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Adaption and Optimisation of the Ferric-nitrilotriacetate Spectrophotometric Method for Quantification of Lactoferrin in Raw Milk

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Abstract:

This study aimed at adapting and optimising the Fe^{3+}-nitrilotriacetate (Fe^{3+}-NTA) spectrophotometric method for determining lactoferrin (LF) content in raw milk and milk fractions. Isolation of LF from acid whey was achieved by using cellulose phosphate resin. LF was quantified from the standard curve based on spectrophotometric reading at 465nm. The purity of LF isolates was established based on lactoperoxidase (LP) activity. The adopted method indicated under estimation of LF content. However, this was modified by incorporation of $NaHCO_3$ and Fe^{3+}-NTA in both the blank for the standard and test samples, which improved the performance of the method. Results generally indicated that modified Fe^{3+}-nitrilotriacetate (Fe^{3+}-NTA) method is a simple, quick and reproducible method that can give a better quantitative estimation of LF. A batch procedure using cellulose phosphate resin was highly effective for the isolation and purification of LF as demonstrated with less contamination of LP.

Keywords: *Lactoferrine, optimising, spectrophotometric, isolation, estimation*

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1 Introduction

Lactoferrin (LF) is a multiple functional whey protein with exceptional properties. It is a glycoprotein of the transferrin family which due to its multifunctional properties is useful for clinical and commercial application (Wahyu *et al.,* 2017). Lactoferrin is regarded as a part of the inborn immune system that plays a vital role in averting bacterial growth, typically via an iron sequestration mechanism (Maika *et al.,* 2013). According to (Niaz *et al.,* 2019) lactoferrin functions as an alternative to antibiotics and acts as a natural antimicrobial for bio preservation of a range of food products, prolonging shelf life, guaranteeing safety and improving health by acting against dangerous diseases like cancer, hepatitis, respiratory infections, and foodborne diseases in humans (Niaz *et al.,* 2019). In view of its importance several studies (Faraji *et al.,* 2017; Wahyu *et al.,* 2017) have been conducted to isolate and purify it from milk whey. Whey, the liquid remnant from dairy processing, is one of the largest reservoirs of food protein available today (Faraji *et al.,* 2017). On the other hand several methods are available for quantifying LF. According to (Pochet *et al.,* 2017) the determination of lactoferrin in dairy products is mostly achieved using immunological methods (ELISA, radio-immunodiffusion and optical biosensing). Radial immunodiffusion technique has been used by several researchers (Welty *et al.,* 1975; Law and Reiter, 1977; Elliot *et al.,* 1984; Tsuji *et al.,* 1990) to quantify LF. Other researchers (Tsakali *et al.,* 2019) used the Reverse-Phase High Pressure Liquid Chromatography (RP-HPLC) method, for the determination of lactoferrin. Furthermore, Majka *et al.* (2013) developed an integrative method for determination of lactoferrin involving several techniques like spectrophotometry, ELISA and inductively coupled plasma–mass spectrometry (ICP-MS). However, according to Pochet *et al.* (2017) these methods (e.g. ELISA, radio-immunodiffusion and optical biosensing) are species-specific and considered to be sensitive (nanogram (ng) on a diluted sample basis) but are expensive and reproducibility is average (Pochet *et al.,* 2017). In this regard this study aimed at adapting and optimising the Fe^{3+}-nitrilotriacetate (Fe^{3+}-NTA) spectrophotometric method of Foley and Bates (1987) because of its simplicity. LF is capable of binding two Fe^{3+}per molecule with bicarbonate taken up per Fe^{3+}. This makes possible the quantification of LF as Fe^{3+}-LF-CO_3^{2-}complex which has a maximum visible absorbance at 465 nm. This part of the study aims at obtaining a simple, accurate and reliable assay procedure for the quantification of LF.

2. Materials and Methods

2.1 Preparation of acid whey

Raw skimmed milk was acidified using 0.5 M HCl (Sigma-Aldrich Chema Gmbh, Germany) to a pH value of 4.6; the resulting whey was separated from the curd by a muslin cloth, centrifuged using a Centaur 2 centrifuge (MSE, UK) at 604 x g for 30 min and then filtered through Whatman filter paper no. 4 (Sigma-Aldrich Chema Gmbh, Germany).

