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Methods to determine denaturation and aggregation of proteins in low-, medium- and high-heat skim milk powders

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Abstract – Skim milk powders (SMPs) of different heat classifications are used in recombined milks and milk products. These SMPs are broadly classified as low-, medium- and high-heat powders, based on their whey protein nitrogen index (WPNI). The WPNI is a measure of undenatured whey protein nitrogen (WPN) content (expressed as milligrams of WPN per gram of powder). This heat classification, based on the WPNI, gives an indirect indication of the denaturation and aggregation of whey proteins and thus the severity of the heat treatments that were used during the manufacture of milk powders. The severity of heat treatment has an impact on the functional properties of the resultant powders or their suitability for different applications. In the present study, we measured the WPNI of a range of SMPs using a dye binding method (reference method) and attempted to correlate these results with the WPNI predicted using Fourier transform near infra-red (FT-NIR) spectra, and with denaturation and aggregation of proteins as analysed using various polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE) methods. In most cases, FT-NIR spectroscopy provided a rapid method for predicting the WPNI of milk powders, with good correlation ($R^2 = 0.985$). The correlation was used to successfully predict the WPNI of a new set of powders, and the method could potentially be used to determine the WPNI routinely using appropriate controls. The denaturation and aggregation of native monomeric whey proteins as analysed by PAGE and CE correlated well with the WPNI of the respective SMP samples. Modified one-dimensional sodium dodecyl sulfate (SDS)-PAGE and two-dimensional SDS- and then reduced SDS-PAGE gave an indication of the type and composition of disulfide-linked protein aggregates and showed some interesting differences between possible protein-protein interactions involved in the manufacture of low-, medium- and high-heat SMPs. The low-heat powder (WPNI 6.76 mg WPN·g⁻¹ powder) retained most of the whey proteins in the native state. In contrast, the high-heat powder (WPNI 0.33 mg WPN·g⁻¹ powder) contained a comparatively small proportion of native whey proteins, although some α -lactalbumin was present. The degree of denaturation of β -lactoglobulin appeared to be crucial and could be related to the WPNI.

skim milk powders / WPNI / protein denaturation and aggregation / 1D and 2D gel electrophoresis / FT-NIR

摘要 – 低、中、高热处理脱脂乳粉蛋白质变性和凝聚的测定方法。摘要 不同热处理程度的脱脂乳粉 (SMPs) 可以用来生产还原奶和其他乳制品。根据脱脂乳粉的乳清蛋白氮指数 (WPNI) 将脱脂乳粉分为低、中、高热处理乳粉。WPNI是一种测定未变性乳清蛋白氮含量的方法 (以每克脱脂乳粉中乳清蛋白氮的毫克数来表示)。根据WPNI值,

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热处理分类可以间接地给出乳清蛋白的变性和凝聚,以及乳粉制造过程中热处理的强度。乳粉热处理的强度影响着最终产品的功能性以及其用途。在本研究中,采用染色法(基准方法)测定了脱脂粉WPNI的变化范围,同时采用傅立叶变换近红外光谱法(FT-NIR)对测定结果进行了校正以及对WPNI值的预测;采用聚丙烯酰胺凝胶电泳(PAGE)和毛细管电泳(CE)法分析了蛋白质的变性和凝聚。在多数情况下,FT-NIR光谱法可以快速地预测乳粉的WPNI,且相关性较好($R^2 = 0.985$)。并且这种相关性成功地用于一批新生产乳粉的WPNI值的预测;如果适当地控制条件,该方法有可能用于WPNI的常规测定。采用PAGE和CE分析的天然单体乳清蛋白的变性与凝聚与脱脂乳样品的WPNI具有较好的相关性。改良的单向十二烷基磺酸钠(SDS)-PAGE和双向SDS-及还原的SDS-PAGE中给出了二硫键蛋白凝聚的组成和类型,而且试验结果表明低、中、高热处理制造的脱脂乳中蛋白-蛋白之间的相互作用可能存在着一些令人感兴趣的差异。低热处理的乳粉(WPNI 6.76 mg WPN·g⁻¹乳粉)保留着较多的天然状态的乳清蛋白。相反,高热处理的乳粉(WPNI 0.33 mg WPN·g⁻¹乳粉)尽管存在一些 α 乳白蛋白,但是还是保留着较小比例的天然状态的乳清蛋白。 β 乳球蛋白的变性程度相当严重,其变性程度与WPNI的变化一致。

