INTENSIFICATION OF MINOR MILK PROTEIN PURIFICATION PROCESSES

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ABSTRACT

The performance of a robotic system that extracts proteins directly from the milk of individual cows on-farm is described. Batch extraction of proteins is demonstrated and extraction yields modelled, indicating that on-farm extraction can significantly outperform centralised, large-scale processing.

INTRODUCTION

Production of purified proteins from whey, originally a means of extracting value from waste streams, has developed to the point where such products generate significant revenues for the dairy industry. Two whey protein products in particular, lactoferrin (LF) and lactoperoxidase (LP), illustrate the potential for commercial exploitation of minor, bioactive milk protein products (1-3). Other proteins, such as immunoglobulins, may be targeted in the future.

Established practice in bioseparation process design seeks to maximise the yield and activity of biological products by minimising the number of separation steps involved (4, 5). However, due to the commodity nature of the dairy industry, where economies of scale are significant, processing is normally carried out in large, centralised factories, and milk typically undergoes many process steps before minor proteins are extracted. Processes that involve shear, high temperatures and prolonged storage, such as pumping, cream separation, pasteurisation and vat storage may degrade proteins. For example, high-temperature processing disrupts non-covalent bonds in macromolecules such as proteins and polysaccharides causing denaturation and gelling of milk components (6).

Pasteurization reduces solubility of casein, decreases chemically-available lysine in whey protein and causes a 56% denaturation of whey protein in the skim milk (7). Acid precipitation of caseins can result in the 4-8 times more LF entrapped in the casein pellet than in the whey fraction (8).

The concentrations of minor milk proteins vary considerably between individual animals, and are affected by diet, milking frequency, stage of lactation, time of the season, stress and genetics (9-13). It is possible, through selective breeding and other farm management practices, to create a herd of animals that produce milk with a higher than average content of a specific protein, such as LF, but the benefits of doing so are largely lost by large-scale pooling of milk prior to centralised production since the pool reflects the average (diluted) concentration from the distributed herd. Also, there is no financial incentive to individual farmers to implement practices to increase the concentration of individual minor proteins if they are paid only on the basis of gross metrics such as milk volume and total solids.

A new paradigm in dairy processing is made possible by modification of Automated Milking Systems (AMS) to direct the milk from each cow to particular streams for processing. Milk containing high concentrations of specific proteins can then be targeted for extraction prior to entering the storage vat, while milk containing little or none of the protein of interest can be passed directly to the vat. Extraction processes should be more efficient in this case because the milk volume associated with low-producing animals need not be processed, while the higher concentrations in the milk targeted for extraction would likely increase yields.

LF production provides a simple example of how process intensification of this kind could add value to the farmer. Milk from high-LF producing cows might have LF concentrations of about 1 g/L (14). At an average volume per milking of 15 L the total LF content would have a wholesale market value of about NZ$7.00 (US$300/kg) (15, 16). This compares well with the NZ$5.00 approximate value of milk solids in the same volume of milk (17). Clearly a farmer able to harvest consistently high levels of LF without reducing the value of the residual milk has the potential to increase revenue significantly.

In this paper, we present details of parameters used to model the batch adsorption process, using the composite nonlinear (CNL) model of Rowe *et al* (18). For a given chromatography media, assuming good mixing, the adsorption rate of a specific protein depends on a number of variables, principally temperature, initial protein concentration and the volume ratio of adsorptive media to the milk. Since temperature is effectively fixed at or near the temperature of milk expression, the latter two variables are critical in predicting protein adsorption yields.