2.2 Isolation of LF from acid whey

Cellulose phosphate resin (3.8 meq/dry weight, sigma Chemical) was essentially prepared as described by Groves (1965) and the LF isolation procedure was basically that of Foley and Bates (1987). Twenty grams of resin was washed on a Buchner funnel (Haldenwanger™ Porcelain Buchner Funnel Fisher Scientific UK Ltd (Leicestershire, UK) with 250 ml 0.1N NaOH, followed by the same amount of distilled water, and then suspended in 250 ml of distilled water. The mixture was then decanted to remove fines. The resin was finally washed on a Buchner funnel with 250 ml 0.1N HCl followed by 250 ml distilled H_2O and suspended in 0.02M Sodium phosphate buffer (pH 7.0) until settled.

The settled resin in 0.02M NaH_2PO_4 pH 7 was mixed with acid whey in a ratio of 1:10 and the pH of the mixture re-adjusted to pH 7.0. The mixture suspension was maintained at 150 shakings /min using a Shaker (ZMD 201, Amersham, UK) for 2 h. The resin was recovered on a Buchner funnel under moderate vacuum taking care not to dry the cellulose pad. The resin pad was then washed with 4 successive volumes of 0.1M NaCl in 0.1M NaH_2PO_4 at pH 7, again under moderate vacuum. The pink cellulose phosphate pad was poured as slurry into a glass column and LF eluted with 0.25M NaCl in 0.2M NaH_2PO_4 at pH 7.5. Fraction with UV A_{280nm} > 1.0 were pooled together and subjected to gel filtration on Sephadex G-100 (Pharmacia, Uppsala Sweden).

2.3 Gel filtration

The Sephadex G-100 was suspended in excess phosphate buffer (0.02M NaH_2PO_4 pH 7) and allowed to swell in boiling water for 5 h and then cooled down at room temperature. The pooled LF bearing fractions with UV A_{280nm} > 1.0 was applied to the column of Sephadex G-100. After gel filtration, LF bearing fractions with UV A_{280nm} > 1.0 were pooled together and dialysed against 50 mM NaCl in 10mM Hepes at pH 7.4. The dialysed LF isolates were used for Reverse-phase HPLC analysis and in LF quantification studies.

2.4. RP-HPLC Analysis of LF

The different fractions were studied by reverse phase HPLC using Agilent 1050 quaternary pump with column oven, vacuum degasser, Agilent 1050 automatic sampler and Agilent 1050 Diode Array detector, Perkin Elmer LC 240 fluorescence detector and Agilent 35900E Dual channel interface with built-in gradient programmer. The Jupiter 5U C18 300A column (4.6 x 250 mm, Phenomenex, UK) was kept at 20°C in a column-oven. Peak detection was at 220 nm. Solvent A consisted of acetonitrile / water / triflluoroacetic acid (100:900:1 v/v); solvent B was acetonitrile / water / trifluoroacetic acid (900:100:0.7 v/v). The flow rate was 1 ml / min. The standards were dissolved in solvent A (1 mg/ml) while the LP fractions (20 µl) were applied after filtration through a 0.22 µm filter onto the column using a gradient program from 15% to 60% B in 30 min and a post run time of 10 min. Samples were automatically injected into the column and the gradient started 5 min after injection.

2.5 Quantification of LF

2.5.1 Reagents

The reagents which included 50 mM dissodium NTA, 50 mM $Fe(NO_3)_3$ in 0.1M HNO_3 and 50 mM Tris base (without adjusting pH) were used to prepare 1.0 mM Fe^{3+}-NTA. The other reagents are 25 mM and 100 mM $NaHCO_3$. One mM Fe^{3+}-NTA was prepared as described by Graham and Bates (1976) such that the NTA to Fe ratio was 4:1. To 8 ml of 50 mM dissodium NTA in a 250 ml beaker, 12.5 ml 50 mM Tris base was added. The mixture was diluted to 80 ml with distilled deionised water. Then, 2 ml of 50 mM Fe $(NO_3)_3$ was drop-wise added to the rapidly stirring NTA-Tris solution. The mixture was again diluted with distilled water to 100 ml. The final pH of the mixture was 7.69.