脱脂乳粉 / 乳清蛋白氮指数 / 蛋白质变性和凝聚 / 单向和双向凝胶电泳 / 傅立叶变换近红外光谱

Résumé – Méthodes de détermination de la dénaturation et de l'agrégation des protéines dans des poudres de lait écrémé « low-heat », « medium-heat » et « high-heat ». Des poudres de lait écrémé de différentes classifications thermiques sont utilisées dans les laits et produits laitiers recombinaés. Ces poudres sont en général classées en « low-heat », « medium-heat » et « high-heat » selon leur indice d'azote des protéines solubles (WPNI). Cet indice mesure la teneur en azote des protéines de lactosérum (WPN) non dénaturées exprimée en mg·g⁻¹ de poudre. Cette classification thermique, basée sur le WPNI, donne une indication indirecte sur la dénaturation et l'agrégation des protéines de lactosérum et ainsi sur la sévérité des traitements thermiques utilisés au cours de la fabrication des poudres de lait, sévérité dont dépendent les propriétés fonctionnelles des poudres et leur aptitude à différentes applications. Dans la présente étude, nous avons mesuré les valeurs de WPNI d'une gamme de poudres à l'aide d'une méthode colorimétrique (méthode de référence) et tenté de corréler ces résultats avec le WPNI prédit à l'aide de la spectroscopie FT-NIR, et avec la dénaturation et l'agrégation des protéines analysées à l'aide de plusieurs méthodes d'électrophorèse PAGE et capillaire (CE). Dans la plupart des cas, la spectroscopie FT-NIR s'avère être une méthode rapide de prédiction du WPNI des poudres de lait, avec une bonne corrélation ($R^2 = 0,985$). La corrélation a été utilisée avec succès pour prédire le WPNI d'un nouveau lot de poudres; la méthode peut être utilisée pour déterminer le WPNI en routine sous réserve des contrôles appropriés. La dénaturation et l'agrégation des protéines de lactosérum natives monomères analysées par PAGE et CE étaient bien corrélées au WPNI des échantillons respectifs de poudre de lait écrémé. Une méthode modifiée SDS-PAGE 1D, SDS-PAGE 2D puis SDS-PAGE 2D en présence d'un agent réducteur donnait une indication du type et de la composition des agrégats de protéines liées par des ponts disulfure et montrait quelques différences intéressantes entre les interactions protéines/protéines possibles impliquées dans la fabrication des poudres « low-heat », « medium-heat » et « high-heat ». La poudre « low-heat » (WPNI 6,76 mg WPN·g⁻¹ de poudre) retenait la majorité des protéines de lactosérum à l'état natif. A l'opposé, la poudre « high-heat » (WPNI 0,33 mg WPN·g⁻¹ de poudre) contenait comparativement peu de protéines de lactosérum natives, mais avec quand même la présence d'un peu d' α -lactalbumine. Le degré de dénaturation de la β -lactoglobuline s'avère être crucial et peut être relié au WPNI.

poudre de lait écrémé / WPNI / protéine de lactosérum / dénaturation / agrégation / électrophorèse / spectroscopie FT-NIR

1. INTRODUCTION

Thermal denaturation and aggregation of whey proteins and their interactions with casein play an important role in determining the functional properties of the resultant milk products [14, 21, 29–31]. Skim milk powders (SMPs) are major trade items in the food industry. Their manufacture often involves different heat treatments to modify the native whey protein content to produce tailored powders with desired functional properties. The degree of denaturation and aggregation of the whey proteins that exist in milk powder depends on the intensity and the duration of the heat treatment that was applied during the manufacture of the powder [15, 21, 24]. The degree of denaturation of the whey protein in milk powder is traditionally indicated by its whey protein nitrogen index (WPNI), which is a measure of the undenatured whey protein nitrogen (WPN) level (expressed as milligrams of WPN per gram of powder). SMPs are broadly classified into low-, medium- and high-heat powders depending on the WPNI. Typical WPNI values for low-heat, medium-heat and high-heat powders are ≥ 6.00 , 1.51–5.99 and ≤ 1.50 mg WPN·g⁻¹ powder respectively [1, 3, 15]. Medium-heat powders are further classified into medium-heat and medium-high-heat powders in the WPNI ranges 4.51–5.99 and 1.51–4.50 mg WPN·g⁻¹ powder respectively [15].

Depending on the extent of whey protein denaturation, milk powders exhibit different functional properties [5, 6, 15] and therefore are suitable for different applications (Tab. I). The WPNI test [1] has been used routinely in the dairy industry to estimate the extent of whey protein denaturation and, in many instances, the end uses of milk powders are decided based solely on the WPNI results. However, the validity of heat classification tests (such as the WPNI) may be influenced by many factors, including variations in the concentrations of

individual whey proteins and non-protein nitrogen (NPN) in raw milk, which may be caused by seasonal fluctuations in milk composition [26, 27]; such variation may be monitored by powder manufacturers to allow more accurate control of the functional properties of the resultant milk powder.

Various methods used to estimate the WPNI, and limitations or relevant comments associated with these methods are listed in Table II. The traditional WPNI test has poor reproducibility because of variable and unstable turbidity. Therefore, it is desirable to develop and evaluate alternative methods to provide reliable and accurate estimations of undenatured whey proteins in milk powders. Also, it is important to ensure that there are good correlations between the WPNI and other methods that might be used for measuring protein denaturation or predicting the WPNI.

In our previous reports [4, 24] and references therein, it was shown that heat treatment causes the native monomer β -lactoglobulin (β -LG) to reversibly interchange into a non-native monomer and non-native disulfide-bonded dimers of β -LG and then to interact with other milk proteins such as α -lactalbumin (α -LA) or κ -casein (κ -CN). Such intermediates may be present in milk powders, but this has not yet been reported. In the present paper, we examine the changes in protein-protein interactions, and discuss the protein conformational changes, induced by heat treatments in different milk powders, using reactive sulfhydryl groups (RSH) and polyacrylamide gel electrophoresis (PAGE) methods supported by capillary electrophoresis (CE), as a potential method for determining the WPNI, and report on a preliminary investigation into the use of Fourier transform near infra-red (FT-NIR) spectroscopy to predict the WPNI of milk powders.

Table I. Heat classification of SMPs based on the WPNI, the typical heat treatments used in their manufacture, their functional properties and suggested applications (adapted from Kelly et al. [15]).

Heat class	WPNI (mg WPN·g ⁻¹ powder)	Typical preheat treatments	Functional properties	Applications or end uses
Low heat	≥ 6.00	70 °C/15 s	Solubility and lack of cooked flavour	Recombined milk, cheesemaking, milk standardization
Medium heat	4.51–5.99 1.51–4.50	85 °C/60 s 90–105 °C/30 s	Emulsification, foaming, water absorption, viscosity	Ice cream, chocolate confectionery
Or Medium- high heat				
High heat	≤ 1.50	90 °C/5 min	Heat stability, water binding, gelation, water absorption	Recombined evaporated milk, sweetened condensed milk, bakery
Or		120°C/1–2 min		
High heat heat stable	≤ 1.50	> 120 °C/4 min		

2. EXPERIMENTAL

2.1. Description of samples

SMP samples (low-, medium- and high-heat SMPs) were manufactured at Fonterra (Waitoa) from a single milk source by applying different preheat treatments prior to evaporation and drying. The typical preheat treatments used for the manufacture of low-, medium- and high-heat SMPs are 70 °C for 15 s, 90–105 °C for 30 s and 120 °C for 1–2 min. These powders, and the milk from which they were made, were analysed for protein denaturation and aggregation using one-dimensional (1D) and two-dimensional (2D) PAGE and CE. The raw skim milk was used as a reference (control) sample, against which the changes (for example, denaturation and aggregation) in the low-, medium- and high-heat powders could be compared.

