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Preparation, characterisation and selected functional properties of sodium caseinate–maltodextrin conjugates

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ABSTRACT

Sodium caseinate (NaCN)–maltodextrin (Md40 or Md100) conjugates were prepared by a Maillard-type reaction by dry heat treatment of mixtures of NaCN and Md at 60 °C and 79% relative humidity for 4 days. Minimal levels of coloured reaction products were formed during conjugate preparation. Conjugation resulted in a 35.6% and a 36.2% loss of available amino groups in the NaCN, and a 17.8% and a 25.7% loss of available reducing groups in Md40 and Md100, respectively. SDS–PAGE and gel permeation chromatography confirmed conjugation. When assessed in the pH range 2.0–8.0 at 20 °C and 50 °C, conjugates had improved solubility compared to NaCN, particularly around the isoelectric point of the protein. The emulsifying properties of NaCN–Md conjugates were assessed in oil-in-water (o/w) emulsions and in model cream liqueurs. The conjugate stabilised o/w emulsions and liqueurs showed improved stability when compared to NaCN stabilised o/w emulsions and liqueurs. These results indicate a potential for these NaCN–Md conjugates as speciality functional food ingredients.

1. Introduction

Sodium caseinate (NaCN) is a soluble mixture of four surface active caseins (α_{s1}-, α_{s2}-, β- and κ-casein), which is widely used as a food ingredient because of its significant nutritional value, viscosity modifying, water-binding, fat-binding, emulsifying and foaming properties (Ennis & Mulvihill, 2000). NaCN is commercially used as an emulsifier in many food systems because of the amphipathic nature of its constituent proteins, which enables them to rapidly absorb at the oil–water interface, lowering the interfacial tension and conferring stability with respect to creaming and flocculation through a combination of electrostatic and steric stabilisation. The utilisation of NaCN as a functional ingredient is restricted in acidic food systems particularly around the isoelectric point of the caseins (pH 4.6) where there is a reduction/loss of the proteins net charge, resulting in a decrease in solubility and emulsifying properties of the protein.

Maltodextrins (Mds) are widely used in the food industry as stabilisers (texture and bulking modifiers) in food emulsions (Loret, Meunier, Frith, & Fryer, 2004). Mds are hydrolysis products of starch consisting of α-(1, 4) and α-(1, 6) linked D-glucose polymers and/or oligomers with a dextrose equivalent less than 20. The dextrose equivalent (DE) is a measure of the reducing power of starch derived polysaccharides/oligosaccharides compared to D-glucose on a dry weight basis (Wang & Wang, 2000) and is an inverse value of the average degree of polymerisation (DP) of anhydro glucose units (Dokic-Baucal, Dokic, & Jakovljevic, 2004). Mds are complex mixtures of high and low molecular weight materials, with Mds of low DE values retaining longer oligosaccharide chains (Kasapis, Morris, Norton, & Clark, 1993). Commercial Mds of different dextrose equivalent values (DE 2-20) possess different physicochemical properties including solubility and viscosity. However, maltodextrins with the same dextrose equivalent may also possess very different physicochemical properties depending on the hydrolysis procedure and source/composition of the starch used in their preparation (Dokic-Baucal et al., 2004).

Milk proteins can be modified by physical, chemical and/or enzymatic treatments to produce speciality ingredients with desirable functional properties (Haard, 2001; Sikorski, 2001). However, few of these approaches to protein modification are suitable for producing modified proteins for food application due to various safety issues. One modification recognised as being suitable for producing speciality ingredients for food applications is protein–polysaccharide conjugation via a Maillard-type reaction (Shepherd, Robertson, & Ofman, 2000). The Maillard reaction can be divided into three stages; initial, intermediate and advanced. The initial stage involves the condensation of the carbonyl group of the reducing sugar with the available ε-amino groups (lysine been the primary reactive amino group) of the protein, resulting in an Amadori product been produced via the formation of a Schiff base with the release of water and the Amadori rearrangement (Ames, 1992). The intermediate stage involves the degradation of Amadori products resulting in a wide range of compounds. The final stage of

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the reaction results in extensively coloured, water insoluble, nitrogen containing polymeric compounds referred to as "melanoidins" (Friedman, 1996).

The initial stage of this reaction has been shown to be sufficient for conjugation to be achieved between the protein and the polysaccharide via the formation of a covalent bond which has been shown to be very stable to changes in pH, ionic strength and temperature (Dickinson & Euston, 1991; Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). These conjugates have been shown to possess improved functional properties, including enhanced emulsifying properties (Akhtar & Dickinson, 2006; Shepherd et al., 2000), increased solubility particularly around the isoelectric pH of the protein (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001; Jiménez-Castaño, López-Fandiño, Olano, & Villamiel, 2005) and increased heat stability compared to the protein itself (Aoki et al., 1999; Chevalier et al., 2001). The conjugates enhanced emulsifying properties has been attributed to the hydrophobic protein being firmly absorbed to the surface of the oil droplet, while the bound hydrophilic polysaccharide is highly solvated by the aqueous phase (Dickinson & Galazka, 1991).

The objectives of this research were to determine the extent of conjugation on dry heating NaCN–Md mixtures under controlled atmospheric conditions; to study the influence of conjugation on solubility as a function of pH, and on emulsifying properties. The NaCN–Md conjugates were also used as the protein emulsifier in a model cream liqueur system, and their influence on liqueur stability, colour and viscosity were compared with that of NaCN.

2. Materials and methods

2.1. Materials

Sodium caseinate (NaCN) containing 90.4% protein, 0.5% fat, 0.85% ash and 5% moisture, (determined as described by AOAC (1995)) was obtained from Kerry Ingredients (Listowel, Co. Kerry, Ireland). The maltodextrins, Maltrin040 (Md40) and Maltrin100 (Md100) with a DE of 4–7 and 9–12, respectively, were obtained from Grain Processing Corporation (Muscatine, IA, USA). Soya oil was obtained from a local supermarket. Dialysis membrane with a molecular weight cut off of 12–14 kDa was purchased from Medicell International Ltd. (Liverpool, UK). Glycerol monostearate obtained from Quest Ltd., UK was supplied by Chemcon Ltd. (Dublin., Ireland). All other chemicals were of analytical grade and were commercially available (Sigma–Aldrich, Ireland).

2.2. Preparation of the NaCN–Md conjugates

Each maltodextrin (Md40 and Md100) was individually dispersed in Milli-Q water at 10% (w/v). The Md solution was placed inside a 12 kDa dialysis membrane and dialysed against Milli-Q water containing 0.01%, w/w, sodium azide at 4 °C for 48 h for the removal of low molecular weight sugars and dextrans. The solution was then freeze dried. The loss of low molecular weight sugars and dextrans on dialysis was determined by the difference in dry weight of the Md before and after dialysis and expressed as a percentage loss of Md.

NaCN–Md conjugates were prepared according to the method of Shepherd et al. (2000). The dialysed, freeze dried Mds prepared as described above and NaCN were individually dissolved in Milli-Q water, containing 0.01%, w/w, sodium azide at 5% (w/v). Equal volumes of the aqueous solutions were mixed together under moderate magnetic stirring at ambient temperature. The mixtures (NaCN–Md40 and NaCN–Md100) were then freeze dried, dry heat treated at 60 °C and 79% relative humidity over saturated KBr solution for 96 h to induce conjugation, after which the conjugates were freeze dried and stored at 4 °C for further analysis.

