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Journal of Chromatography A, 947 (2002) 307–311

JOURNAL OF CHROMATOGRAPHY A

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Short communication

# Detection and quantitation of lactoferrin in bovine whey samples by reversed-phase high-performance liquid chromatography on polystyrene–divinylbenzene

Kate P. Palmano[a,*], David F. Elgar[b]

[a]*Institute of Fundamental Sciences, Massey University, Private Bag 11 222, Palmerston North, New Zealand*
[b]*New Zealand Dairy Research Institute, Private Bag 11 029, Palmerston North, New Zealand*

Received 5 October 2001; accepted 26 November 2001

**Abstract**

An existing RP-HPLC method for the measurement of the major bovine whey proteins in bovine whey samples and powders has been extended to include the analysis of the minor bovine whey protein lactoferrin. Lactoferrin could be detected and quantitated at levels down to 0.2 μg and linear calibration was observed between 0.2 and 30 μg. Reliable quantitation of lactoferrin in whey samples could be achieved provided the bovine serum albumin to lactoferrin ratio did not exceed 10:1. Quantitative data obtained by the RP-HPLC method compared favourably with data obtained by Mono S analytical chromatography. 

*Keywords:* Whey; Milk; Food analysis; Lactoferrin; Whey proteins; Proteins

## 1. Introduction

In a previous paper, a reversed-phase (RP) high-performance liquid chromatography (HPLC) method for the analysis of the major proteins appearing in a comprehensive range of bovine whey types and preparations was described [1]. In this report, the applicability of the existing method to analysis of the bovine milk protein lactoferrin (Lf), which is a minor whey protein of considerable commercial interest and value [2–4] is discussed. In particular, the possibility of accurate determination of lactoferrin in commercial whey streams and powders was investigated.

*Corresponding author. Tel.: +64-6-356-9099x3527/3537; fax: +64-6-350-5682.

*E-mail address:* k.p.palmano@massey.ac.nz (K.P. Palmano).

## 2. Conditions

### 2.1. Materials

S Sepharose Fast Flow (FF) and S Sepharose Big Beads (BB) were from Amersham Pharmacia Biotech (Uppsala, Sweden). All other materials were as previously described [1].

### 2.2. Whey protein standards and samples

Whey protein standards and whey protein concentrate (WPC) solutions were as described previously [1] except that WPC solutions were prepared from different batches of source material and made to ~4 mg/ml for RP-HPLC analysis. Acid whey was prepared by threefold dilution of fresh skim milk

0021-9673/02/$ – see front matter 
PII: S0021-9673(01)01563-1

with 0.2 *M* sodium acetate, pH 3.9, followed by centrifugation to remove the caseins. A milk fraction containing the basic (high isoelectric point, p*I*) proteins was prepared by mixing 50 ml fresh skim milk with 5 ml S Sepharose BB for 2 h at room temperature after which the resin was recovered, washed with water and packed into a 10-ml disposable plastic column. Bound protein was then eluted with 25 ml 2 *M* NaCl to give the basic fraction. This was used undiluted for RP-HPLC and diluted 1 in 3 with water for Mono S HPLC. Lf was purified from fresh bovine skim milk using a cation-exchange protocol based on previous methods [5–7]. Briefly, 5 l of skim milk were passed through a 300-ml column of S Sepharose FF which had been equilibrated with 25 m*M* sodium phosphate buffer at pH 6.5, at a flow-rate of 5 ml/min and at 4 °C. After a water wash, a lactoperoxidase (Lp)-containing fraction was eluted from the column by passage of 0.35 *M* NaCl followed by elution of the Lf-containing fraction with 1.0 *M* NaCl. This fraction was dialysed, freeze-dried and the resulting powder subjected to chromatography on S Sepharose FF at pH 6.5 using the above buffer and a salt gradient to 1.0 *M* NaCl.

Fractions containing Lf of sufficient purity as judged by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were pooled, dialysed and freeze-dried as the Lf standard. Identity was confirmed by N-terminal amino acid sequencing and protein content was determined by quantitative amino acid analysis.