For FT-NIR analysis, several powder samples were obtained from Northland Co-operative Dairy Company (Kauri) and Kiwi Co-operative Dairies (Hawera). These samples covered a range

of specifications and WPNI values (see Tab. III).

2.2. Chemicals

The electrophoresis chemicals were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The reducing agent 2-mercaptoethanol (2-ME) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical grade from BDH Laboratory Supplies, Poole, England. Artesian bore water was purified by reverse osmosis treatment followed by carbon treatment and was deionized using a Milli-Q apparatus (Millipore Corporation, Bedford, MA, USA).

2.3. Analysis

2.3.1. Whey protein nitrogen index

The WPNI of the unheated milk and the powder samples was determined using the reference laboratory method (dye binding

Table II. Various methods used in the past for estimating the WPNI and criticisms or comments associated with each of these methods.

Method	Comments
Harland and Ashworth [10]	Simpler than multiple Kjeldahl nitrogen assays. Problem of relating turbidity to whey protein content. Poor reproducibility of samples in interlaboratory comparisons.
Harland et al. [12]	Identified that natural variation of milk serum proteins, NPN, etc., posed limitations.
Kuramoto et al. [16]	Variation in the development of turbidity and poor correlation with the final pH. Low reliability and accuracy.
Leighton [17]	It was found that the small error in acid addition results in pH outside the region of maximum turbidity. This problem was overcome by use of a saturated salt solution, so that the final pH was always between 2.7 and 3.1. Thus this method overcame variability by maintaining the final pH between 2.7 and 3.1 in the saturated salt solution.
Sanderson [26]	This method considered the limitations of previous methods. In this method, casein and denatured whey proteins are precipitated from the reconstituted milk powder using sodium chloride. Undenatured whey proteins are precipitated using amido black dye, and excess dye is determined spectrophotometrically. Thus, the undenatured whey protein is estimated by binding with amido black dye to form a protein-dye precipitate, followed by measuring the optical density of the supernatant in a flow-through cuvette with a 0.36-mm path length. This method can minimize the variation contributed by NPN and consequently can avoid greater assay variance, and therefore can give comparatively better reproducibility. However, great care in following the protocols accurately is still necessary. It is used routinely in New Zealand.
Manji and Kakuda [19]	Comparison of whey protein denaturation using differential scanning calorimetry (new), fast protein liquid chromatography (FPLC) (new), WPNI [17] and Kjeldahl (AOAC, 1980) methods on a range of heated milks.
Sikand and Tong [28]	Use of an FPLC column is reliable, and the data correlate with the WPNI method for determination of heat denaturation.

Table III. Performance of the calibrations and correlation between the WPNI measured using the laboratory (dye binding) method and the WPNI predicted using FT-NIR spectra of various milk powders and their standard error of cross validation (SECV).

	No. samples	WPNI range	R ²	SECV
WMP	22	0.7–2.9	0.94	0.28
SMP	20	0.8–6.5	0.74	0.26

method) of Sanderson [26]. In this method, casein and denatured whey proteins are precipitated from the reconstituted milk powder using sodium chloride solutions. Undenatured whey proteins are precipitated using amido black dye, and excess dye is determined spectrophotometrically. Thus, the undenatured whey protein is estimated by binding with amido black dye to form a protein-dye precipitate, followed by measuring the optical density of the supernatant in a flow-through cuvette with

**Glass scintillation vial
containing sample**



Figure 1. Photograph of the FTIR spectrometer showing the powder sampler accessory.

a 0.36-mm path length. This method can minimize the variation contributed by NPN and consequently can avoid greater assay variance, and therefore can give comparatively better reproducibility. However, great care in following the protocols accurately is still necessary.

2.3.2. FT-NIR instrument and sample preparation

A laboratory-scale Fourier transform infra-red (FTIR) spectrometer (model MB 154S, Bomem, Quebec, Canada, with a spectral range of 510–7500 cm^{-1} using a silicon carbide source with zinc selenide optics) was used for the experiments. The best resolution is claimed as 0.7 cm^{-1} , with an accuracy of 0.4 cm^{-1} at 7300 cm^{-1} and a repeatability of 0.001 cm^{-1} . Resolution is switchable from 1 to 64 cm^{-1} . For optimum detection sensitivity in the NIR, an indium arsenide (InAs) detector with a

cooled power supply was used. The instrument is designed with an open sample bay that can accommodate a variety of sampler accessories. These are held in place by two thumb screws and are easily changed. The powder sampler accessory (Fig. 1) is a device that is designed to facilitate the presentation of finely divided samples to the instrument.

The powder samples were poured via a funnel into glass scintillation vials to a set depth (2 cm) and were then settled with gentle tapping. A minimum of 2 g of sample was added to a glass scintillation vial. Any physical discontinuity within the sample was found to affect the result. Care was taken to ensure that the samples were brought to ambient temperature before introduction to the instrument.

2.3.3. FT-NIR spectroscopy

The InAs detector was set at Hi/E for both the background spectra and the

sample spectra. Partial least squares (PLS) regression with multiplicative scattering correction (MSC) was used to produce calibration data. In some cases, a Savitsky Golay smoothing algorithm was also used to pretreat the data. A calibration set of 201 samples of whole milk powder (WMP) and SMP was supplied from various New Zealand dairy ingredient production sites. They covered a range of manufacturing specifications and WPNI values. The key areas of the total spectrum identified, via spectral weightings, as having an important effect on the calibration were 1130–1230, 1265–1380, 1415–1490, 1675–1700, 1800–1850, 1890–2060 and 2145–2270 nm. These wavelengths indicate the maximum areas of spectrum linked to the changes in observed WPNI reference values.