2.3. Conjugate characterisation

2.3.1. Determination of available amino groups

The concentration of available amino groups in NaCN, NaCN–Md conjugates and mixtures was determined by the trinitrobenzenesulphonate method (TNBS) as described by Habeeb (1966). A lysine stock solution (2.7×10^{-4} M) was freshly prepared in Milli-Q water. A lysine standard curve was constructed by placing 0.1–0.6 ml of the lysine stock solution into a glass test tube and making the volume up to 2 ml with sodium hydrogen carbonate buffer (4%, w/v, adjusted to pH 8.5 with 0.2 M NaOH). One millilitre of trinitrobenzenesulphonate (0.02%, w/v) was then added to each test tube, vortexed and then incubated at 40 °C for 2 h in a covered water bath. SDS (1 ml, 10%, w/v) and HCl (0.5 ml, 0.1 M) were then added to the solutions to terminate the reaction. The concentration of ε-triphenyl-lysine was determined by measuring the absorbance of the resultant yellow solutions at 340 nm using a Milli-Q water treated blank as a reference. The standard curve was constructed by plotting the absorbance at 340 nm against the known lysine concentration.

NaCN (0.2%, w/v), NaCN–Md conjugate or mixture (0.4%, w/v) was reconstituted in sodium hydrogen carbonate buffer at room temperature with moderate magnetic stirring for 1 h, followed by centrifugation at 4000*g* for 2 min at 20 °C (Sorval RC-5b Refrigerated Super-speed Centrifuge with GSA rotor) to remove any insoluble material present in the solution. Each supernatant was recovered and its protein concentration determined by measuring its absorbance at 280 nm and calculating its protein concentration by reference to a calibration curve of absorbance at 280 nm of NaCN in a sodium hydrogen carbonate buffer. The concentration of available amino groups in each supernatant was measured by replacing the lysine stock solution with a 0.2 ml aliquot of the supernatant and proceeding as previously described above. The concentration of the available amino groups in NaCN, NaCN–Md conjugates and mixture was measured by reference to the lysine standard curve. The change in the concentration of available amino groups in the NaCN–Md conjugate and mixture relative to unreacted NaCN (assumed to represent 100% available amino groups) was calculated per gram of soluble protein and expressed as a percentage loss of available amino groups compared to NaCN.

2.3.2. Determination of available reducing groups

The concentration of reducing groups in the Mds, NaCN–Md conjugates and mixtures was determined using a modified chloramine-T assay (IDF, 1974). Md (0.4%, w/v), NaCN–Md conjugate or mixture (0.8%, w/v) was hydrated in Milli-Q water. The protein in each solution (30 ml) of conjugate and mixture was precipitated by addition of 40 ml tungstate acid [sodium tungstate (0.7%, w/w), H_2SO_4 (7%, w/w, 1 N) and orthophosphoric acid (0.01%, w/v)]. The formation of a covalent bond on conjugation between NaCN and Md enables all of the covalently bound Md to be precipitated with the NaCN, while the remaining non covalently bound Md, with its available reducing groups, remains soluble. The resulting solutions were filtered using Whatman No. 1 filter paper. The filtrate (20 ml) was mixed with 20 ml of Milli-Q water and 10 ml of 6.8 N HCl, and was then hydrolysed for 2.5 h on a reflux condenser. After hydrolysis, the pH was adjusted to 7 with NaOH; the solution was then filtered (as previous) and diluted to 100 ml. Aliquots (10 ml) of filtrate were transferred to a conical flask with a glass stopper; potassium iodide (5 ml, 10%, w/v) and chloramine-T solution (20 ml, 0.042 N) were added and the solution was left to stand for 2 h in the dark at room temperature. The reaction was terminated by the addition of HCL (5 ml, 2 N) and the concentration of

reducing groups was determined by redox titration with sodium thiosulphate (0.042 N) using starch as an indicator. The filtrate was replaced with water for the blank. The difference in concentration of the total reducing groups in the prepared hydrolysed filtrate of the NaCN–Md conjugate or mixture relative to that of the prepared hydrolysed Md filtrate (assumed to represent 100% available reducing groups) was calculated per gram of Md and expressed as a percentage loss of available reducing groups compared to Md.

2.3.3. Colour measurement following conjugation

The influence of membrane dialysis of Md on the colour developed on conjugation was determined by measuring Hunter chromaticity coordinates (L^* a^* b^*) with a Minolta chromometer CR-100 (Minolta Ltd., Milton Keynes, UK). The instrument was calibrated with a standard white tile ($Y = 88.2$, $x = 0.309$ and $y = 0.316$) before measurement. Dry NaCN, NaCN–Md conjugate or mixture, was placed into a petri-dish and its L^* a^* b^* colour coordinates measured. In the L^* a^* b^* colour space system, L^* values quantify lightness/darkness, while positive a^* values quantify redness, negative a^* values quantify greenness, positive b^* values quantify yellowness and negative b^* values quantify blueness (Francis & Clydesdale, 1975).

2.3.4. SDS–PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970) using a 10%, w/v, acrylamide separating gel and a 3%, w/v, stacking gel both containing 0.1%, w/v, SDS. Solutions of NaCN, NaCN–Md mixture or conjugate (1%, w/v, protein, based on protein concentration of the dried materials, determined by the macro-Kjeldahl method (AOAC, 1995)) were prepared in 0.0625 M Tris–HCl buffer (pH 6.8) containing 2%, w/w, SDS, 5%, w/w, mercaptoethanol and 0.012%, w/w, bromophenol blue and heated at 95 °C for 4 min prior to loading in the gel slots. Electrophoresis was carried out at a constant voltage (200 V) for 5 h using a tris–glycine buffer (pH 8.3) containing 0.125%, w/w, SDS. The gel was removed from the electrophoresis unit, fixed in 50:10:40 methanol:acetic acid:water; and stained with either Coomassie brilliant blue R-250 or Periodic Acid Stain/Fuchsin reagent (Thornton, Carlstedt, & Sheehan, 1994) for protein and carbohydrate detection, respectively.

2.3.5. High-performance gel permeation chromatography

The influence of conjugation on the molecular weight distribution was determined by high-performance gel permeation chromatography of NaCN and NaCN–Md conjugates using a TSK Gel G5000PW$_{XL}$ column and a TSK Gel G4000PW$_{XL}$ column (Tosoh Corporation, Tokyo, Japan) connected in series (Morris, Sims, Robertson, & Furneaux, 2004). The columns were equilibrated with a 4 M guanidine hydrochloride buffer (pH 6.7), prepared with Milli-Q water containing 0.01%, w/w, sodium azide (Hattori, Numamoto, Kobayashi, & Takahashi, 2000). NaCN and NaCN–Md conjugates were dissolved in the buffer (5 mg protein/ml) and centrifuged as described above (Section 2.3.1). The supernatant was filtered (0.2 μm Whatman filter), applied to the column (80 μl) and eluted at a flow rate of 1 ml/min at ambient temperature. The eluate was monitored for UV absorbance at 280 nm.