The CNL model for batch ion exchange adsorption of proteins is shown in equation (1), where q is the solid-phase adsorbed protein concentration per mL of resin (mg/mL); q_k is a kinetic parameter (mg/mL); C is the solution-phase protein concentration (mg/mL); C_o the starting solution-phase concentration (mg/mL); k is a rate constant (min^{-1}) and $y(0)$ is the zero-time intercept when the term on the left of Equation (1) is plotted against time, t (min). The time constant, a (min^{-1}), accounts for the deviation from straight line behaviour at small times.

$$\ln\left(1 - {q}/{q_k}\right)\ \ln\left({C}/{C_o}\right) = k\,t + y(0)\left[1 - e^{(-a.t)}\right] \qquad (1)$$

As suggested by Rowe *et al*, variations of fitted parameters *a, q_k, y(0)* and *k* over the range of operating conditions encountered in practice were examined with the objective of determining predictive methods for their estimation. In this way, we sought to predict the rate of protein adsorption from initial conditions and from this, to estimate the amount bound after a pre-determined time of adsorption.

The authors have previously described an automated protein fractionation robot (PFR) that is capable of extracting proteins directly from raw, whole milk (Fee & Chand) (19, 20). The prototype PFR currently uses cation exchange chromatography via a batch contacting method to bind LF and LP without affecting the gross milk composition, and is able to maintain traceability of protein product directly back to individual animals.

EXPERIMENTAL METHODS

Adsorption rates of LF and LP were determined in the laboratory as previously described (19). Briefly, known amounts of chromatography resin were contacted with milk at 35 ^{o}C under gentle stirring and samples of the milk were removed at timed intervals to determine the residual protein concentration in solution. Adsorbed protein was then calculated by difference between residual and initial concentrations.

A Protein Fractionation Robot (PFR) prototype (20) was used for on-farm capture of LF and LP. When a signal from the AMS was received, a cassette containing 250 mL of SP Sepharose Big Beads (GE Healthcare Technologies, Uppsala, Sweden) was picked up from a stack held in a refrigerator (4^{o}C) by pneumatic rams and delivered to the load position. Once milking was completed, the AMS sent a signal to the PFR and a reversible pump (Fristam Pumps Inc., Middleton, Wisconsin, U.S.A.) pumped the milk into the receiver can with an upward flow which suspended the resin with the milk for adsorption. Stirring was applied for 10 minutes at a constant rate (150 rpm). After 10 minutes, the pump drained the milk (in reverse direction from which it was initially pumped), assisted by gravity, hence retaining the resin on the 44 μm sieve of the cassette. The resin was rinsed twice with warm water (40-50^{o}C) and drained. The cassette was then transferred automatically to the stacking position in the fridge to await manual elution of proteins. Cow identifications, volume produced, pH and conductivity were noted and samples were taken for LF, LP, milk composition and protein analyses. Processing temperature after completion of milking and after adsorption were noted.

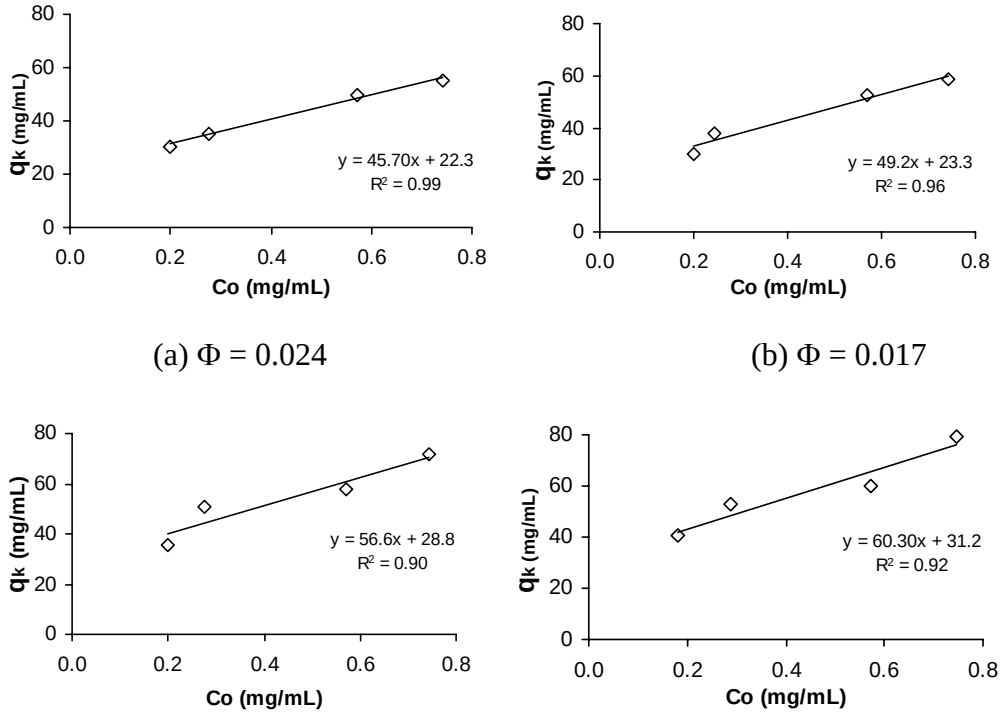
The amount of protein adsorbed after 10 minutes was determined indirectly by measuring the amount obtained after elution. LF and LP were eluted consecutively by washing the resin with 1 litre of 0.4 M NaCl followed by 1.5 litre of 1.0 M NaCl. In all experiments, LP was analyzed using an activity assay using 2,2'-azinobis[3-ethyl-benzothiazoline-6-