2.5.2 Lactoferrine Standard curve

The following concentrations 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml of LF were prepared from the stock solution of 0.5 mg/ml (in 0.2M NaH_2PO_4 at pH 7.5) by dilution with the same buffer. The assay was completed by mixing 4.5 ml aliquot of commercial LF with 0.5 ml of 25mM $NaHCO_3$. To 3 ml of the mixture, 200 µl of Fe^{3+}-NTA was added and incubated at room conditions for 30 minutes and the absorbance read at 465 nm.

2.5.3 Sample assays for LF

2.5.3.1 Colostrum /milk / whey samples

The aliquot of sample (4.5 ml) was mixed with 0.5 ml of 100 mM $NaHCO_3$. To 3 ml aliquot of the mixture, 100 µl of 1 mM Fe^{3+}-NTA was added and incubated for 30 min at room temperature. The absorbance at 465nm was read against the sample blank.

2.5.3.2 Purified LF

The aliquot of purified LF (4.5 ml) was mixed with 0.5 ml of 25 mM $NaHCO_3$. To 3 ml of the mixture, 200 µl of Fe3+-NTA was added and incubated at room conditions for 30 min and the absorbance measured at 465 nm against the sample blank.
The actual concentrations were determined from the standard curve.

2.6 Sample assay for LP activity

To 0.1 ml of milk in phosphate buffer, pH 6.7 (dilution factor = 5) in a 4.5 cm^3 cuvette, 2 ml ABTS solution was added, mixed well and left for 5 minutes at room temperature to allow dispersion / solubilisation of the casein micelles. One ml of 0.3 mM H_2O_2 was added and mixed quickly. The first absorbance at 412 nm was recorded at exactly 15 seconds after addition of H_2O_2, and the second absorbance was taken after 1 minute and 15 seconds of the reaction time. The activity [E] was calculated using the equation below

$$[E]_{milk} = \left\{ \frac{(R + R_o)(V_s + V_a)}{V_s} \right\} - 96$$

Where $[E]_{milk}$ = LP activity (µM product/minute); *Vs* = Sample volume (0.1 ml); *Va* = Total volume of an assayed sample (3.1 ml); *Ro* = 3µM product/minute; *R* = Initial rate of generation of oxidized product which is given by this relation $(\Delta A/\Delta t)$/ (32.4 x 10^{-3}) µM product/minute; Where 32.4 x 10^{-3} is the extinction coefficient of the ABTS oxidation product at 412nm; ΔA = change in absorbance; Δt = change in time).
The numerical constant 93 was changed to 96 to take care of the 0.1ml increase in assay volume. The total assay volume was 3.1 mL. All measurements were carried out at room temperature.

3 Results and Discussion

3.1 Modification of the method

The method revealed underestimation of LF concentration (Fig 1 Line B1). The recommended preparation of solution yielded lower LF value. This was due to the inconsideration of the effect of Fe^{3+}-NTA, as the absorbance readings was taken against the blank composed of buffer and water alone. This gave a trend line with a Y-intercept far away from the zero point of the X /Y-axes (Fig. 1 Line B1).To account for this, the blank sample was made to include Fe^{3+}-NTA and $NaHCO_3$.
The incorporation of Fe^{3+}-NTA in the blank improved the alignment of the trend line (Fig. 1, Line B2) to start from around zero. This was even further improved when both $NaHCO_3$ and Fe^{3+}-NTA constituted the blank (Fig. 1 Line B3). The blank for the standards was thus modified to constitute of buffer, $NaHCO_3$ and Fe^{3+}-NTA, while the blank for the test samples, constituted of the sample itself, $NaHCO_3$ and Fe^{3+}-NTA. The absorbance for the sample blank was determined in a three step spectrophotometric procedure to avoid the reaction between the sample LF and Fe^{3+}-NTA as follows (i) A_1= water absorbance, A_2= water + Fe^{3+}-NTA absorbance, and A_3 = absorbance for the mixture of a test sample and $NaHCO_3$. The sample blank was thus determined from the following formula (A_2 - A_1) + A_3. In other trials, the replacement of $NaHCO_3$ with distilled H_2O gave more or less similar results (SD = ± 0.002). It is thus possible to further modify the sample blank to include the mixture of the test sample and H_2O + Fe^{3+}-NTA and hence read the absorbance in a single step spectrophotometric procedure. This value was subtracted from the absorbance reading of the test sample. The standard variations of each point (n=2) was very low (≤ 0.4%), therefore standard errors are not shown.