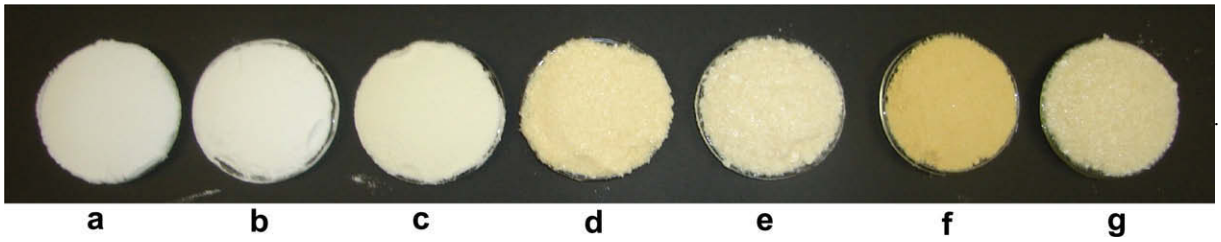
Table 1
Model cream liqueur composition.

Components	Liqueur base (A) g/100 g	Liqueur base (B) g/100 g	Alcohol sugar base g/100 g	Cream liqueur (A) g/100 g	Cream liqueur (B) g/100 g
NaCN	6.5	–	–	2.8	–
NaCN–Md mixture or conjugate	–	13.0	–	–	5.6
Fat (soya oil)	29.2	29.2	–	12.5	12.5
GMS	0.7	0.7	–	0.3	0.3
Sucrose	–	–	33.6	19.2	19.2
Alcohol (95% pure)	–	–	22.2	12.7	12.7
Water	63.6	57.1	44.2	52.5	49.7

Table 2
The loss of available amino groups in the NaCN and the loss of available reducing groups in the Md on conjugation and the effect of heating at 60 °C, 79% relative humidity for 4 days on colour (L^* a^* b^*) of NaCN and NaCN–Md conjugates.

Protein–polysaccharide	Loss of available amino groups (%)	Loss of available reducing groups (%)	L^*	a^*	b^*
(a) Md40	–	0[b]	97.9 ± 0.5	0.37 ± 0.02	0.66 ± 0.06
(b) Md100	–	0[b]	99.6 ± 0.3	−0.76 ± 0.03	0.54 ± 0.04
(c) NaCN (untreated)	0[a]	–	96.1 ± 0.1	−1.63 ± 0.04	8.47 ± 0.09
(d) NaCN–Md40 conjugate	Not measured	Not measured	89.7 ± 0.2	1.15 ± 0.05	20.79 ± 0.19
(e) NaCN–dialysed Md40 conjugate	35.6 ± 0.7	17.8 ± 1.0	91.7 ± 0.2	−0.13 ± 0.03	16.14 ± 0.16
(f) NaCN–Md100 conjugate	Not measured	Not measured	83.2 ± 0.3	1.34 ± 0.05	27.54 ± 0.24
(g) NaCN-dialysed Md100 conjugate	36.2 ± 0.4	25.7 ± 0.4	89.4 ± 0.3	0.74 ± 0.08	17.63 ± 0.21

[a] Unreacted NaCN assumed to represent 100% available amino groups.
[b] Untreated Md assumed to represent 100% available reducing groups.



2.4. Determination of conjugate functionality

2.4.1. Solubility

The solubility of NaCN and NaCN–Md conjugates as a function of pH was determined according to the method of Mohanty, Mulvihill, and Fox (1988). NaCN or the NaCN–Md conjugate was dispersed (1%, w/v, protein) in Milli-Q water at 20 °C or 50 °C under moderate magnetic stirring conditions for 1 h. The pH was adjusted to values in the range of 2.0–8.0 (0.5 unit intervals) using 0.1 N HCl or 0.1 N NaOH as required. After 30 min of moderate magnetic stirring, the pH of each sample was rechecked and re-adjusted, if necessary. The samples were then centrifuged at 1000*g* for 20 min at 20 °C. After centrifugation, each supernatant was decanted and filtered (Whatman No. 1 filter paper) and its protein content was determined by macro-Kjeldahl (AOAC, 1995). Solubility was calculated as the protein content of each supernatant expressed as a percentage of the total protein content of the initial dispersion.

2.4.2. Emulsifying properties

2.4.2.1. Conjugates as emulsifiers in model o/w emulsions. Model oil-in-water (o/w) emulsions were prepared by reconstituting NaCN, NaCN–Md conjugate or mixture (0.8%, w/w, protein) in Milli-Q water, containing 0.01%, w/w, sodium azide (aqueous phase) at ambient temperature under moderate magnetic stirring conditions for 1 h. The oil phase (20%, w/w) was pure soya oil. Each emulsion was prepared by adding the oil phase to the aqueous phase and allowing the mixture to temper at 50 °C for 30 min. The blend was then pre-homogenised using an Ultra-Turrax (10,000 rpm for 15 s), and homogenised by passing the pre-emulsion through a two stage laboratory scale homogeniser (APV 1000 Homogeniser, APV AS, Albertslund, Denmark) for five passes at 50 °C at a first stage pressure of 20 MPa and a second stage pressure of 5 MPa.

The fat globule size distribution of each NaCN, NaCN–Md conjugate or mixture stabilised emulsion was determined on emulsion formation and on storage of the emulsion in glass containers under accelerated shelf life testing conditions at 45 °C for 20 days (Lynch