Stock solutions of Lf were prepared at ~3 mg/ml in Milli-Q water and stored in aliquots at −20 °C. Standards containing Lf only or Lf in combination with other whey proteins were prepared freshly from the stock solutions on the day of use to give working concentrations of ~0.3 mg/ml for Lf and values for the other whey proteins similar to those previously described [1] (see also Fig. 1). Standard curves with typically eight points were constructed from either single or mixed standards using injection volumes between 10 and 100 μl. To assess the limit of detection for Lf, mixed standards containing very low amounts of Lf were prepared. In this case, stock Lf which had been diluted 10-fold with Milli-Q water was added to the mixed standard to give final concentrations of ~0.008–0.03 mg/ml.

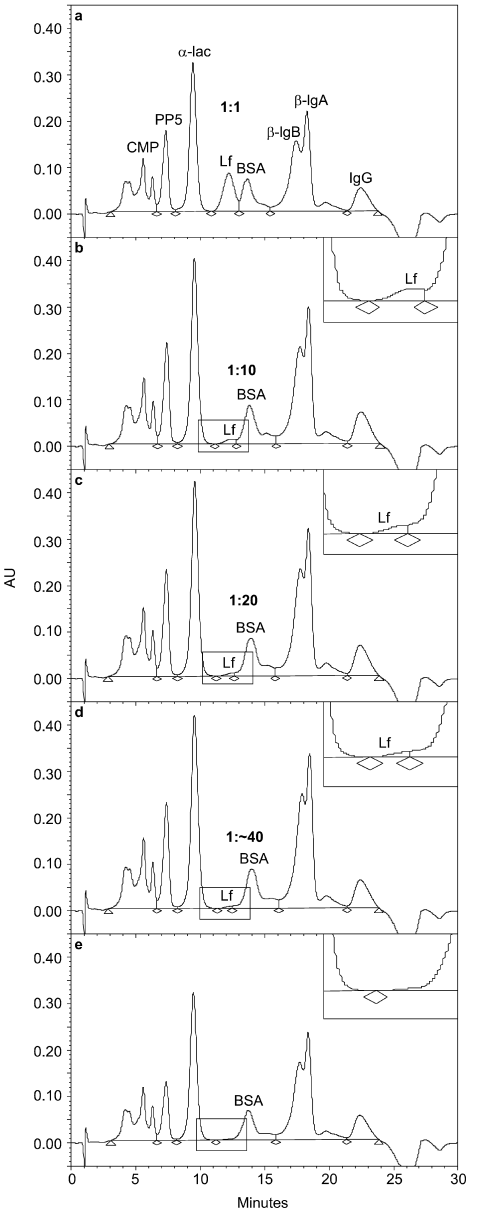


Fig. 1. RP-HPLC chromatograms of mixed bovine whey protein standards with Lf present (a–d) or absent (e). Protein concentrations were ~0.5 mg/ml for α-lac, β-lgs A and B and casein-macropeptide (CMP), and ~0.3 mg/ml for BSA, immunoglobulin G (IgG) and proteose peptone (PP5). Lf was included at (a) 0.3 mg/ml, (b) 0.027 mg/ml, (c) 0.015 mg/ml and (d) 0.008 mg/ml. Injection volume was 20 μl for (a) and 25 μl for (b)–(e). The Lf/BSA ratios at each Lf dosage are indicated on the diagrams. Inserts in (b)–(e) represent amplification of the chromatograms in the region of Lf elution.

### 2.3. Methods

All experimental methods and systems used in this work were as described previously [1] with the exception of Mono S HPLC which was an adaptation of the method of Francis et al. [8] for analysis of Lf. Citrate buffer was replaced by sodium phosphate buffer pH 7, to enable detection at 214 nm and the gradient to 1 *M* NaCl was modified as follows: 0–5 min, isocratic at 5% B; 10 min to 50% B; 2 min to 100% B; hold 3 min at 100% B; 1 min to 5% B; 5 min isocratic at 5% B. Lf standards (~0.3 mg/ml) were prepared from stock solutions as for RP-HPLC and standard curves were constructed using 10–100-μl injections.

## 3. Results and discussion

Preliminary analysis of Lf in the Resource RP-HPLC system showed that Lf emerged as a single peak between the elution positions previously established for α-lactalbumin (α-lac) and bovine serum albumin (BSA) [1]. Furthermore, linear calibration plots with $R^2 > 0.99$ over the range 2–25 μg protein loaded could be consistently achieved with RSD for response factor (slope of standard curve) being <2%. The applicability of such analysis to mixed whey protein standards and samples was then addressed.