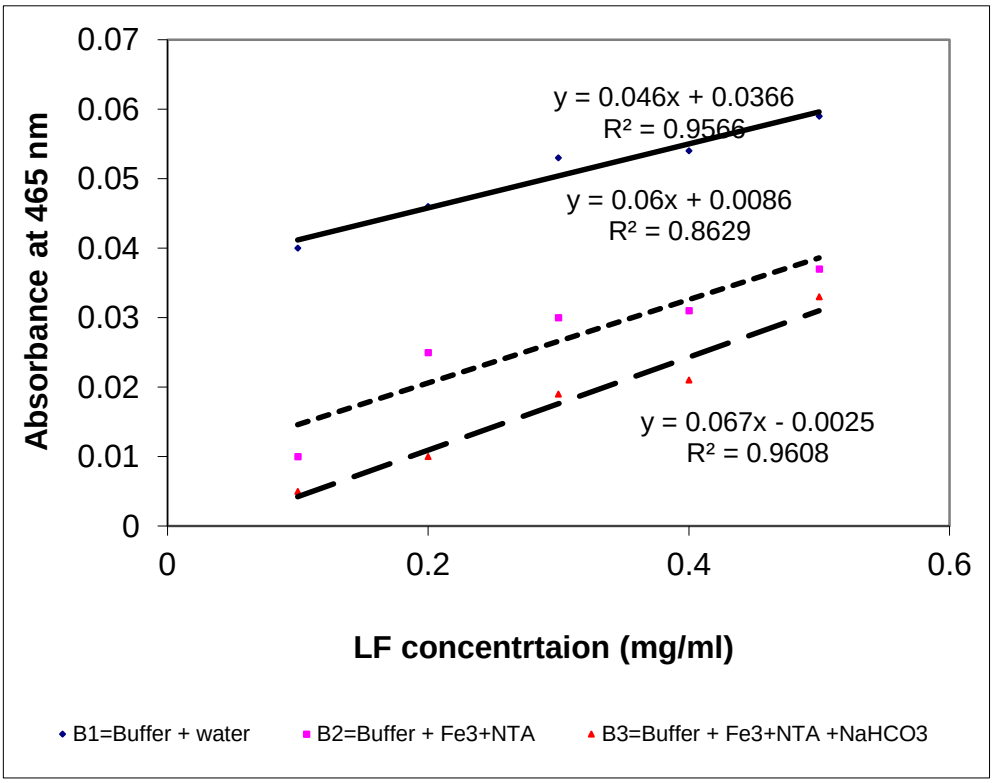

Figure 1. Shows the effect of blank composition on the alignment of the trend line.

3.2 Application of the method

To examine the potential of the method in quantifying LF, a variety of samples were used and results compared with available literature data as discussed below.

3.2.1 LF concentration in colostrums

In order to prevent interferences by fat on absorbance reading, the fat fraction in colostrum was removed by centrifugation using a Centaur 2 centrifuge (MSE, UK) at 604 x g for 30 min. Wide variations in LF concentration were recorded between individual cows' but the differences were negligible within individual cows during the first five post parturition days (Table 1). The concentration varied from non-detectable levels to 1.53 mg/l. The inability to detect LF in some samples could be due to its low content or poor sensitivity of the method. However, Tsuji *et al.* (1990) using single radial immunodiffusion method similarly reported high variability in LF content among individuals of dairy breeds compared to beef breeds varying from undetectable levels to 11.77 mg/ml. The average LF content they recorded in colostrums of dairy breeds was 2 mg/ml (i.e. 1.96 ± 0.27 mg/ml for Holstein-Friesian and 2.11 ± 0.36 mg/ml for Jersey) and in colostrums of beef breeds 0.5 mg/ml (Japanese black 0.56 ± 0.31 mg/ml and Japanese brown 0.4 ± 0.30 mg/ml). The concentration of LF in bovine colostra according to other researchers is approximately 1 mg/ml (IDF 191/1985), 2 - 5mg/ml (Paulsson *et al.*, 1993).