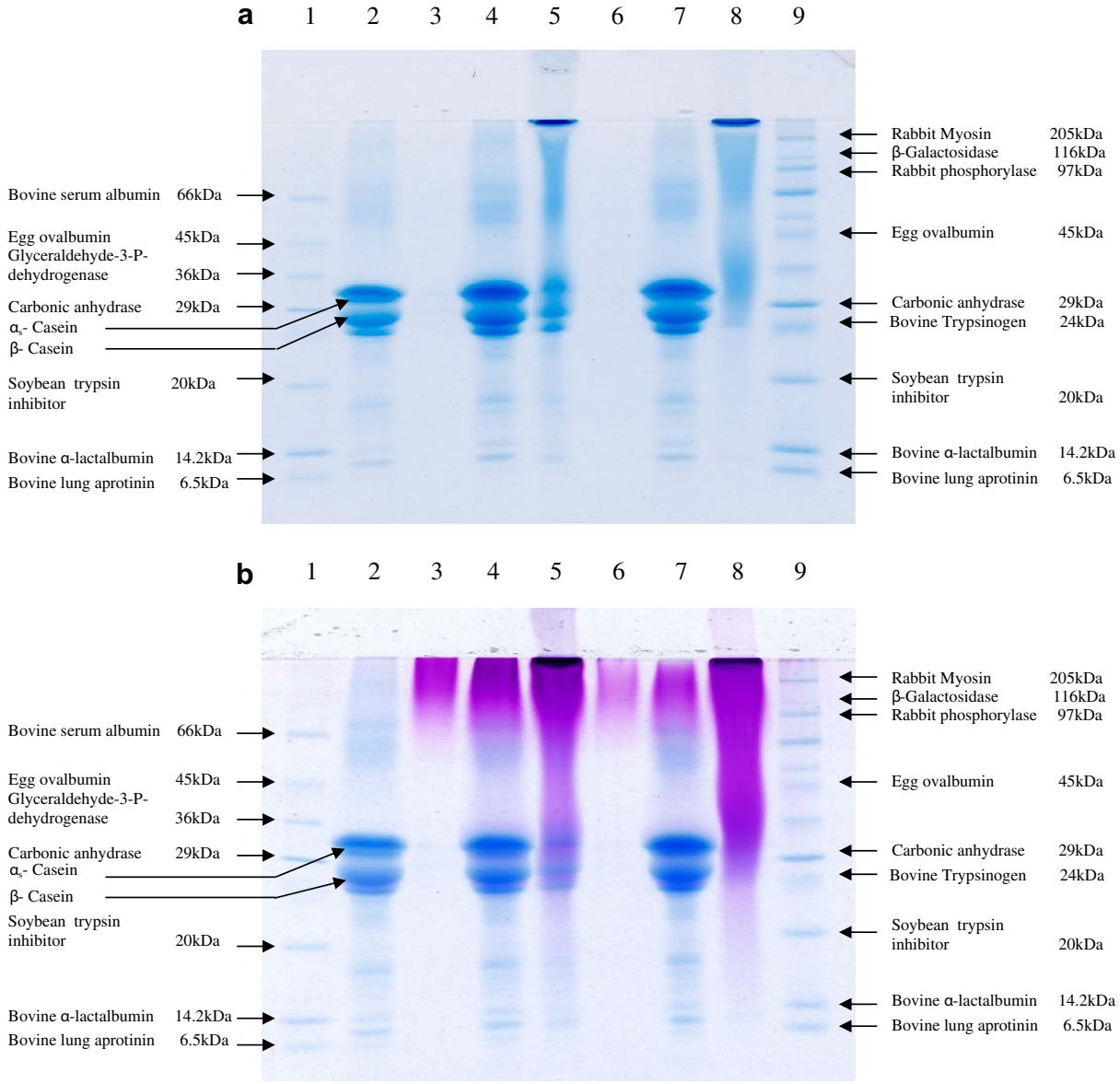


Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoretogram stained for protein with Coomassie brilliant blue R-250 (a) and stained for protein (as above) and carbohydrate with Periodic Acid/Fuschin reagent (b). The labelled lanes are (1) molecular weight marker (low range); (2) NaCN; (3) Md40; (4) NaCN–Md40 mixture; (5) NaCN–Md40 conjugate; (6) Md100; (7) NaCN–Md100 mixture; (8) NaCN–Md100 conjugate; (9) molecular weight marker (wide range).

& Mulvihill, 1997). The fat globule size distribution was measured by dynamic light scattering using a Malvern Particle Size Analyser (Mastersizer 2000S, Malvern Instruments Ltd., UK) equipped with a He–Ne laser (λ = 623). The optical parameters selected were, a dispersed phase refractive index of 1.449, a droplet absorbance of 0.001 and a continuous phase refractive index of 1.333. The results are reported as a fat globule size distribution and a mean globule size (size range measured from 0.05–878.67 μm), reported as d_{43}.

$$d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

where n_i is the number of droplets of a diameter d_i.

2.4.2.2. Conjugates as emulsifiers in model cream liqueurs. Model cream liqueurs (Table 1 outlines their composition) were prepared as follows: the liqueur base was composed of an aqueous phase and an oil phase which were prepared separately. The aqueous phase was prepared by hydrating NaCN, NaCN–Md conjugate or mixture in Milli-Q water at 50 °C under moderate magnetic stirring conditions. The oil phase was prepared by heating glycerol monostearate (GMS) at 75 °C to a molten state and mixing with soya oil, after which the mixture was cooled to 50 °C. The oil phase was then added to the aqueous phase and the mixture was pre-emulsified using an Ultra-Turrax (10,000 rpm for 15 s). An alcohol sugar base was prepared separately by mixing water, sucrose and alcohol at 40 °C under moderate magnetic stirring

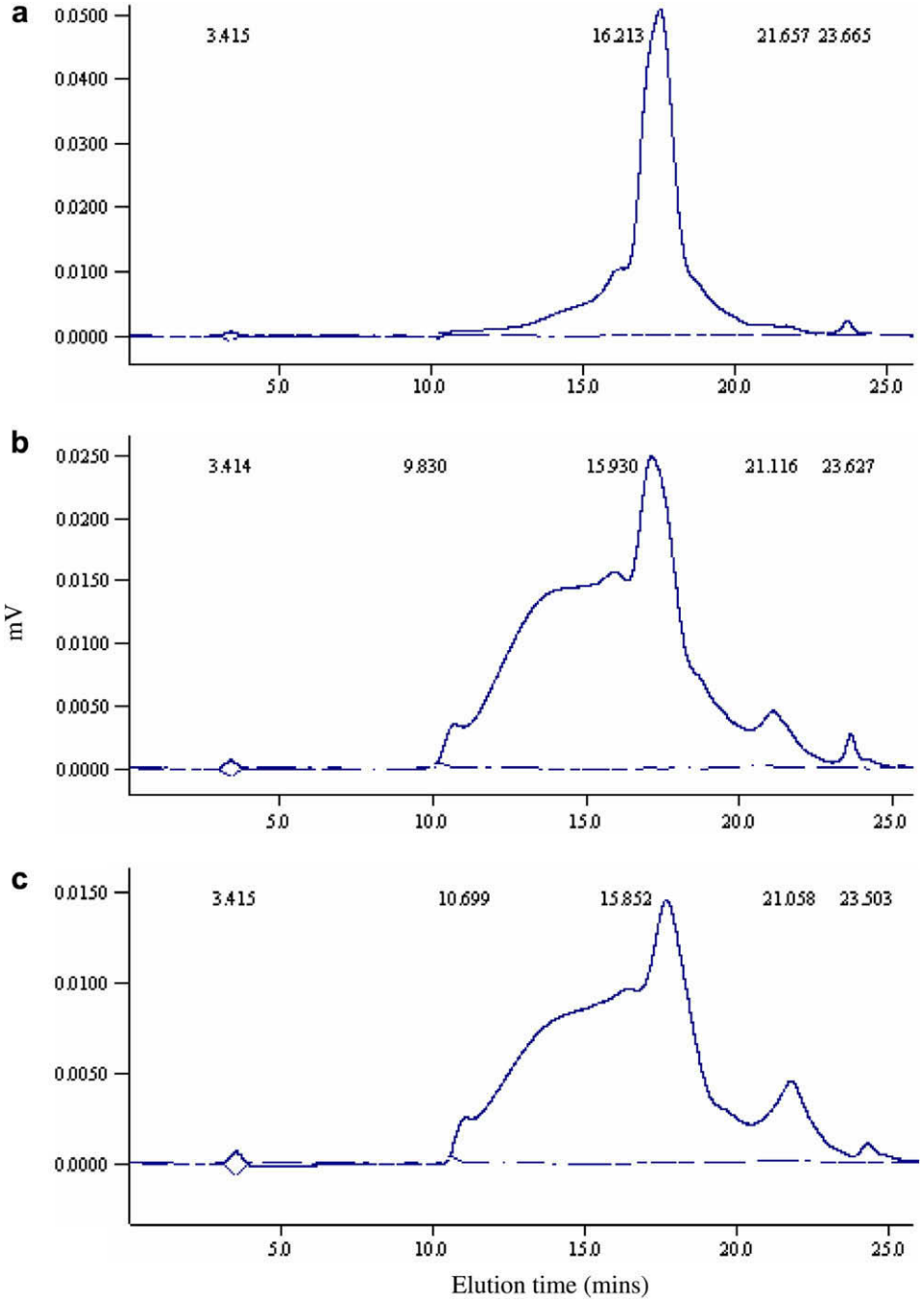


Fig. 2. Gel permeation chromatography elution profiles of (a) NaCN, (b) NaCN–Md40 conjugate and (c) NaCN–Md100 conjugate from a TSK gel G5000PW$_{XL}$ and TSK gel G4000PW$_{XL}$ column connected in series. Protein was eluted using a 4 M guanidine hydrochloride buffer, pH 6.7 and the UV absorbance of the eluate was monitored at 280 nm (see Section 2.3.4). The void and total volumes of the columns are 3.41 and 23.6 min, respectively.

conditions. This alcohol sugar base was added to the liqueur base at a ratio of approximately 0.75:1, which was mixed and pre-homogenised using an Ultra-Turrax (10,000 rpm for 15 s) and homogenised by passing the mixture through a two stage laboratory scale homogeniser (APV 1000 Homogeniser, APV AS, Albertslund, Denmark) for five passes at 50 °C at a first stage pressure of 10 MPa and a second stage pressure of 5 MPa to give a final model cream liqueur.

