Application Note

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A Simple, Accurate, and Reliable Method for Lactoferrin Analysis in Human Milk

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Abstract

Lactoferrin is the second most abundant and multifunctional protein in human milk. Some interesting therapeutic benefits are attributed to this compound. For this reason, research on this protein requires reliable quantification methods for its determination in natural sources. This application note presents an analytical method for lactoferrin using the Agilent 1260 Infinity II Prime LC System. The method showed excellent analytical performance according to the results obtained for linearity, precision, LOD, LOQ, recovery, and selectivity.

Introduction

Human lactoferrin is an 80 kDa iron-binding glycoprotein of the transferrin family. It is the second most abundant and multifunctional protein in human milk.[1,2] Human lactoferrin is considered one of the most important bioactive components in breast milk, and its concentration changes depending on many factors. It has been reported at a concentration of approximately 1.5 mg/mL in mature milk.[3]

There are several analytical and biological techniques to determine lactoferrin in different species, including enzyme-linked immunosorbent assays (ELISA), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and liquid chromatography-coupled tandem mass spectrometry (LC/MS).[4,5] These techniques are sensitive but expensive, and are not used as a routine method to determine lactoferrin. High-performance liquid chromatography (HPLC) coupled with UV-VIS detectors have become a very effective tool to quantify different components in dairy products, using simple, selective, and robust methods.[6]

The commercial interest in employing the therapeutic value of lactoferrin, including antimicrobial and immunomodulatory benefits[7], require reliable quantification methods for its determination at endogenous levels in natural sources.

This application note presents the use of an Agilent 1260 Infinity II Prime LC System in the development and optimization of an effective method for the quantitative analysis of purified human lactoferrin from breast milk samples to demostrate the reliability, repeatibility, and reproducibility of the analytical method.

Experimental

Equipment

The 1260 Infinity II Prime LC instrument comprises the following modules:

- Agilent 1260 Infinity II Flexible Pump (G7104C).
- Agilent 1260 Infinity II Multisampler (G7167A).
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A).
- Agilent 1260 Infinity II Diode Array Detector HS (G7117C), equipped with an Agilent Max-Light Cartridge Cell of 60 mm and 4.0 µL (G4212-60007).

Software

Agilent OpenLab CDS version 2.7.

Column

An Agilent InfinityLab Poroshell 120 SB-C18 (150 × 4.6 mm, 5 µm particle size) chromatography column was used.

Chemicals

Solvents were HPLC grade. Human lactoferrin reference standard and trifluoride acetic acid were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Disodium phosphate (Na_2HPO_4) and Sodium chloride (NaCl) were purchased from J.T. Baker (Radnor, Pennsylvania, USA) and Sigma-Aldrich (St. Louis, Missouri, USA), respectively. The 1 mL Cytiva HiTrap HP Heparin affinity column was acquired from GE Healthcare Life Sciences (Chicago, Illinois, USA).

Standards preparation

A 0.2 mg/mL lactoferrin stock solution was prepared. For this, 20 mg of standard was weighed and transferred to a 10 mL volumetric flask. The standard was dissolved with 5 mL of HPLC water by stirring for 30 seconds, followed by sonication for two minutes. Finally, the volume was adjusted to 10 mL. To generate a calibration curve, the stock solution was diluted in water to obtain the following concentration levels: 40, 60, 80, 100, 120, and 140 µg/mL.

Samples

To verify the method performance with real samples, breast milk samples were obtained from the human milk bank at Civil Hospital Fray Antonio Alcalde in Guadalajara, Mexico. The sample collection was made with the informed consent of the donors and the approval of the hospital ethics committee (Number 268/17, ruled on 15 October 2019). The samples were transported at a refrigeration temperature of 4 °C and stored at −20 °C until analysis.

For HPLC analysis, milk samples were thawed and centrifuged at 4,000 rpm for 20 minutes to discard the lipid fraction.

Lactoferrin was extracted, adding 1 mL of buffer 0.2 M Na_2HPO_4 (pH 8) to 1 mL of sample. Next, the sample was mixed for 30 seconds, and centrifuged at 4,000 rpm for 20 minutes at 4 °C. The supernatant was recovered.

After that, lactoferrin purification was carried out using the HiTrap Heparin affinity column, which was conditioned, adding 5 mL of 0.2 M Na_2HPO_4 buffer (pH 8). The supernatant extracted was injected into the column, followed by a wash with 5 mL of the conditioning buffer. The lactoferrin eluted from the column with 3 mL of buffer, 0.05 M Na_2HPO_4, and 1.0 M NaCl (pH 8). The obtained solution was injected into the HPLC system.

Method

Table 1. Chromatographic analysis conditions.