Table 1: LF concentration of colostrum milk from individual cows during the first five days after parturition.

Days	**LF concentration (mg / ml)**			
	Cow A	**Cow B**	**Cow C**	**Cow D**
Day 1	N.D	N.D	N.D	N.D

Day 2	1.33	N.D	N.D	N.D
Day 3	1.53	N.D	0.05	0.20
Day 4	1.20	0.39	N.D	N.D
Day 5	1.04	0.46	0.05	N.D
Average	1.28 ± 0.2	0.43± 0.05	0.05± 0.0	0.20±0.0

ND = not detected

3.2.2 LF concentration in milk, whey and LF isolates

Changes in LF concentration were monitored during fractionation of milk and isolation of LF from acid whey so as to further asses the sensitivity of the method. The procedure for the purification of LF is as described in subsection 2.2 and 2.3.

3.2.2.1 LF concentration in milk and milk fractions

Results in Table 2 summarises the amount of LF quantified in different fractions and the corresponding LP activity. LP activity can be used to gauge the efficiency of the purification process of LF due to the close similarities of the physicochemical properties of these two proteins which poses great difficulties in their isolation. The recorded LF concentration in bovine milk (0.05 mg/l) was increased 7 folds by precipitation of the casein fraction in acid whey and 14 folds through purification / isolation. LF in normal bovine milk occurs at > 0.02- 0.2 mg/ml (Steijns and Van Hooijdonk, 2000), 0.1- 0.3 mg/ml (Paulsson et al., 1993), 0.02 - 0.35 mg/ml (IDF, 191/1985) depending on the stage of lactation. In normal milk whey the concentration is 0.03-0.73 mg/ml (Welty *et al.*, 1975). Based on LF concentration of acid whey used in the present study (0.35 mg/ml), about 80% of this was recovered during isolation. Foley and bates (1987) similarly reported the average yield of 80%. On the other hand, LP activity was reduced by almost 99 % in LF isolate (i.e. purified fraction) which reflects the efficiency of the batch extraction method using cellulose phosphate resin in the isolation of LF from other whey protein particularly LP. The purity of recovered LF recorded in previous studies (Foley and Bates, 1987) was 96%. Although purity was not quantitatively analysed, R-phase HPLC analysis (Fig 2 a & b) show that the isolated protein was LF (Retention time ≈10 as for the standard Fig. 2.c) and confirms the high purity of the recovered LF isolates. No LP peak (Fig. 2 d) was resolved in the chromatogram of LF isolate which indicates its presence in amounts below the detection level of the R-phase HPLC. The only contaminant observed was the low molecular weight compound with a retention time of about 4 min. While Foley and Bates (1987) successfully further purified the LF isolate by gel filtration. However, further purification by gel filtration was less successful in this study. Although the purity of gel filtered LF isolate (Fig. 2 a) was slightly higher than that of the non-gel filtered LF isolate (Fig.2 b), the low molecular weight impurity was not completely removed. This implies that regardless of the efficiency of the purification method it is hardly possible to achieve 100% purity.

Table 2: Variation of LF concentration and LP activity with fractionation

Samples	Lactoferrin (mg/ml)	% concentration	LP activity μM product/min	% activity
Raw milk	0.05	-	-	-
Acid whey	0.35	100	1831	100
Depleted whey	0.07	20	1015	55.5
Purified LF	0.68	194	21.7	1.19