The fat globule size distribution of each NaCN, NaCN–Md conjugate and mixture stabilised model cream liqueur was determined on liqueur formation and following storage of the model cream liqueur under accelerated shelf life testing conditions at 45 °C for 90 days (Lynch & Mulvihill, 1997) as described above. The results are reported as a fat globule size distribution and a mean globule size, reported as d_{43}.

The viscosity of each model cream liqueur was determined in a CarriMed CSL-100 rheometer (TA Instruments, Surrey, UK). Model cream liqueur (~12 ml) was transferred to the double-concentric-cylinder measuring system and allowed to equilibrate to 20 ± 1 °C for 10 min prior to analysis. The sample was pre-sheared by increasing the shear rate from 0 to 200 s^{-1} over 75 s to establish a uniform shear history. The shear rate was maintained at 200 s^{-1} for 75 s while the viscosity data (MPas) was recorded; the mean viscosity was determined from this data.

The colour of model cream liqueurs on manufacture and following storage for up to 90 days at 45 °C was determined by pouring the model cream liqueur (~20 ml) into a petri-dish and measuring chromaticity coordinates (L^* a^* b^*) as described above. These L^*, a^* and b^* values were used to calculate colour difference (ΔE) values $[\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}]$, between the NaCN stabilised and NaCN–Md conjugate and mixture stabilised cream liqueurs on manufacture and following storage.

All experiments were carried out in triplicate and the results presented are representative of the trends observed.

3. Results and discussion

3.1. Conjugate formation and colour development

Moderate browning was observed following conjugation indicating the formation of Maillard reaction products (Table 2). Dialysis resulted in a loss of 16.8 ± 1.23 and 40.5 ± 3.55% of low molecular weight sugars and dextrans for the Md40 and Md100 respectively, prior to mixing with NaCN. Dialysis of the Mds significantly reduced colour development on conjugation as reported previously by Shepherd et al. (2000).

3.2. Conjugate characterisation

3.2.1. The effect of conjugation on the available amino groups in sodium caseinate and available reducing groups in maltodextrin

Conjugation resulted in a loss of 35.6 ± 0.7% and 36.2 ± 0.4% of the available amino groups in the protein for the NaCN–Md40 and NaCN–Md100 conjugates, respectively, and a loss of 17.8 ± 1.0% and 25.7 ± 0.4% of the available reducing groups in the Md for the NaCN–Md40 and NaCN–Md100 conjugates, respectively (Table 2).

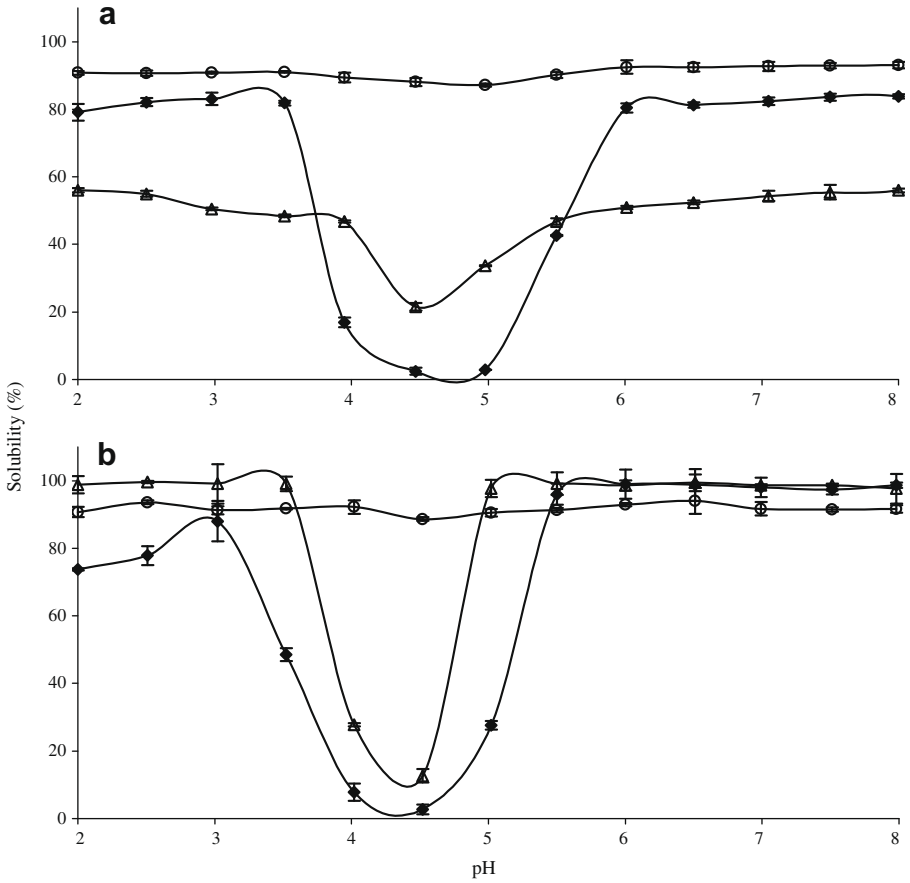


Fig. 3. The solubility of NaCN (♦), NaCN–Md40 conjugate (Δ) and NaCN–Md100 conjugate (o), at 20 °C (a) and at 50 °C (b), as a function of pH (2–8).

3.2.2. SDS–PAGE profile of conjugates

SDS–PAGE was performed to confirm conjugation and also to estimate the molecular size of the conjugates. The SDS–PAGE gel, stained for protein with Coomassie brilliant blue R-250, is shown in Fig. 1a. The typical monomeric caseins (α_s-, β- and κ-casein) were observed in the NaCN and in both NaCN–Md mixtures (lanes 2, 4 and 7). The characteristic native casein bands disappeared on conjugation, with a distinct shift to a broad range of high molecular weight conjugated proteins (lanes 5 and 8). However, not all of the native casein bands completely disappeared in the NaCN–Md40 conjugate, indicating that the reaction between the NaCN and Md40 may not have gone to completion. A portion of the conjugate also remained immobile in the loading wells of the gel, which suggests the presence of a high molecular weight conjugated protein that was too large to penetrate the separating gel.

