dieses Verfahren kann bei den meisten flüssigen Proben ohne jede vorherige Vorbereitung angewendet werden. Der Hauptvorteil besteht in der Ersparnis von Zeit und Analysenkosten und in der Anwendbarkeit des enthalpiemetrischen Verfahrens zur Automatisierung.