sulphonic] diammonium salt (ABTS) substrate and LF was analyzed using the surface plasmon resonance (SPR) assay of Indyk & Filzoni (2005) (14) with minor modifications (19, 20). Yields of LF and LP were calculated. Values for *a*, q_k, *y(0)* and *k* were determined**,** following the procedure of Rowe *et al* (18), by fitting the CNL model to laboratory adsorption rate experimental data for a range of initial LF concentrations and resin to milk volume ratios, Φ.

RESULTS AND DISCUSSION

Kinetic capacity, q_k

The effect of initial protein concentration on q_k was shown to be linear for all values of Φ (Figures 1a-d).



(a) Φ = 0.024 (b) Φ = 0.017

(c) Φ = 0.012 (d) Φ = 0.010

Figure 1: The effects of Φ and C_o on q_k.

Values for the intercept and gradient in Figures 1 (a) – (d) were plotted against the corresponding values of Φ (Figure 2), showing that both are linearly related to Φ. q_k is therefore found to be a function of *Co* and Φ, as given by equation (2):

$$q_k = (66.1 - 882\Phi)\ Co\ +\ (33.9 - 516\Phi) \qquad (2)$$

Rowe *et al* (18) expressed uncertainty as to the physical meaning of q_k, which appears to be unrelated to the maximum equilibrium capacity of the resin. However, the form of equation (2) is consistent with the behaviour expected for batch adsorption in the concentration-dependent portion of the equilibrium curve. In this region, the specific equilibrium capacity is affected both by initial concentration of protein and by the amount of resin present. To determine the amount of material bound at equilibrium from a known starting concentration, solution volume and amount of adsorbent, the intersection point between a line determined by mass balance and the equilibrium curve is found (21). The equilibrium capacity of the resin for LF follows the Langmuir isotherm overall (19) but for the LF concentrations typically found in milk (<1 g/L), capacity is highly dependent on solution concentration. For a process where the resin initially contains no bound material, a mass balance on LF shows that at equilibrium

$$q^* = \frac{\left(C_o - C^*\right)}{\Phi} \qquad (3)$$

; where the * denotes the equilibrium value. Therefore we expect the y-intercept of the mass balance line to increase with C_o and both the y-intercept and the slope of the mass balance line to decrease with Φ. The specific equilibrium capacity of the resin under the operating conditions therefore also increases with C_o and decreases with Φ. Thus q_k is likely related to the specific equilibrium capacity of the resin that, together with the initial LF concentration in solution determines the driving force for adsorption. We have not attempted to determine a relationship between q_k and q^* but this may a worthwhile objective in the future.