Fig. 1b shows the SDS gel stained for polysaccharide with Periodic Acid/Fuchsin reagent. The Md40 (lane 3), Md100 (lane 6), and both of the NaCN–Md mixtures (lanes 4 and 7) appear to have remained relatively immobile and migrated close to the loading well. The Md present in the NaCN–Md conjugates (lanes 5 and 8) migrated through the gel with the protein as indicated by the formation of a purple stain, confirming that conjugation had occurred and that the Md was covalently attached to the NaCN. Covalent linkage formation upon conjugation by controlled atmosphere heating of casein/caseinate–dextran (Kato, Mifuru, Matsudomi, & Kobayashi, 1992), caseinate–maltodextrin (Morris et al., 2004; Shepherd et al., 2000) and other protein–polysaccharides mixtures (Akhtar & Dickinson, 2006; Jiménez-Castaño et al., 2005) has previously been verified by SDS–PAGE.

3.2.3. High-performance gel permeation chromatography elution profile of conjugates

To further confirm that conjugation had occurred, high-performance gel permeation chromatography of the NaCN and NaCN–Md conjugates was performed. The NaCN eluted as a main peak with an elution time of 16.2 min (Fig. 2). The NaCN–Md40 and NaCN–Md100 conjugates eluted as much broader peaks with a large amount of the protein eluting at shorter elution times (Fig. 2), as a result of an increase in the hydrodynamic diameter upon conjugation. These elution profiles also indicate that a portion of the protein was un-reacted suggesting that conjugation has not gone to completion.

3.3. Conjugate functionality

3.3.1. Conjugate solubility

It is well established that the solubility of NaCN in solution is influenced by pH; as the pH of a caseinate solution is adjusted to near the isoelectric pH of the caseins (pI ~ 4.6), the net charge on the proteins is reduced resulting in precipitation of the caseinate. The solubilities of the NaCN and NaCN–Md conjugates hydrated in Milli-Q water at 20 °C and 50 °C, as a function of pH (2–8), are shown in Fig. 3a and b, respectively. When the NaCN and NaCN–Md conjugates were hydrated in Milli-Q water at 20 °C, the NaCN–Md100 conjugate was almost completely soluble (>90%) over the entire pH range measured, including around the pI of the protein (Fig. 3a). The NaCN–Md40 conjugate had a significantly lower solubility of ~55% between pH 2–4 and 6–8, but had improved solubility in comparison to NaCN near the pI of the protein,

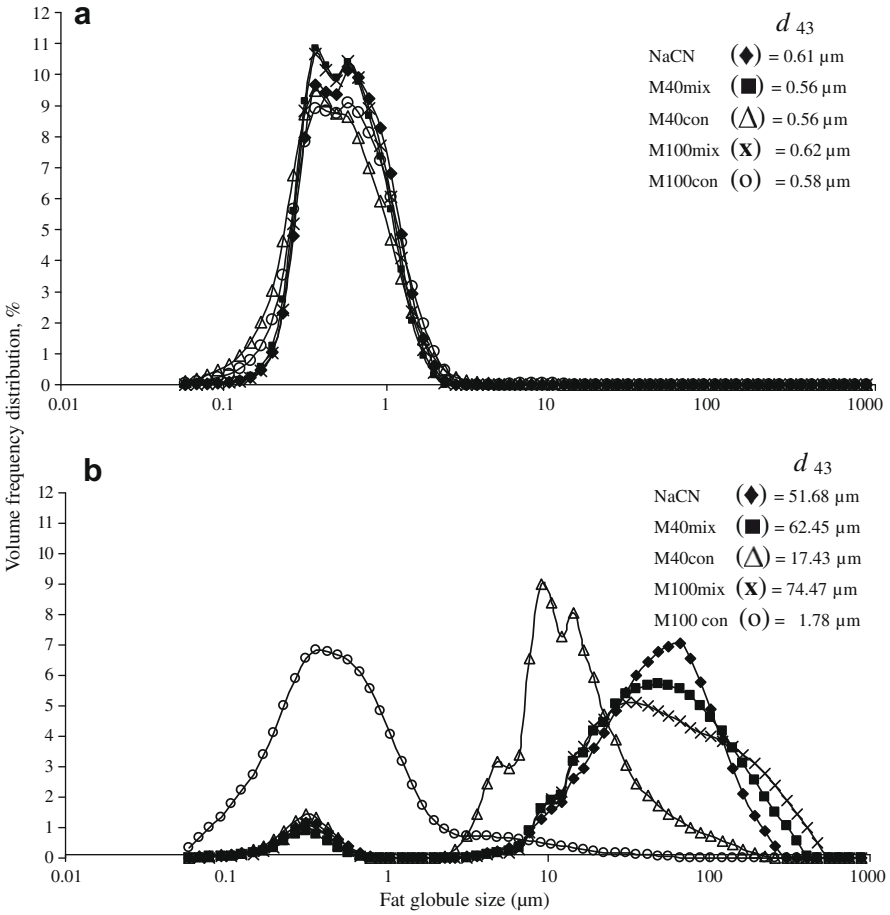


Fig. 4. Fat globule size distribution and mean fat globule size (d_{43}) of model oil-in-water emulsions stabilised by NaCN (♦); NaCN–Md40 mixture (■); NaCN–Md40 conjugate (Δ); NaCN–Md100 mixture (x); NaCN–Md100 conjugate (o) immediately after emulsification (a) and following storage at 45 °C for 20 days (b).

with a solubility of ~21.5% and ~2% observed at the pI of the protein for the NaCN–Md40 conjugate and NaCN, respectively.

When hydrated in Milli-Q water at 50 °C, the NaCN–Md100 conjugate was almost completely soluble over the entire pH range studied. The NaCN–Md40 conjugate showed improved solubility of >90% between pH 2–3.5 and pH 5–8 in comparison to the solubility of the same conjugate hydrated at 20 °C. However, at the pI of the protein the solubility of the NaCN–Md40 conjugate was reduced (~12.8%) on hydrating the conjugate at 50 °C in comparison to the same conjugate hydrated at 20 °C (~21.5%), but it still had higher solubility than the NaCN (~2%).

The results indicate that the solubility of the NaCN–Md100 conjugate was independent of pH at both temperatures suggesting that conjugation confers a protective effect against precipitation in the isoelectric region of the protein (Chevalier et al., 2001). The solubility of the NaCN–Md40 conjugate was influenced by the temperature at which the conjugate was hydrated; this is attributed to the necessity to heat an Md40 dispersion to ensure that it goes fully into solution and also the conjugation may have led to a more tightly packed network which restricts the access of the sugar residues to the aqueous medium (Oliver, Melton, & Stanley, 2006). The improvement in solubility of both conjugates