The glass scintillation vials containing the powder samples were placed on to the accessory. Background spectra were taken using a Labsphere (Spectralon, Springfield, IL, USA) reflectance standard and were automatically subtracted from the sample spectra. Spectra were collected over the range from 3996 to 10002 cm^{-1} (770–2000 nm) using 4 or 8 cm^{-1} resolution with 64 scans per sample. All results reported are the average of two successive measurements on discrete samples. Data processing was handled by the Bomem/GRAMS (Galactic Industries Corporation, Salem, NH, USA) software package with chemometrics performed by the PLS plus/IQ application (Galactic Industries).

2.3.4. 1D and 2D PAGE

Reconstituted skim milk samples (10% w/v) were prepared by dissolving 10 g of low-, medium- or high-heat SMP in about 80 mL of Milli-Q water. After complete dissolution, the final volume was made up to 100 mL with Milli-Q water. These

reconstituted skim milk samples were used for various 1D and 2D PAGE assays.

The samples were analysed using a Mini-Protean II dual cell (Bio-Rad Laboratories, Hercules, CA, USA) discontinuous PAGE system for both 1D and 2D PAGE. The standard 1D (native and sodium dodecyl sulfate (SDS)) PAGE as described by Havea et al. [13] and Manderson et al. [18] was used. The gels were scanned and photographed, as described by Manderson et al. [18], using a computing laser densitometer (Molecular Dynamics Model P. D., Sunnyvale, CA, USA) and the integrated intensities of the bands corresponding to β -LG and α -LA were determined using Molecular Dynamics Image-Quant software (Version 5.0).

The standard native- and SDS-PAGE methods as described by Havea et al. [13] and Patel et al. [23] were not suitable for analysing the very high molecular weight aggregates (molecular weight > 500 $\text{kg}\cdot\text{mol}^{-1}$) that were generated by the severe heat treatments involved in the manufacture of the medium- and high-heat SMPs. These aggregates did not enter the gel during electrophoresis or were caught up in the sample loading well. These very large aggregates were subsequently lost during the staining and destaining procedures, which made them difficult to characterize. Therefore, this method was modified by mixing the samples with a polyacrylamide gel of similar composition to the stacking gel and setting them in the sample loading well as described by Davis [7]. This strategy trapped the large protein aggregates (molecular weight > 500 $\text{kg}\cdot\text{mol}^{-1}$) within the set sample gels and prevented their loss during electrophoresis and during the gel staining and destaining procedures. Consequently, an estimate of the qualitative composition of these aggregates could be made using the 2D SDS- and then reduced SDS-PAGE procedure (as described by Patel et al. [24]). This modified technique was

then used in the present study for qualitative determination of the protein aggregates present in the low-, medium- and high-heat powders.

2.3.5. *Capillary electrophoresis*

For CE analysis of the reconstituted milk samples made from the different SMPs, the method described by Paterson et al. [25] using a CE system (model 270A-HT; Applied Biosystems, San Jose, CA, USA) was followed.

3. RESULTS AND DISCUSSION

3.1. **Correlation between residual native-like whey proteins using PAGE assays and the WPNI and the RSH content**

The PAGE assay separates protein molecules on the basis of their charge and molecular size. Although these assays are somewhat time consuming compared with WPNI and RSH assays, it is possible to obtain detailed quantitative information on the level of denaturation of the individual whey proteins. The PAGE pattern of heat-treated samples gives an indication of the decrease in the concentration of the native-like or SDS-monomeric whey proteins and the consequent formation of protein aggregates of various sizes. However, it was useful to confirm that the results of these assays correlated well with each other.

In a previous study [2], we reported on a comparison of alternative methods to analyse whey protein denaturation and attempted to correlate the results of different methods. There was a strong positive correlation ($R^2 = 0.98$) between the WPNI of various SMPs, as measured using the dye binding assay [26], and the concentration of residual native-like β -LG and total whey

protein respectively, as determined using the native-PAGE assay. As the WPNI assay is a measure of the total undenatured (native) whey protein, this strong correlation was expected. Similarly, there was a strong negative correlation ($R^2 = 0.97$) between the WPNI and the RSH content of different SMP samples. It is known that, in unheated milk or whey, most of the free sulfhydryl (SH) groups are buried within the native globular structure of β -LG or bovine serum albumin (BSA); therefore, these SH groups remain unreactive. Upon heating, the native structure unfolds (denatures), exposing the free SH groups and thus they become reactive. Severe heat treatment gives more RSH and vice versa. Therefore, a negative correlation between RSH and the amount of undenatured whey protein (WPNI) was expected.

However, it is important to mention that the WPNI assay determines the residual native whey protein present in the samples after a particular heat treatment whereas the RSH assay provides an indication of the level of denaturation of those whey proteins (for example, β -LG and BSA) that have a free SH group in their native structure.

The RSH assay is therefore primarily a measure of the level of denatured β -LG and BSA and not of denatured α -LA, as SH groups are absent in α -LA. This should be an important consideration when interpreting the results of RSH assays in studies of mixed whey protein systems.

3.2. **Correlation between the WPNI predicted using FT-NIR spectra and the WPNI measured using the reference laboratory method**

A number of calibrations were created using the calibration set of samples (see Sect. 2.3.3 for detail). An independent set of 66 samples not used in the calibration set was used to validate different results.

The performance of the calibrations on this validation set is shown in Table III. The standard error of cross validation (SECV) data show the standard deviation of the differences between the FT-NIR-predicted WPNI and the WPNI of the same samples measured using the laboratory dye binding method [26] and thus give an indication of the accuracy of the calibration. The lower the value for the SECV, the better the calibration will be in predicting WPNI values of milk powders using FT-NIR spectra. The SECV obtained for the analysis of about 40 milk powder samples (Tab. III) suggested that there was a good correlation for WPNI analysis using these two methods.