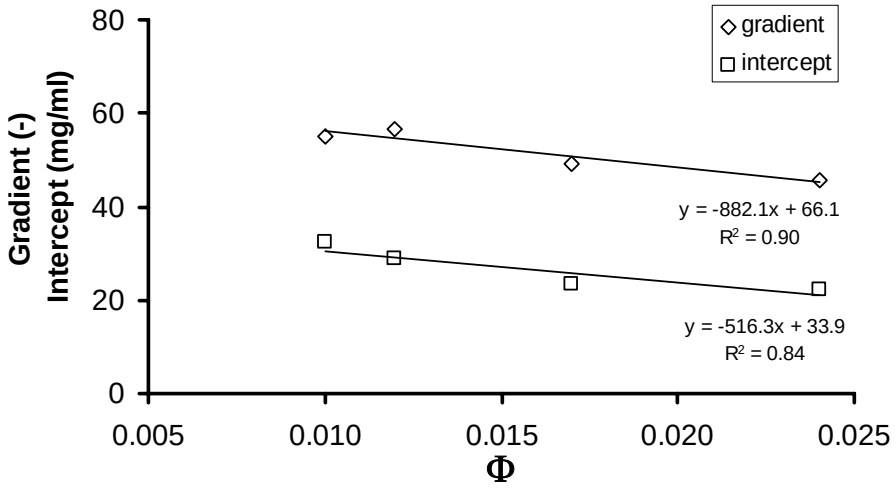
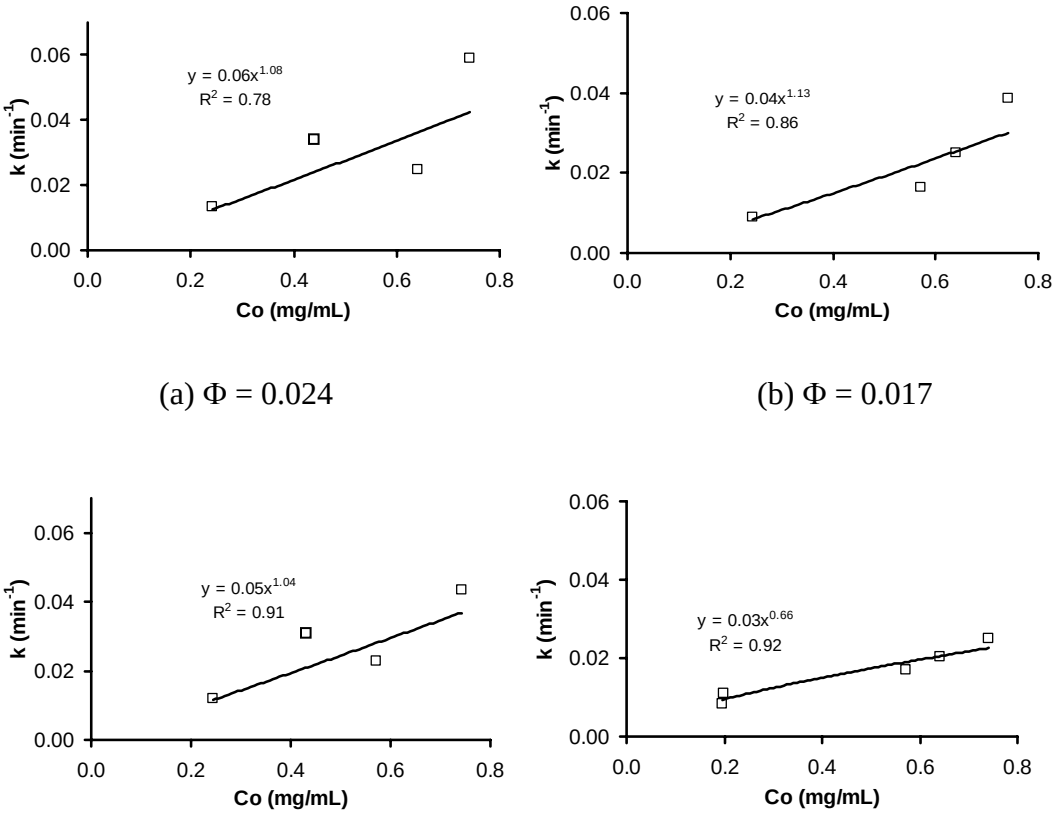


Figure 2: Gradient and intercept derived from Figure 2.