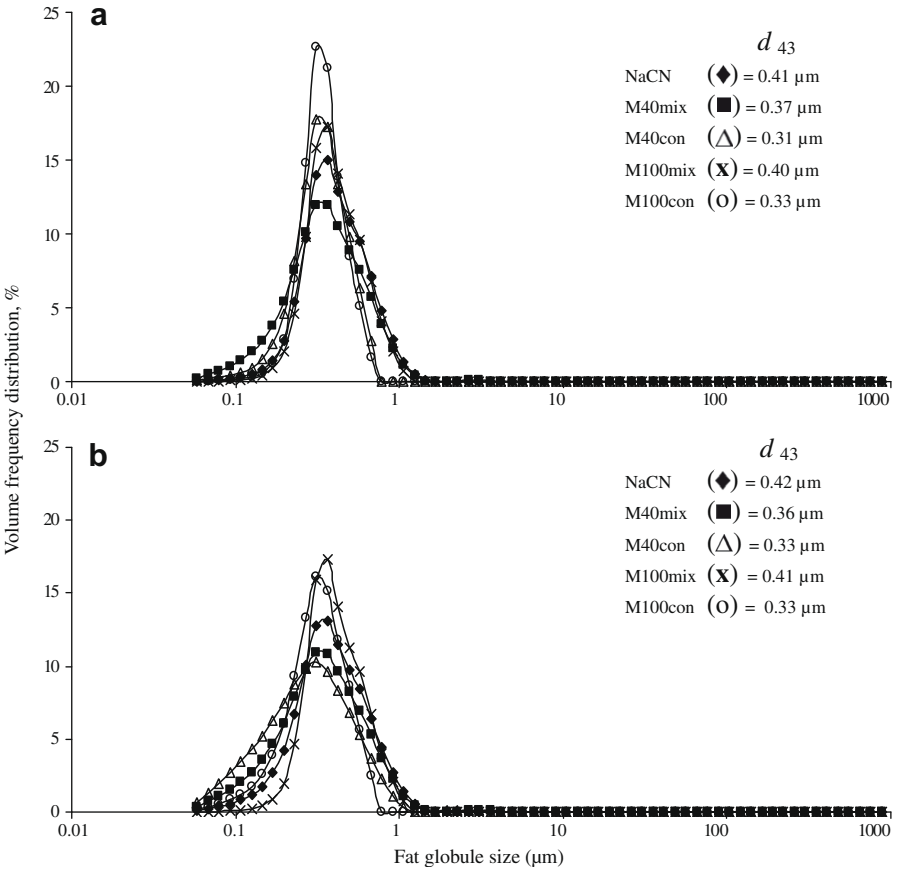


Fig. 5. Fat globule size distribution and mean fat droplet size (d_{43}) of model cream liqueurs stabilised by NaCN (♦); NaCN–Md40 mixture (■); NaCN–Md40 conjugate (Δ); NaCN–Md100 mixture (x); NaCN–Md100 conjugate (o), after manufacture (a) and after 90 days of storage at 45 °C (b).

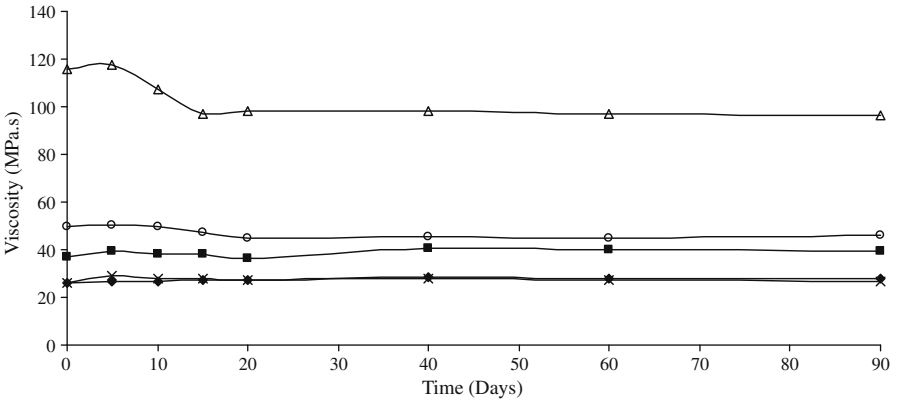


Fig. 6. The viscosity of model cream liqueurs stabilised with NaCN (♦); NaCN–Md40 mixture (■); NaCN–Md40 conjugate (Δ); NaCN–Md100 mixture (x); NaCN–Md100 conjugate (o), on the day of manufacture (day 0) and during storage of the liqueurs for 90 days at 45 °C.

when compared to NaCN near the pI of the protein corroborates the findings of Shepherd et al. (2000), and is attributed to a change in the net charge of the protein and also hydration due to the attached sugar residues.

3.3.2. Emulsifying properties

3.3.2.1. Emulsifying properties of conjugates in model o/w emulsions. The fat globule size distribution and mean globule size of model emulsions stabilised with the NaCN, NaCN–Md conjugates and mixtures were similar immediately after emulsification (Fig. 4a). After 20 days of storage at 45 °C a shift in the fat globule size distribution to larger fat globules was observed for all emulsions (Fig. 4b); the shift to larger fat globule size observed for both of the NaCN–Md conjugate stabilised emulsions was much smaller than that for the NaCN and NaCN–Md mixture stabilised emulsions. The improved stability of the conjugate stabilised emulsions in comparison to the NaCN stabilised emulsions is attributed to the conjugated protein molecule forming a bulkier polymeric layer than the non conjugated protein on the droplet surface, with the Md portion extruding outwards into the continuous phase providing better steric stabilisation, thus preventing droplet aggregation and coalescence (Akhtar & Dickinson, 2006). The NaCN–Md mixture stabilised emulsions were the least stable of the model emulsions; this may be attributed to the presence of non-absorbed Md in the continuous phase of the emulsion causing attractive forces between droplets resulting in destabilisation of the emulsions by depletion flocculation (de Kruif & Tuinier, 1994; Dickinson & Galazka, 1991).

3.3.2.2. Conjugates as emulsifiers in model cream liqueurs. One commercial application of NaCN is as an emulsifying ingredient in alcoholic cream liqueurs (Banks & Muir, 1988). On manufacture of model cream liqueurs stabilised with the NaCN, NaCN–Md conjugates or mixtures, all liqueurs had generally similar fat globule size distributions but both of the conjugate stabilised liqueurs had a slightly lower mean fat globule diameter (0.31 and 0.33 μm for NaCN–Md40 and NaCN–Md100 conjugates, respectively) than the NaCN and NaCN–Md mixture stabilised cream liqueurs (ranging