A plot (not shown here) of the FT-NIR-predicted WPNI results versus the WPNI results obtained using the dye binding method [26] for different milk powder samples suggested a strong correlation ($R^2 = 0.985$) between the WPNI values obtained using these two methods. The results indicated that this system will afford a rapid and efficient means of predicting the WPNI of milk powders, provided the calibration is performed using accurate WPNI results, obtained using the laboratory method [26]. A generic calibration that encompasses multiple specifications from different sites indicates that the instrument can predict WPNI values with an error of $\pm 0.2 \text{ mg}\cdot\text{g}^{-1}$ over a WPNI range from 0.7 to $6.5 \text{ mg}\cdot\text{g}^{-1}$ (Tab. III). It may be possible to obtain even more accurate and practical results by constructing separate calibrations for each specification or milk powder type.

3.3. 1D and 2D PAGE

3.3.1. 1D PAGE

The 1D native- and SDS-PAGE patterns of representative samples of control (untreated milk), low-heat, medium-heat

and high-heat SMPs are presented in Figures 2A and 2B respectively. The WPNIs of these samples were 7.10, 6.76, 2.67 and $0.33 \text{ mg}\cdot\text{g}^{-1}$ respectively. For the same samples, the modified SDS-PAGE pattern with the set sample is presented in Figure 2C.

The bands corresponding to the various milk proteins (monomeric lactoferrin (LF), BSA, immunoglobulin G (IgG), α_{s1} -CN, α_{s2} -CN, β -CN, κ -CN, β -LG and α -LA) were clearly identified on the native- and SDS-PAGE patterns by comparison with previous reports [8, 24] and were marked appropriately on each gel (Figs. 2A–2C). Some large aggregates marked as X0 were also present in the PAGE patterns of the control samples (Lane 1, Figs. 2A and 2B). A marker of known molecular weights (Mr) is also appended with these PAGE patterns, where possible.

Comparison of the native- and SDS-PAGE patterns of low-heat SMP (Lane 2, Figs. 2A–2C) with those of the respective control samples (Lane 1, Figs. 2A–2C) shows that the intensities of the bands corresponding to β -LG and α -LA were not affected in the low-heat SMP samples. However, the intensities of the bands corresponding to monomeric IgG, LF and BSA decreased slightly.

In contrast, the native- and SDS-PAGE patterns of the medium-heat SMP (Lane 3, Figs. 2A–2C) and the high-heat SMP (Lane 4, Figs. 2A–2C) were significantly different from those of the unheated control sample (Lane 1, Figs. 2A–2C) and the low-heat SMP (Lane 2, Figs. 2A–2C). The bands corresponding to IgG, LF and BSA were essentially absent in the native- and SDS-PAGE patterns of the medium-heat (Lane 3, Figs. 2A–2C) and high-heat (Lane 4, Figs. 2A–2C) SMP samples and the intensities of the bands corresponding to both monomeric β -LG and α -LA decreased significantly; simultaneously, a significant proportion of high molecular weight aggregates was also observed on

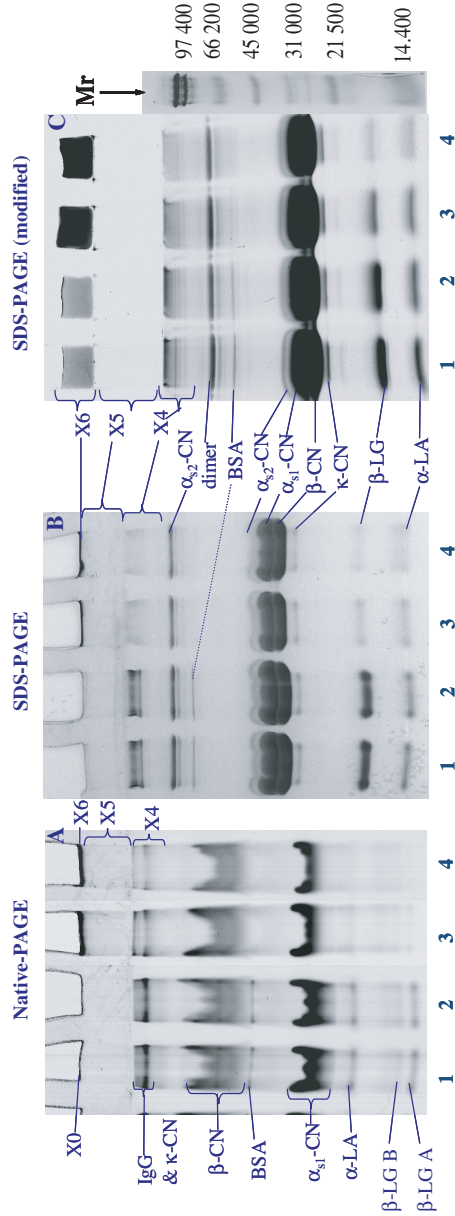


Figure 2. 1D native-PAGE (A), SDS-PAGE (B) and modified SDS-PAGE (C) patterns of raw skim milk (Lane 1), low-heat SMP (Lane 2), medium-heat SMP (Lane 3) and high-heat SMP (Lane 4). The identities of the major protein bands are marked on each PAGE pattern.

the PAGE patterns of the medium-heat and high-heat SMP samples. These high molecular weight aggregates were classified into three groups, that is, those caught up at the top of the resolving gel, those caught up within the stacking gel and those that could not enter the gel or were caught up in the sample loading well, and were marked as regions X4, X5 and X6 respectively. The modified SDS-PAGE patterns of the medium-heat and high-heat SMP samples (Lanes 3–4, Fig. 2C) showed an increased density or dark-staining portion in the set sample gels (X6), indicating that a significant proportion of high molecular weight aggregates that could not enter the gel were formed during the manufacture of the medium-heat and high-heat SMPs.