Rate constant, *k*

For all Φ values investigated there was a positive power law relationship between the rate constant, *k*, and *Co* (Figure 3).



(a) Φ = 0.024 (b) Φ = 0.017

(c) Φ = 0.012 (d) Φ = 0.010

Figure 3: The effect of Φ and C_o on the value of *k*.

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At each value of Φ the exponent of the power law function is close to unity, so *k* is approximately linearly related to C_o and the constant of proportionality was found to be a linear function of Φ (Equation 4).

$$k = (2.01\,\Phi + 0.013)\,Co \qquad (4)$$

The function shown in equation (4) is in line with expectations, since the rate of adsorption (given adequate mixing) will be related to the difference between actual and equilibrium solution concentration. Increasing both C_o and Φ both increase this difference, the latter by decreasing the specific equilibrium solution concentration. Therefore *k* increases with both C_0 and Φ.

Time Constant, *a*

Values for the time constant, *a*, were generally small (0.12 to 0.30) with no significant relationship between *a* and either C_o or Φ (Figure 4).

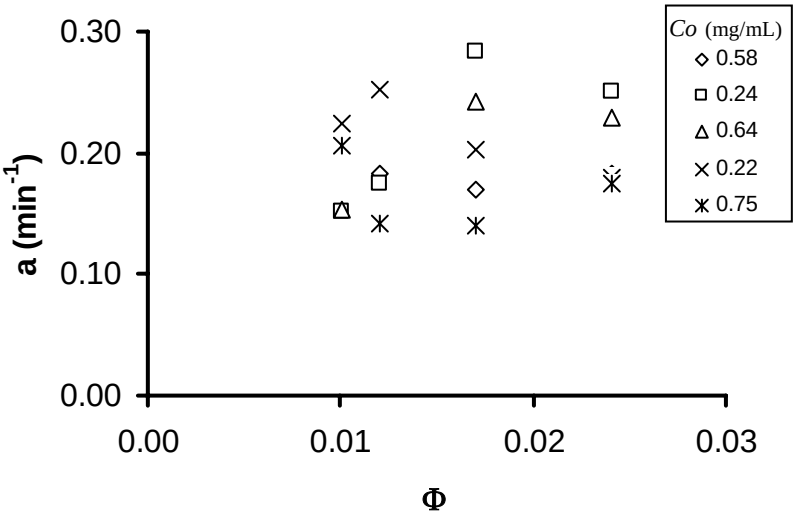

Figure 4: Effect of Φ and C_o on *a*.

The fit between the CNL model and experimental data was not affected over the range $0.18<a<0.22$ min $^{-1}$ so *a* was approximated as 0.2 min^{-1}. This was slightly lower than the range 0.3 - 0.8 min^{-1} obtained by Rowe *et al*. (18) for anion exchange of BSA at concentrations up to 3 mg/mL. They used a second order equation to describe the effect of Φ on *a*, whereas data obtained in the current experiments for cation exchange of LF can be approximated as a single value. Raw whole milk has LF concentrations between 0.07 and 1.0 mg/mL, significantly lower than the protein concentrations use by Rowe *et al*.

Linear Fit Intercept, *y(0)*

Rowe *et al*. (18) found a relationship between *y(0)* and *k/a*. However, scatter plots for the current data showed no such relationship (data not shown), with *y(0)* varying only between -0.01 and -0.07. This may be because a much smaller range (0.1 to 1.0 mg/mL) of C_o values were used in the current studies. The value *y(0)* = -0.023 worked consistently when the value of *a* was set at 0.2 min^{-1}.