### 3.1. Assayability of Lf

A typical chromatogram for mixed whey protein standard inclusive of Lf at a dosage similar to BSA is shown in Fig. 1a. Peak shapes were consistent over the range 10–100 μl injection volume. Lf eluted after and discreet from α-lac but ahead of BSA. Although there was some convergence of the tail of the Lf peak with the leading edge of the BSA peak, a clear valley could be distinguished and for integration purposes a vertical drop was made from the lowest point in the valley to the baseline. Calibration plots were constructed by using a line fit forced through zero (Millennium 32 software) and it was found that a linear response between protein mass and absorbance at 214 nm was obtained for Lf with $R^2 > 0.99$. Furthermore, integrated data for Lf obtained from the mixed standards fitted extremely well into calibration plots obtained from the single standard ($R^2 > 0.99$). This was also the case for BSA and α-lac where integrated data from the mixed standards with Lf present was fitted into that from the mixed standards with Lf absent.

### 3.2. Limit of detection for Lf

The concentration of Lf in bovine milk and whey is relatively low. Values are milk-seasonal-dependent but generally fall within the range 0.02–0.4 mg/ml [7,9–13], although they can increase to as much as 1.22 mg/ml during mastitis [10]. Since Lf may be present in some whey samples at very low levels and in quantities only 10–20% of that of BSA [7], it was important to estimate not only limit of detection for Lf but also its assayability in the presence of much greater BSA concentrations. The mixed whey protein standards containing very low levels of Lf were used for this purpose. Lf was discernible as a small, reasonably well-defined peak on the shoulder of the BSA peak when included at 0.027 mg/ml (~0.7 μg load) and at an Lf/BSA ratio of 1:10 (Fig. 1b). At even lower loads and decreasing Lf/BSA ratios (Fig. 1c and d) Lf was still visible and quantifiable. However, the loss of clear definition of the Lf peak from the BSA peak made placement of integration markers more difficult. For comparison, a chromatogram of mixed whey standards exclusive of Lf is shown in Fig. 1e. Lf peak data from these chromatograms was combined with data obtained from the higher dosage Lf mixed standard runs and it was found that calibration was linear between 0.2 and 30 μg Lf with $R^2 \geq 0.99$. Although it is possible to quantitate Lf at very low levels using the RP-HPLC method, it is obvious that care should be exercised in delineating Lf from BSA (where present) and that highly sensitive electronic data acquisition is necessary to ensure reliability. Furthermore, it is likely that reproducibility of measurement will decrease as the ratio of Lf/BSA decreases below 1:10.