From the results of the 1D PAGE analysis, a comparison between the denaturation and aggregation of whey proteins and the severity of the heat treatment used in the manufacture of particular milk powders could be made. For example, the mild pre-heat treatments (70–75 °C for 15 s) used in the manufacture of low-heat SMP ([15], see Tab. I) denatured only the heat-labile whey proteins (IgG, LF and BSA); the major whey proteins (β -LG and α -LA) were unaffected. In contrast, typical pre-heat treatments used for medium-heat SMP (90–105 °C for 30 s) and high-heat SMP (120 °C for 1–2 min) had severe effects and led to much more denaturation and aggregation of all the whey proteins including β -LG and α -LA.

3.3.2. 2D PAGE

Various changes in the proteins, including the decrease in the intensity of monomeric proteins and the simultaneous formation of high molecular weight aggregates (particularly disulfide-bonded aggregates), as observed on the 1D SDS-PAGE (Fig. 2C) pattern of selected low-, medium- and high-heat SMP samples,

were further characterized using 2D (SDS- and then reduced SDS-) PAGE. Almost all the changes observed on 1D PAGE (Fig. 2C) were reflected in the 2D PAGE patterns of the corresponding samples.

The 2D PAGE pattern of the unheated control (raw milk) sample (Fig. 3A) had a series of spots that lay diagonally from the lower right to the upper left of the gel. These protein spots were identified by comparison with the results of Patel et al. [24]. The 2D PAGE patterns of the low-heat SMP (Fig. 3B) and the control sample (Fig. 3A) were almost identical (except for a slight decrease in the intensity of the spots corresponding to BSA, LF and IgG), suggesting that the mild pre-heat treatment (70–75 °C for 15 s) involved in the manufacture of low-heat SMP did not have much effect on the major whey proteins (β -LG and α -LA). Also, it seems that this mild pre-heat treatment did not affect the distribution of κ -CN. In contrast, the medium-heat SMP (results not shown) and the high-heat SMP (Fig. 3C) showed the presence of several new spots, corresponding to reduced monomeric whey proteins (including LF, BSA, IgG, β -LG and α -LA) and caseins (κ -CN and a small proportion of α _{s2}-CN), indicating that these proteins were involved in the formation of disulfide-linked casein-whey protein complexes, which were resolved by disulfide-bond reduction of the protein aggregates caught up in the set sample gel region (that is, resolved from the region marked as X6 on the 1D SDS sample gel strip (a')). Interestingly, the distribution of κ -CN was affected significantly in the PAGE pattern of the high-heat SMP (Fig. 3C). The 2D PAGE patterns of medium-heat SMP and high-heat SMP were similar, except that very little α -LA was involved in the disulfide-linked aggregation in the PAGE pattern of the medium-heat powder (results not shown) whereas a significant amount of α -LA was involved in the disulfide-linked

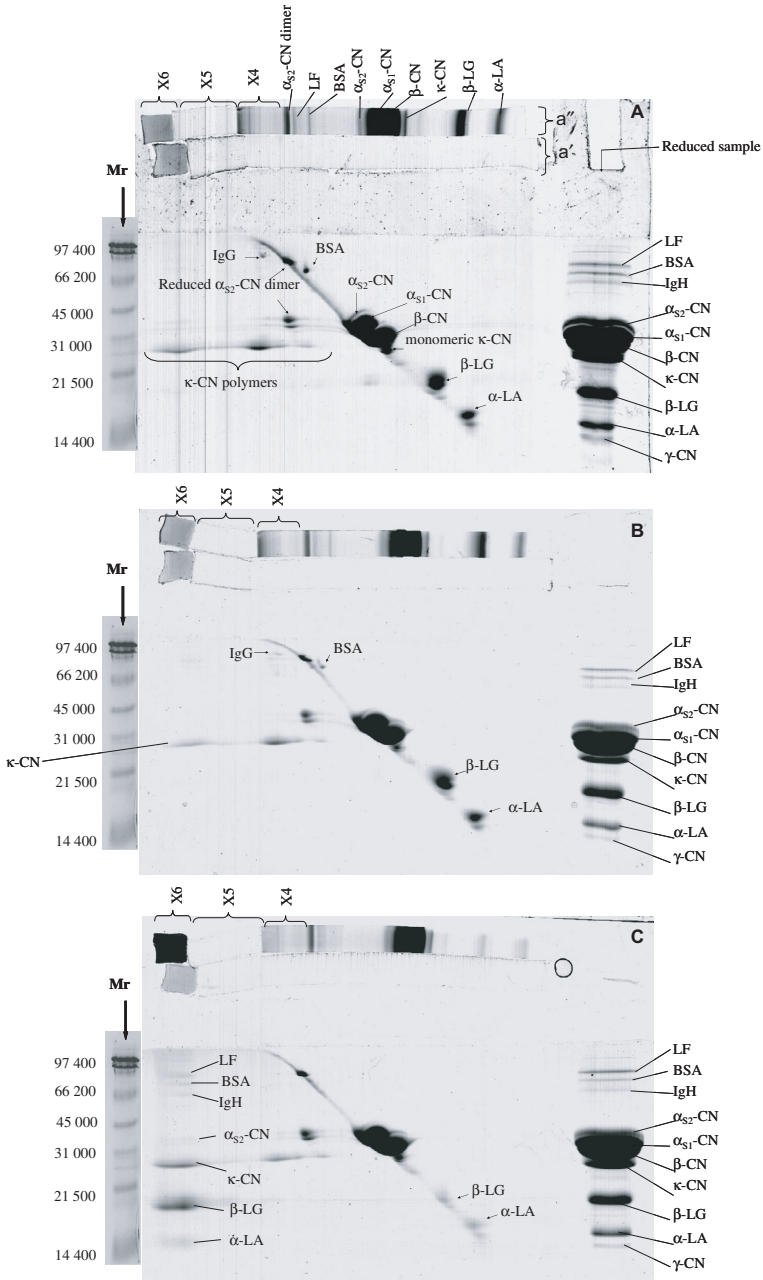


Figure 3. 2D SDS- and then reduced SDS-PAGE patterns of raw skin milk (A), low-heat SMP (B) and high-heat SMP (C).

aggregation in the PAGE pattern of the high-heat powder (Fig. 3C).