Overall Adsorption Kinetics

Using the relationships found above, the overall kinetics of LF adsorption are given by equation (5).

$$\ln\left[1-\frac{q}{q_k}\right] \ln\left[\frac{C}{C_0}\right] = \quad kt \; -0.0234\left[1-e^{-0.2t}\right] \qquad (5)$$

Equation (5), together with equations (2) and (4), allows prediction of LF adsorption versus time, if C_o and Φ are known. The second term on the right hand side of equation (5) is small compared to the magnitude of *kt*. Thus variations in the values of *a* and *y(0)* have relatively little effect on the model predictions.

There was good agreement between predicted values for LF adsorption with time for Φ=0.017 (corresponding to the average volume of milk produced by an individual cow and 250 mL of resin) and experimental data (Figure 5). Similar agreement was found with other values of Φ (data not shown).

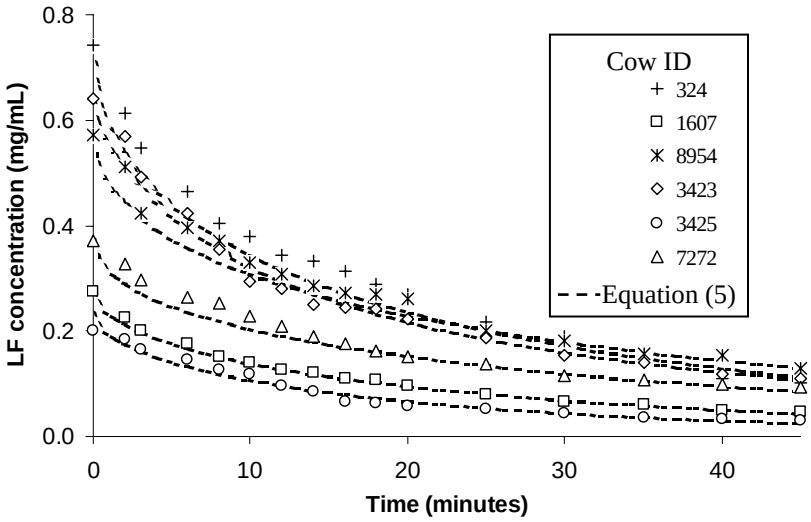

Figure 5: Comparison of experimental and simulated data (Equation (5)) for laboratory-scale experiments with Φ=0.017.

On-Farm Yields Prediction

Equation (5) was used to predict the amount of LF adsorbed on-farm by the PFR after a 10-minute adsorption time. The value of Φ is easily calculated at the beginning of adsorption from the volume of milk (measured on-line) and the resin volume in the cassette. However, currently no method exists to measure C_o on-line. Therefore samples of the feed milk were taken from the AMS and values of C_o measured by SPR were used later in calculations. If a method of determining C_o within the time-frame of milking were available, C_o and Φ could together be used to control the adsorption time to attain a desired extraction percentage. In the absence of such an assay, historical data could instead be used to predict the likely LF concentration in individual cows, identified at the time of milking by radio-frequency identification tags.

Table 1 shows the predicted versus measured adsorption, the latter being estimated by the yield after elution from the resin.

Table 1: Experimental (on-farm) and predicted (Equation (5)) adsorption of LF after 10 minutes of contacting.