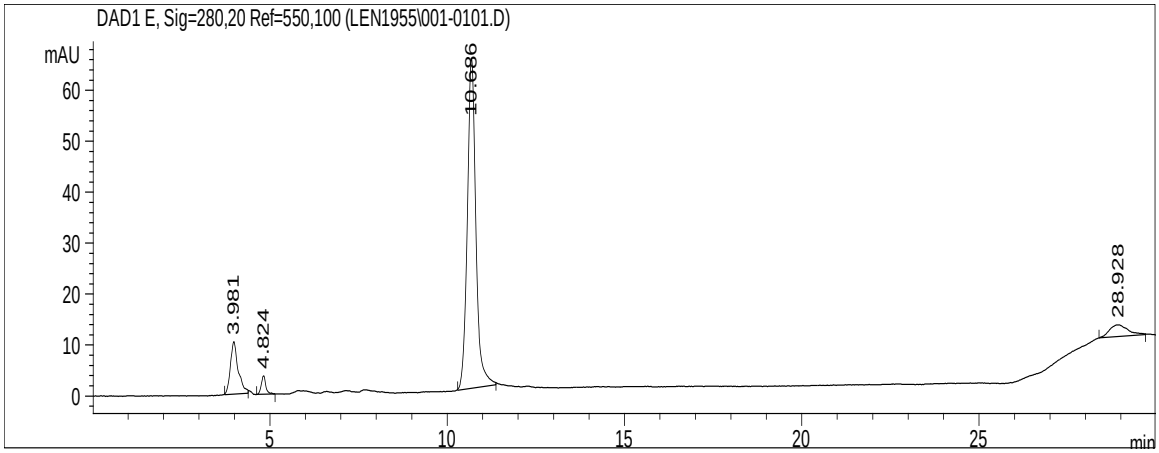

Figure 2 (a): Chromatogram of LF isolated by cellulose phosphate resins followed by gel filtration using Sephadex G-100

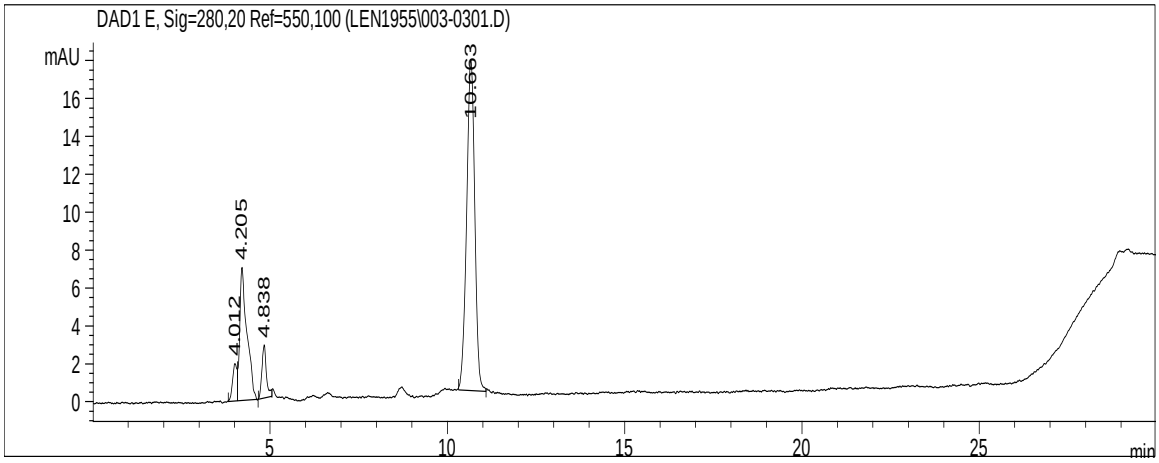

Figure 2 (b): Chromatogram of LF isolated by cellulose phosphate resins without gel filtration