Parameter	Value
Solvent	A) 0.1% (v/v) Trifluoroacetic acid in water B) Acetonitrile
Gradient	Time (min) %A %B 0 75 25 7 30 70 8 75 25
Flow Rate	0.6 mL/min
Temperature	40 °C
Detection	280 nm
Injection	Injection volume: 50 µL Sample temperature: 25 °C Needle wash: 3 sec in water

During chromatographic method optimization, the lactoferrin peak shows less tailing at the 0.6 mL/min flow rate.

Results and discussion

Figure 1 shows the chromatogram for lactoferrin standard; the retention time (RT) obtained is 5.907 minutes. During method performance verification, a blank of water was injected between each standard or sample.

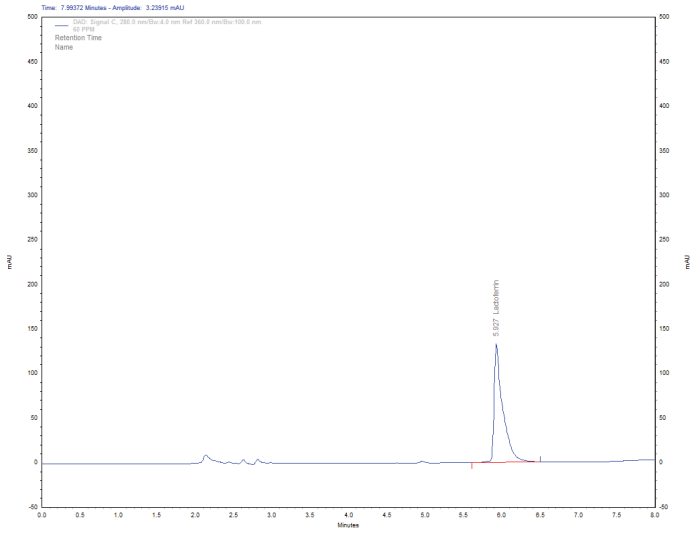

Figure 1. Lactoferrin chromatogram (60 µg/mL).

The validation parameters showed performance in accordance with what was established for HPLC analytical methods, exceeding expectations for linearity, precision, limits of detection (LOD), limit of quantification (LOQ), and recovery.

The method linearity was excellent (R^2 > 0.999) in the range of 40 to 140 µg/mL (Figure 2).

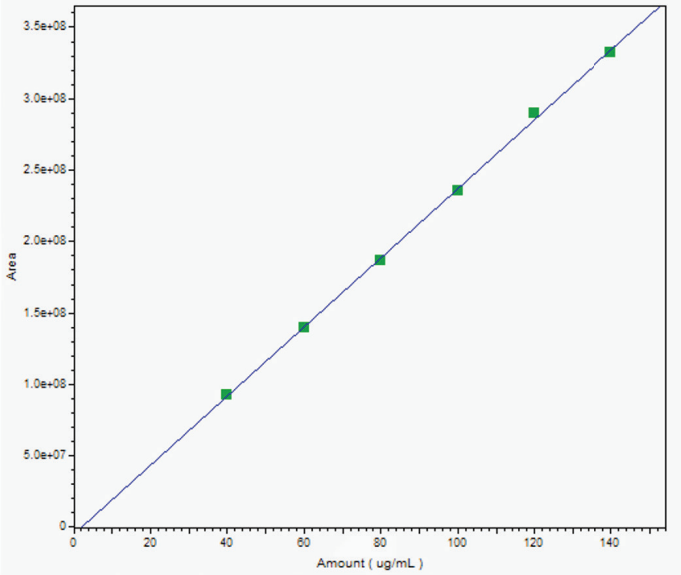

Figure 2. Lactoferrin calibration curve.

Repeatability and intermediate precision of the method were evaluated by calculating the relative standard deviation (RSD) of RT and area. Repeatability was performed with data from a single analyst, obtained on different days (N = 2). Intermediate precision was assessed with data from two analysts. In both parameters, an excellent RSD result was obtained, showing a minimal variation of results (Table 2).

Table 2. Method precision results.

Parameter	RT	Area
Repeatability (RSD, %)	0.12	0.79
Intermediate Precision (RSD, %)	0.14	1.30

The LOQ and LOD were calculated using the calibration curve with standard deviation of y-intercept (Sb_0). Limits were calculated with the following equations:

Equation 1.

$$LOD = \frac{3.3 \cdot Sb_0}{slope}$$

Equation 2.

$$LOQ = \frac{10 \cdot Sb_0}{slope}$$

Results are shown in Table 3. The LOQ value is 300 times lower than the average concentration of lactoferrin in real milk samples.