Cow Identification	C_o (mg LF /mL)	% Adsorption (predicted)	% Adsorption (experimental)	% Difference
3532	0.254	58.7	55.2	6.0
3022	0.373	60.1	46.0	23.5
9570	0.157	48.9	48.9	0.1
3109	0.374	46.8	25.6	45.3*
1922	0.239	41.2	27.6	33.0
1922	0.408	46.1	47.8	3.8
3402	0.392	44.6	23.4	47.5*
1607	0.330	40.6	36.1	11.1
2409	0.078	33.9	36.7	8.1
9564	0.240	36.8	43.5	18.1
694	0.145	32.5	35.3	8.7
5710	0.333	39.6	29.5	25.6
480	0.478	40.4	34.9	13.7
1401	0.260	34.0	28.5	16.2
7656	0.101	26.7	19.7	26.1
Average difference between predicted and experimental values** (%)				14.1

* Unusually high fat content

** Excluding Cow 3109 and 3402 data

The data in Table 1 show that values predicted by equation (5) were, on average, 14.1% higher than experimental values. However, experimental yields were measured after elution, whereas equation (5) predicts amount adsorbed. Therefore product loss during elution may account for the lower observed values.

We did not seek to optimise the process by selecting only milk from cows with high LF productivity for processing. Even so, we obtained a LF yield of approximately 11.6 g/100 L of milk, which is 16% higher than that reported for an industrial (centralised) LF extraction process (22). Selecting only high-producing cows for on-farm extraction would have a dramatic, positive impact on protein yield. Equation (5) predicts that 57% of LF could be extracted in 10 minutes from a cow having C_o = 1000 mg/L and producing an average milk volume (15 L), corresponding to 8.6 g of LF product per milking. Such an intensified process would compare very well with the nominal 1.5 g that might be extracted per 15 L in a centralised factory (22). Using a resin with faster uptake rates might improve the process still further. In laboratory trials using Sepharose FF SP™ resin (GE Healthcare Technologies, Uppsala, Sweden), we adsorbed up to 75% of LF in

10 minutes (data not shown) from randomly selected milk (23), suggesting that over 75 g LF/100 L of milk might be possible from high LF content milk.

Furthermore, LF extracted in a centralised factory would have passed through many potentially damaging processes and holding steps prior to capture, likely resulting in loss of bioactivity. Potential differences in bioactivity between LF extracted on-farm and that extracted in centralised processing sites have not been addressed in this study but any differences may affect market value for the respective products.

An important factor, also not accounted for in the current study, is the effect of fat content in raw, whole milk on adsorption rates and/or capacity. We did observe that unusually low protein adsorption occurred when high levels of fat were present in milk and that this was often accompanied by excessive foaming of the milk during adsorption. The milk from cows 3109 and 3402 (Table 1) were in this category, so were excluded when calculating the average differences between predicted and experimental yields. The effect of fat content on the parameters of the CNL model has yet to be determined. However, we found that resin cleaned according to the manufacturers instructions for high-fat applications (including an isopropanol wash step) did not lose adsorptive capacity over 50 cycles. Therefore the effect of milk fat on adsorption, if any, appears to be fully reversible by cleaning.

CONCLUSIONS

An intensified process, on-farm extraction of minor milk proteins directly from the raw, whole milk of individual cows by batch, cation exchange chromatography, has been demonstrated. The extraction yields can be modelled by the composite nonlinear model of Rowe *et al* (18). Fitted parameters of the CNL model were shown to be either constant or functions of initial target protein concentration and resin:milk volume ratio across the range of conditions expected in practice. Process intensification through extraction of proteins immediately after milking can significantly improve yields compared to large, centralised processing. On-farm extraction also has potential to provide direct financial incentives to farmers who implement management practices (including selective breeding) to increase the content of commercially valuable minor proteins from their herd.

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NOTATION

a time constant, min^{-1}

C_o initial liquid-phase LF concentration, mg/mL

C instantaneous liquid-phase LF concentration, mg/mL

C^* equilibrium liquid-phase LF concentration, mg/mL

Φ solid-phase to liquid-phase volume ratio, -

k rate constant, min^{-1}

q instantaneous solid-phase LF concentration, mg/mL of resin

q_k kinetic capacity parameter in CNL model, mg/mL

q^* equilibrium solid-phase LF concentration, mg/mL of resin

t time, min

$y(0)$ y-intercept at zero time of linear portion of CNL curve, -

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KEYWORDS

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