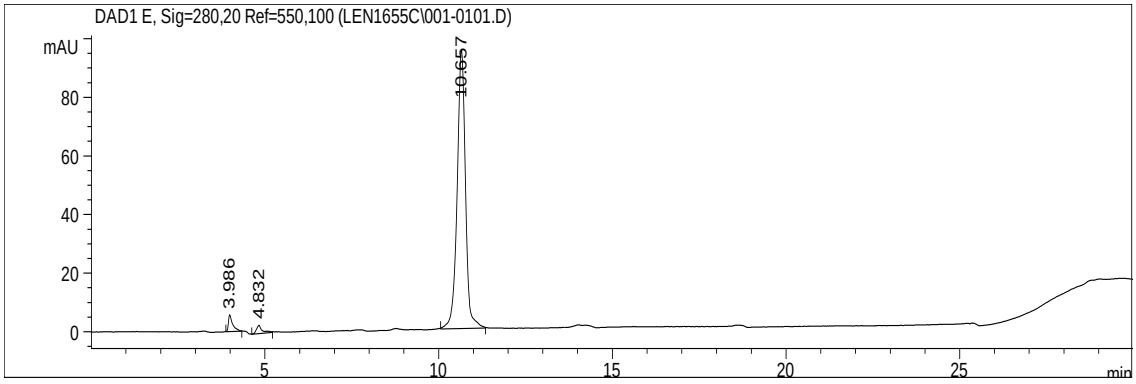

Figure 2 (c): Chromatogram for LF standard

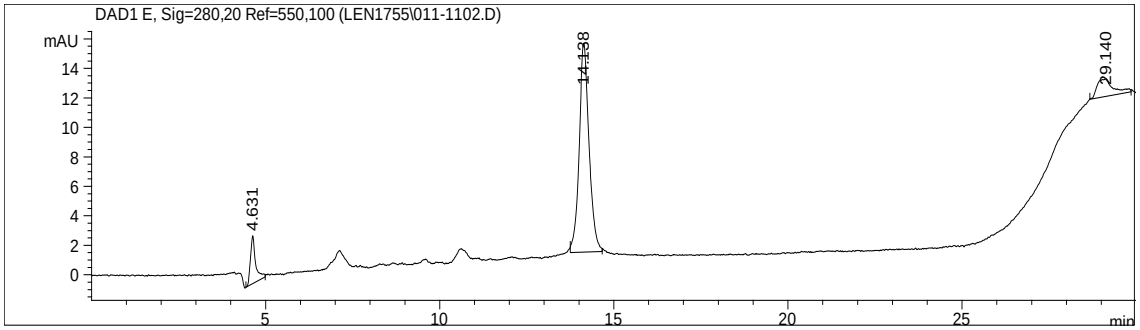

Figure 2 (d): Chromatogram for LP standard

3.3 Effect of 0.2 µm filters on LF concentration

In an attempt to reduce turbidity and enhance the clarity, whey was vacuum filtered through 0.2 µm filters. Preliminary results demonstrated the decline in LF content of whey filtrate. The results prompted further investigation to find out if filtration caused decrease in activity. Three experiments were thus conducted on different whey samples to examine the effect of application of micro-filters on LF concentration. Table 3 summarises the average concentrations of LF in whey prior and after filtration through 0.2 µm filters. Surprisingly, massive reductions in LF concentration were recorded in all experiments. Whey filtrate exhibited extremely lower LF concentration than the corresponding unfiltered whey samples with an overall mean reduction of 69%. This huge reduction seems to suggest the selective exclusion of LF based on molecular size. Further examination of the possible exclusion of some LF through micro-filtration was investigated by using commercial LF and isolated LF. The solutions were syringe filtered through 0.2 µm filters. Similar massive decreases in concentrations were observed in both isolated and commercial LF (Table 4). This observation reinforces the possibility of selective filtration of LF when filters with such small mesh sizes are used. Two different molecules of LF of large and small sizes are reported in bovine milk (Tsuji *et al.*, 1989**).** Filtration on the other hand, resulted into slightly higher LP activity in whey filtrate than in whey (Table 3). These results signal the possibility of separating these two proteins (LF and LP) by a simple filtration process. However, this observation requires further investigation for conclusive remarks to be made.

Table 3: Mean LF concentration and LP activity of whey and whey filtrate (0.2 µm filters) obtained from three separate experiments.

Sample	Mean LF ± SD (mg/ml)	Mean % reduction	Range % reduction	LP activity ± SD µM product/ml
Whey	0.71 ± 0.46			1432 ± 38
Whey filtrate	0.22 ± 0.18	69%	38% - 89%	1612 ± 49

Table 4: Concentration of commercial LF and isolated LF prior and after filtration (0.2 µm filters)

Sample	**mg/ml**	**% decrease**
Commercial LF	0.77	
Commercial LF (filtered)	0.37	52 %
Isolated LF	3.50	
Isolated LF (filtered)	0.28	92 %

4. Conclusions

The modified Fe^{3+}-nitrilotriacetate (Fe^{3+}-NTA) method is a simple, quick and reproducible method that can give a good quantitative estimation of LF. However, evaluation of the method against other standard methods is crucial for assessing its sensitivity. Although LF and LP have similar molecular weight and isoelectric point which complicates their separation, a batch procedure using cellulose phosphate resin is a highly effective method for the isolation and purification of LF with less contamination of LP. The reduction of LF concentration by micro-filtration (0.2um filters) provides the base for further investigation of the possible isolation of LP and LF through filtration.

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