Considering the complexity of breast milk samples, other compounds can interfere with lactoferrin analysis. Therefore, to ensure the effective performance of the present HPLC method, lactoferrin recovery from breast milk samples was assessed. For this purpose, samples were cleaned up as described in the experimental section. The recovery obtained was satisfactory (Table 3).

Table 3. LOD, LOQ, recovery, and selectivity for lactoferrin analysis.

Parameter	Value
LOD	1.4 µg/mL
LOQ	4.1 µg/mL
Recovery from breast milk sample	90.0 %
Selectivity	No interference observed at the same retention time as the analyte.

Finally, selectivity was evaluated by comparing chromatograms of lactoferrin standard, extraction buffer blank, and breast milk sample. The standard, buffer, and samples were subjected to the cleanup procedure prior to HPLC analysis. Figure 3 shows that there was no sample interference in the retention time of the lactoferrin peak; therefore, this method is selective.

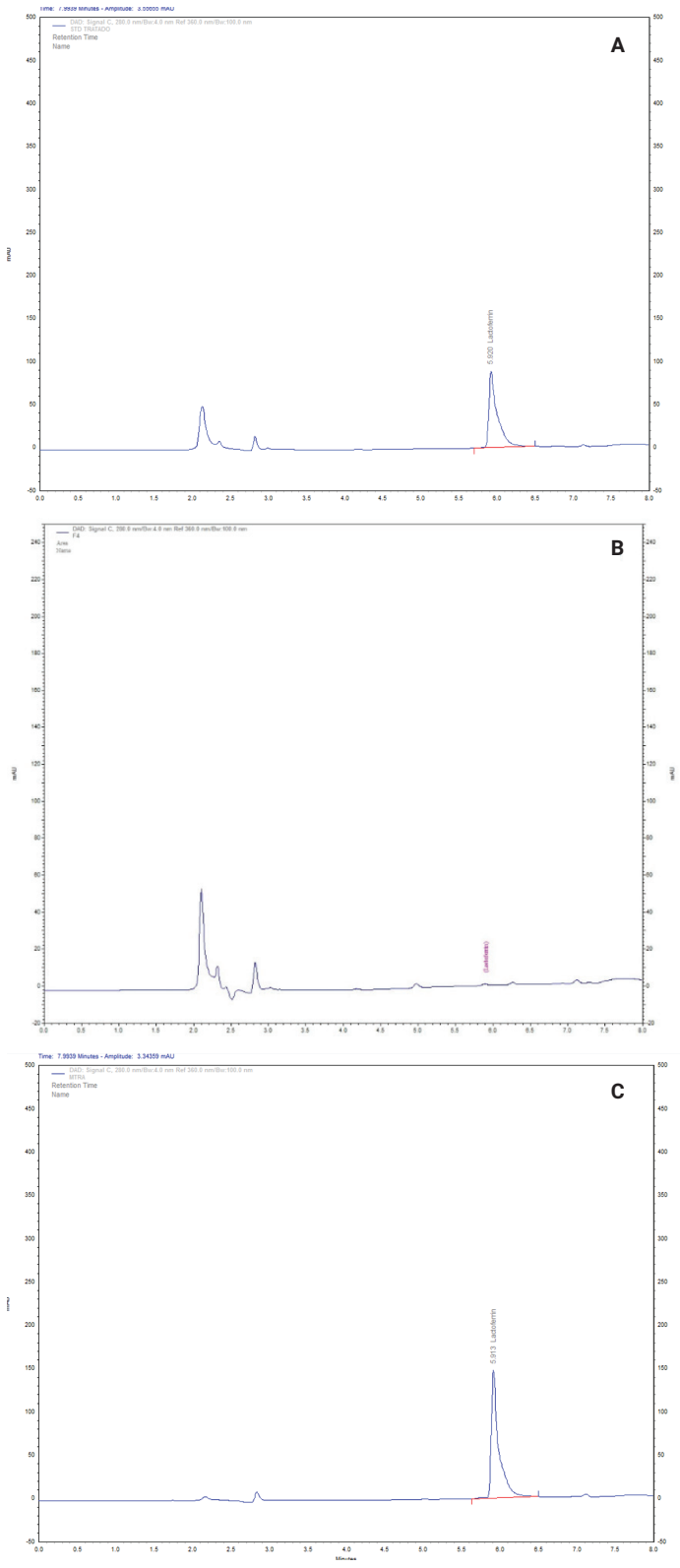

Figure 3. Chromatograms of (A) lactoferrin standard, (B) extraction buffer blank, and (C) breast milk sample after cleanup procedure.

Conclusion

The present HPLC analytical method for the determination of lactoferrin in breast milk, using an Agilent 1260 Infinity II Prime LC System, showed excellent performance. This demonstrates that this method is a good option for lactoferrin analysis in real samples for routine work.

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