These results of 2D PAGE characterization suggested that there were significant differences between the low-heat SMP and the high-heat SMP, in terms of denaturation and aggregation of whey proteins, and their subsequent complex formation with caseins. These differences in the denaturation and aggregation were as expected, because of the different intensities of the heat treatments (time and temperature combinations) involved in the manufacture of these milk powders. This study demonstrated that characterization of powder samples using 1D and 2D PAGE was very useful in providing detailed information about the denaturation and aggregation of individual proteins and the protein-protein interactions that are present in particular powder samples. This detailed information may be useful in predicting the functionality of the powders in the final products.

3.4. Capillary electrophoresis (CE)

The amount of native (undenatured) β -LG and α -LA present in the raw skim milk and the low-, medium- and high-heat SMP samples (WPNI 7.10, 6.76, 2.67 and 0.33 mg WPN·g⁻¹ powder respectively) was also analysed using CE, and the electropherograms of these samples are presented in Figures 4A, 4B, 4C and 4D respectively.

Comparison of the electropherogram of the low-heat SMP (Fig. 4B) with that of unheated skim milk (Fig. 4A) shows that these two patterns were almost identical. These results confirmed the earlier 1D and 2D PAGE results (Figs. 2 and 3); that is, minimal changes in β -LG and α -LA occurred in the low-heat SMP. In contrast, the electropherogram of the medium-heat SMP (Fig. 4C) showed that very small peaks corresponding to β -LG A and

β -LG B were present, whereas the peak area corresponding to α -LA decreased to about half and eluted between 12 and 13 min, and that a new major peak corresponding to high molecular weight protein aggregates eluted between 16 and 18 min. Comparison of the CE pattern of the high-heat SMP (Fig. 4D) with that of the control sample (Fig. 4A) suggested that the peaks corresponding to β -LG A, β -LG B and α -LA were absent, whereas only the new major peak corresponding to heat-induced protein aggregates eluted between 17 and 19 min was present, confirming the results of 1D and 2D PAGE (Figs. 2 and 3).

Although the results of application of CE are based on a preliminary study, they suggest good potential of this method to provide quick and reliable (i.e. free from manual error) indication of changes in native proteins and the formation of protein aggregates in milk powder samples. This method has been used successfully in the past for fast and precise determination of the degree of denaturation of bovine α -LA during the heat treatment of whey [20]. This method also has the potential to determine the concentrations of specific proteins, by quantifying the CE peaks [20]. However, detailed work will be necessary to validate these results and calibrations in order to generate more meaningful results for the purpose of specific analyses.

3.5. General discussion

If we take a glance back at milk powder research, it can be seen that efforts to develop methods to determine whey protein denaturation began in the 1940's and 1950s. When using SMPs for bakery applications, Harland and Ashworth [9–11] recognized that the extent of whey protein denaturation in the milk powder was a reflection of the heat treatment that the milk had received during manufacture of the powder. They reported [9–11] the

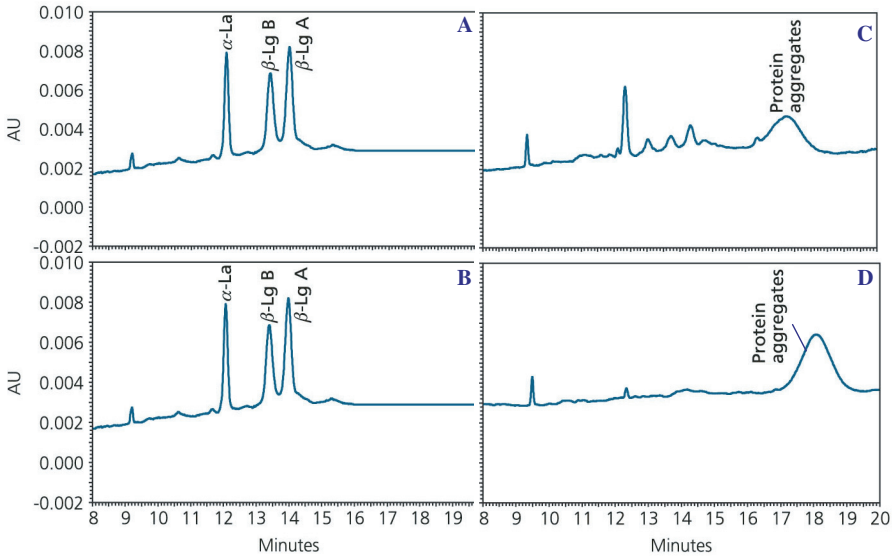


Figure 4. CE electropherograms of the whey portion obtained from raw skim milk (A), low-heat SMP (B), medium-heat SMP (C) and high-heat SMP (D).

relationship between whey protein denaturation and functional properties in bakery applications, such as water absorption of the dough and production of the maximum loaf volume, and attempted to devise an early turbidimetric method that was appropriate for a simple determination of the WPNI. This original method for estimating the WPNI was suitable for determining whether skim milk had received a sufficiently high heat treatment during manufacture for it to be suitable in bread-making, but did not cover the determination of a wide range of WPNIs. An improved version of this method was used to explore the effects of various steps in the manufacture of SMP on whey protein denaturation and to suggest that the natural variations in individual whey proteins, NPN, etc., in the milk supply contributed to the variations in WPNI [12]. However, these methods had poor reproducibility and a lack of agreement among different laboratories analysing the same samples. Fur-

ther modifications to the earlier methods were suggested by Kuramoto et al. [16] and Leighton [17], mostly by identifying the variables that should be more strictly monitored for more precise determination of the WPNI.