### 3.3. Assay of whey samples

The applicability of the existing RP-HPLC method

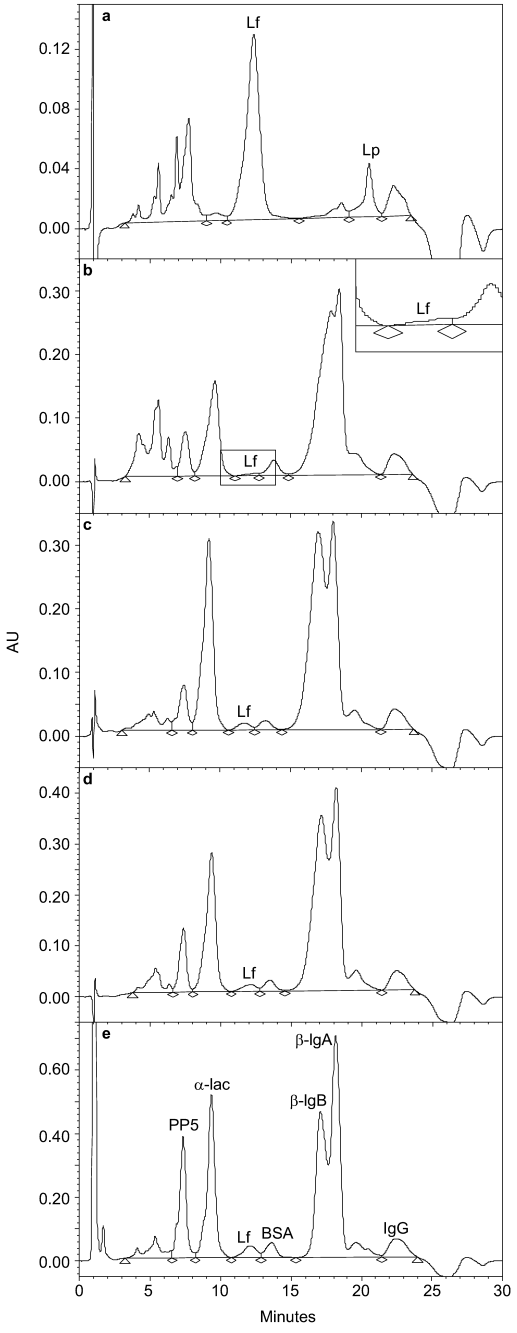


Fig. 2. RP-HPLC chromatograms of (a) skim milk basic fraction, (b) cheese WPC, (c) lactic acid WPC, (d) mineral acid WPC and (e) acid whey. Injection volume was 25 μl for the WPC samples and basic fraction, and 60 μl for the acid whey. Insert in (b) represents amplification of the chromatogram in the region of Lf elution.

to assay of Lf in fractionated whey streams enriched in the basic proteins is illustrated in Fig. 2a. The elution of Lp in this sample is also indicated. However, no attempt was made to quantitate this protein using the current RP-HPLC method as resolution of Lp from the β-lactoglobulin (β-lg) dimer peak (see Ref. [1]) was not always clear (see e.g. Fig. 2b and c). RP-HPLC profiles for cheese, lactic and mineral acid WPCs and a fresh batch of mineral acid whey are shown in Fig. 2b–e. In the cheese WPC sample, Lf appeared as a flat peak on the shoulder of the BSA peak (Lf/BSA ratio ~1:5) but in the other samples it was well defined and in comparable levels to BSA. These profiles closely resembled those obtained for similar, but not identical, samples in our previous work [1] and it can now be concluded that the small peak eluting ahead of BSA in those earlier reported samples was indeed Lf and not a BSA heteroform as assumed. In this context it is interesting that Luf and Rosner [14] reported BSA eluting on the tail of the α-lac peak and ahead of Lf in separation of whey proteins using a $C_4$ RP column.

### 3.4. Quantitation of Lf in whey samples

Results for quantitation of Lf in the above whey samples using the RP-HPLC method are given in Table 1 along with values obtained for the same samples by Mono S HPLC. It can be seen that the

Table 1
RP-HPLC determination of lactoferrin in bovine WPC samples, skim milk whey and skim milk basic fraction

| Sample | Mean[a] | SD | RSD (%) |
|---|---|---|---|
| Cheese WPC | 0.30 (0.35)[b] | 0.033 | 11.2 |
| Acid WPC | 0.99 (0.99) | 0.033 | 3.32 |
| Lactic WPC | 0.78 (0.82) | 0.025 | 3.14 |
| Basic fraction | 0.40 (0.31) | 0.004 | 1.03 |
| Skim milk whey | 0.15 (0.15) | 0.001 | 0.85 |

[a] Values represent the mean of 11 separate determinations for WPC samples and basic fraction, and the mean of six separate determinations for skim milk whey. Values for WPC powders are expressed as % powder mass while those for the basic fraction and whey are in mg/ml.

[b] Values in brackets represent the average of duplicate determinations of Lf by Mono S HPLC.

RSD for RP-HPLC analyses was within 4% for all samples except cheese WPC. In the latter case, the higher variability might be accounted for by the comparatively low content of Lf in combination with a higher relative amount of BSA. The value obtained for fresh whey fell within the mid range of values reported in the literature for both bovine whey [9–11] and bovine milk [12,13]. It can be seen from Table 1 that the RP-HPLC results were in reasonable accord with those from Mono S analysis, even in those samples containing relatively low amounts of Lf.

## 4. Conclusions

In conclusion, an existing RP-HPLC method for the separation and quantitation of the major bovine whey proteins can also be applied to the analysis of Lf in whey samples and whey protein powders. This method can provide useful data on Lf without compromising the analysis of the other whey proteins, in particular BSA, thus enabling multiple analyses in a single run. At best, reliable quantitation of Lf can be achieved within defined limits, while in the worst case the method can serve as a useful qualitative screen for presence or absence of Lf. In a similar context, the method would be particularly suited to the monitoring of Lf extraction processes to assess both yield and purity.

## Acknowledgements

The authors are grateful to Garnett Davy of the New Zealand Dairy Research Institute for his excellent contribution to data formatting. This work was funded by the New Zealand Dairy Research Institute.

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