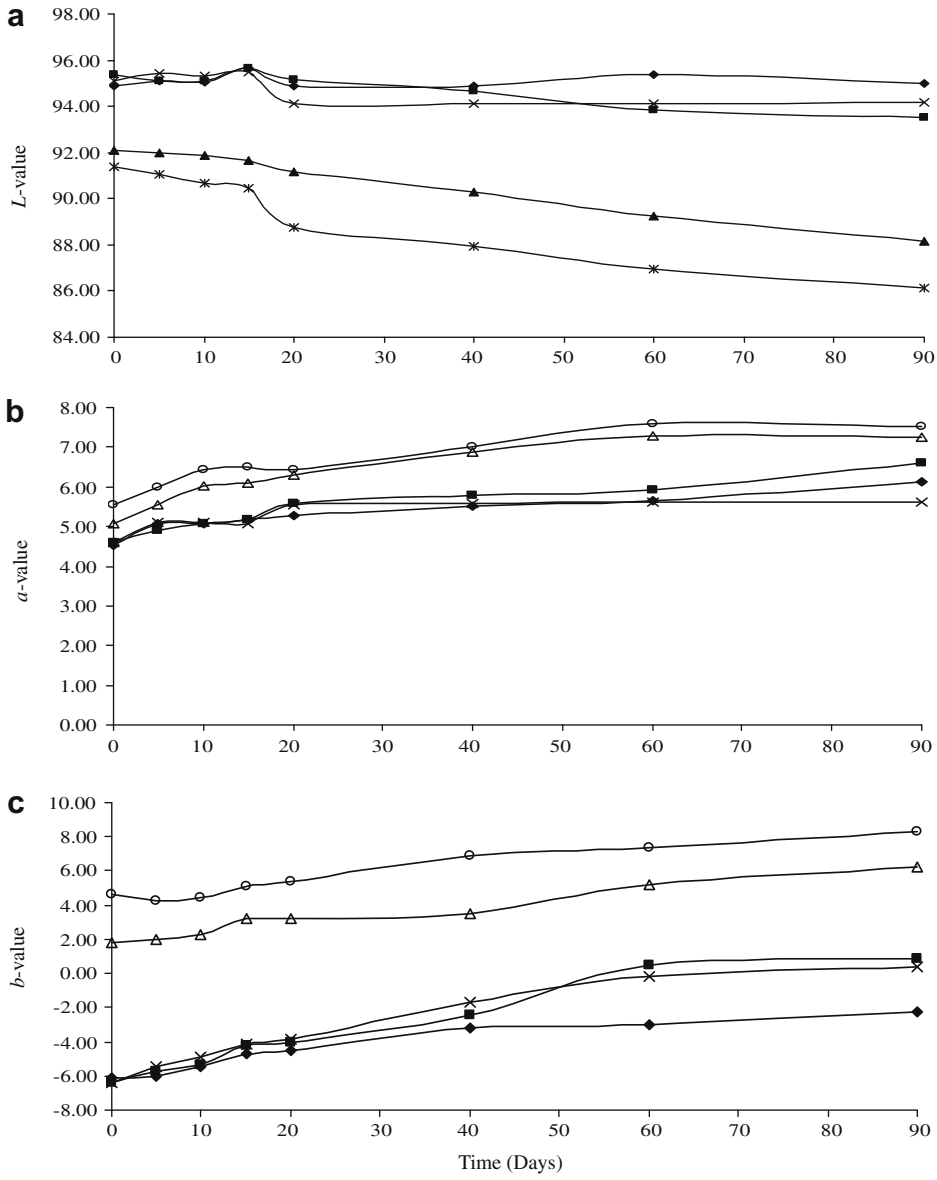


Fig. 7. Chromaticity coordinates (L^* a^* and b^* values) of model cream liqueurs stabilised with NaCN (♦); NaCN–Md40 mixture (■); NaCN–Md40 conjugate (Δ); NaCN–Md100 mixture (x); NaCN–Md100 conjugate (o), on manufacture (day 0) and on storage for 90 days at 45 °C.

from 0.37 to 0.41 μm) (Fig. 5). After 90 days of storage at 45 °C, minimal changes were observed in the fat globule size distribution for all of the cream liqueurs, as the mean fat globule size remained in the range of 0.33–0.41 μm; this demonstrates that NaCN–Md conjugates are capable of maintaining the stability of model cream liqueur emulsions.

The viscosity of an emulsion is an important characteristic since it influences the rate of creaming, the physical shelf life of the product and the organoleptic properties of the product (McClements, 1999). NaCN–Md conjugate stabilised cream liqueurs had higher viscosities on formation, when compared to the NaCN and NaCN–Md mixture stabilised liqueurs (Fig. 6). The NaCN–Md40 conjugate and mixture stabilised liqueurs had higher viscosities than the NaCN–Md100 conjugate and mixture stabilised liqueurs possibly due to Md40 containing longer oligosaccharide chains than Md100. During the 90 days of storage under accelerated shelf life testing conditions, there were minimal changes in viscosity in each of the cream liqueurs. The results suggest that the high viscosity of conjugate stabilised liqueurs was primarily due to the conjugated protein molecule forming a bulky polymeric layer on the droplet surface. This high viscosity may also hinder propensity to creaming and gravitational induced flocculation during storage (Dickinson, Golding, & Povey, 1997).

NaCN and NaCN–Md mixture stabilised liqueurs had a similar colour (L^*, a^* and b^* values) on model cream liqueur manufacture (Fig. 7a–c); the NaCN–Md40 and NaCN–Md100 mixture stabilised liqueurs had ΔE values of 1.3 and 1.1, respectively, in comparison with the NaCN stabilised liqueurs, which showed that the differences in colour on manufacture were barely discernable (Hutchings, 1994). Both the NaCN–Md40 and the NaCN–Md100 conjugate stabilised liqueurs, had lower L^* values and higher a^* and b^* values in comparison with the NaCN and NaCN–Md mixture stabilised liqueurs, on manufacture, and had ΔE values of 5.2 and 11.3, respectively, when compared to the NaCN stabilised liqueurs, indicating that the difference in colour on liqueur manufacture could be visually perceived (Hutchings, 1994). This can be attributed to the colour that developed during the preparation of the conjugates (Table 1), which in turn directly influenced the colour of the conjugate stabilised liqueurs.

On storage of the liqueurs for 90 days at 45 °C, colour changes were evident for each of the liqueurs. The ΔE value for the NaCN stabilised liqueur was 4.2 and the change in colour was visually perceptible; the ΔE values for the NaCN–Md40 and NaCN–Md100 mixture stabilised liqueur were 7.5 and 6.8, respectively, and the ΔE value for NaCN–Md40 and NaCN–Md100 conjugate stabilised liqueur were 6.3 and 6.7, respectively. While these results show a larger change in colour on storage of both NaCN–Md conjugate stabilised cream liqueurs in comparison with the NaCN stabilised cream liqueur, the change in colour observed was not considered extensive enough to be perceived as a defect. It is generally known that various flavourings and colourings such as caramel, chocolate and coffee are used in the manufacture of cream liqueurs. The enhanced colouring of the conjugate stabilised liqueurs may present an opportunity to produce liqueurs of appropriate colour using conjugates as the stabilising protein and using a lower concentration of added colourant to achieve the requisite cream liqueur colour.

Although most of the data concerning the composition and concentration of ingredients in cream liqueurs is propriety, it has been suggested that maltodextrins have been added to improve mouthfeel in cream liqueurs (Rule, 1981). Production of liqueurs with NaCN–Md conjugates could be cost effective as the conjugated protein could act to effectively stabilise the fat droplets, while any non conjugated maltodextrin could contribute to the desired mouthfeel of the liqueur.

4. Conclusion

NaCN–Md conjugates of a broad molecular weight distribution were produced *via* a non-toxic, naturally occurring Maillard-type reaction. In comparison to NaCN, the NaCN–Md conjugates had improved solubility, particularly around the isoelectric pH of the protein. NaCN–Md conjugate stabilised o/w emulsions showed improved stability in comparison to NaCN stabilised emulsions after storage for 20 days under accelerated shelf life testing conditions. NaCN–Md conjugates were found to be suitable emulsifiers for stabilising model cream liqueurs; on manufacture, liqueurs stabilised by the NaCN–Md conjugates had slightly lower mean fat globule diameters than NaCN stabilised liqueurs. After 90 days of storage at 45 °C, the NaCN–Md conjugate stabilised liqueurs maintained their stability.

Overall, the minimal colour development during their preparation and their improved solubility, particularly around the isoelectric pH of the protein, and their improved emulsifying properties in o/w emulsions and model cream liqueurs, indicates a potential for NaCN–Md conjugates as speciality functional food ingredients.

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