These modified methods were suitable for a wider range of heat classification. However, all the above methods relied heavily on the measurement of the turbidity of solutions using a spectrophotometer and it was noted that the development of turbidity was unstable and variable (not uniform) and was dependent on the pH. Therefore, it was difficult to produce accurate and reproducible results using the above methods. Subsequently, a major modification to the above assay was instituted by Sanderson [26], in which the undenatured whey protein was estimated by binding with amido black dye to form a protein-dye precipitate, followed by measuring the optical density of the supernatant in a flow-through cuvette

with a 0.36-mm path length. Therefore, this method minimized the variation contributed by NPN and consequently avoided greater assay variance, and gave comparatively better reproducibility. However, great care in following the protocols accurately was still necessary in order to obtain accurate and reproducible results.

It is clear from the above reports that several factors may contribute to the variations in the WPNI results of milk powders. The ratio of different proteins (for example, the ratio of β -LG to α -LA), the protein to lactose ratio and the NPN content of the milk vary with the season and it was confirmed that the seasonal variation affects the WPNI of milk powders [27]. Therefore, depending on the initial composition of the milk, the amount of undenatured WPN will be different in powders manufactured using particular sets of process parameters. Also, there is a genetic variant effect, because the A variant of β -LG is expressed at a higher concentration than the B variant and consequently the whey protein to casein ratio is different [22]. Recently, Tong [33] indicated that Codex Alimentarius allows for downwards standardization of SMP with lactose or milk permeate. Protein standardization using permeate has the potential for greater consistency in product composition and performance for end users and will improve SMP manufacturing profitability and/or provide a means of cost reduction for suppliers; however, it will increase the need for improved methods of classifying milk powder to communicate performance needs, as milk permeate contains significant amounts of minerals and lactose, and therefore the “standardization” of milk powders to particular protein contents (for example, 35% protein) by the addition of lactose has an effect on the WPNI of the milk powder [28], because of the dilution effects on whey protein content. This dilution effect is likely to affect the WPNI range in the final powder and may shift the WPNI value from low heat to

medium heat or medium heat to high heat (see Tab. I). This may lead to false classification of different powders (particularly when permeate is used from milk powder standardisation). This suggests the need for more reliable methods to determine whey protein denaturation and aggregation, compared with the traditional method (turbidimetric method to determine the WPNI). Moreover, as SMPs are selected for specific functional applications and end uses depending on their WPNI results, the accuracy and reliability of the WPNI analysis has an impact on the functionality and suitability of the powders for different applications. As discussed earlier, the current turbidimetric method [1] to determine the WPNI will impact on the use of permeate (because of the contribution of lactose, minerals, etc., and whey protein dilution). Tong [32] also suggested that the variation in the protein and moisture contents of the powder will influence the performance of the powder and the perceived heat classifications. They therefore proposed analysing whey protein denaturation using fast protein liquid chromatography (FPLC) column [32]. It is obvious from the above reports that the WPNI is a complex phenomenon that is affected by several factors. Therefore, predicting the functional properties of a particular milk powder based solely on its WPNI measured using the traditional WPNI test may not be reliable. Some previous reports also draw similar conclusions [19].

The present study used some recently developed methods to measure the quantities and the states of individual soluble whey proteins in a range of milk powders. A typical “high-heat” SMP with a WPNI of $0.33 \text{ mg}\cdot\text{g}^{-1}$ was found to contain only a small proportion of whey protein that was monomeric as analysed by native- and SDS-PAGE. In contrast, raw skim milk (WPNI approximately $7 \text{ mg WPN}\cdot\text{g}^{-1}$ powder) and a low-heat SMP (WPNI approximately $6.8 \text{ mg WPN}\cdot\text{g}^{-1}$ powder)

contained all of the major whey proteins (α -LA, β -LG and BSA) as monomeric or largely native in structure. The strong correlation obtained between the WPNI and the residual monomeric β -LG or total whey protein or the RSH [2] suggested that these assays can be used for more precise estimation of undenatured whey proteins, irrespective of the seasonal variation and the variation in the composition of the milks, NPN, etc. The protein-protein reactions that occur as a consequence of heat treatment have also been studied for many decades. Successful application of 1D and 2D PAGE for characterization of protein aggregates in powder samples provided an indication of the differences in the composition and the type of protein-protein interactions in the low-, medium- and high-heat SMPs. Differences in the protein-protein interactions as characterized using 1D and 2D PAGE can also be correlated with the functional properties of different powders. β -LG seems to be the most important protein, with a single thiol on Cys121 in its native structure, as, during heat treatment, this thiol becomes available to react with disulfide bonds, initially with β -LG Cys119–SS–Cys106, followed by β -LG Cys160–SS–Cys66 and other disulfide bonds in α -LA and κ -CN [4,13,18,24]. CE and FT-NIR spectroscopy can be used for rapid and accurate predictions of undenatured whey proteins after calibration and standardization of these methods for a particular set of process parameters. For the use of FT-NIR for the rapid determination of approximate WPNI values, a generic calibration covering a wide range would be sufficient. When more accurate results are necessary, a calibration unique to the manufactured product specification would be necessary. The effects of particle size or agglomeration of powder samples on calibration accuracy would also require further investigation. However, the early methods for WPNI [1,26] are widely used for routine analysis of powders and

are incorporated in the legislation of some dairying countries and therefore may not be easy to change.

4. CONCLUDING REMARKS

The WPNI remains an important attribute for milk powders that are to be used for recombining applications. WPNI results and the heat classification of milk powders can be influenced by seasonal variations in the protein content, the type of protein of the raw milk, as well as by the moisture and protein contents of the powders. These factors should be taken into account for more accurate control of their functional properties.

The present study showed some promising results that merit further investigation. The successful application of modified 1D and 2D PAGE methods for the qualitative analysis of low-, medium- and high-heat SMPs was found to be useful for identifying protein aggregates and the composition of heat-induced disulfide-linked protein aggregates. The newly developed FT-NIR method will provide a rapid and efficient means of predicting the WPNI of milk powders, provided the calibration is performed accurately. It is expected that successful applications of this knowledge will be helpful for close control of the functional properties of milk powders. After all, the early methods for WPNI are enshrined in the legislation of some dairying countries and will not be easy to change.

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