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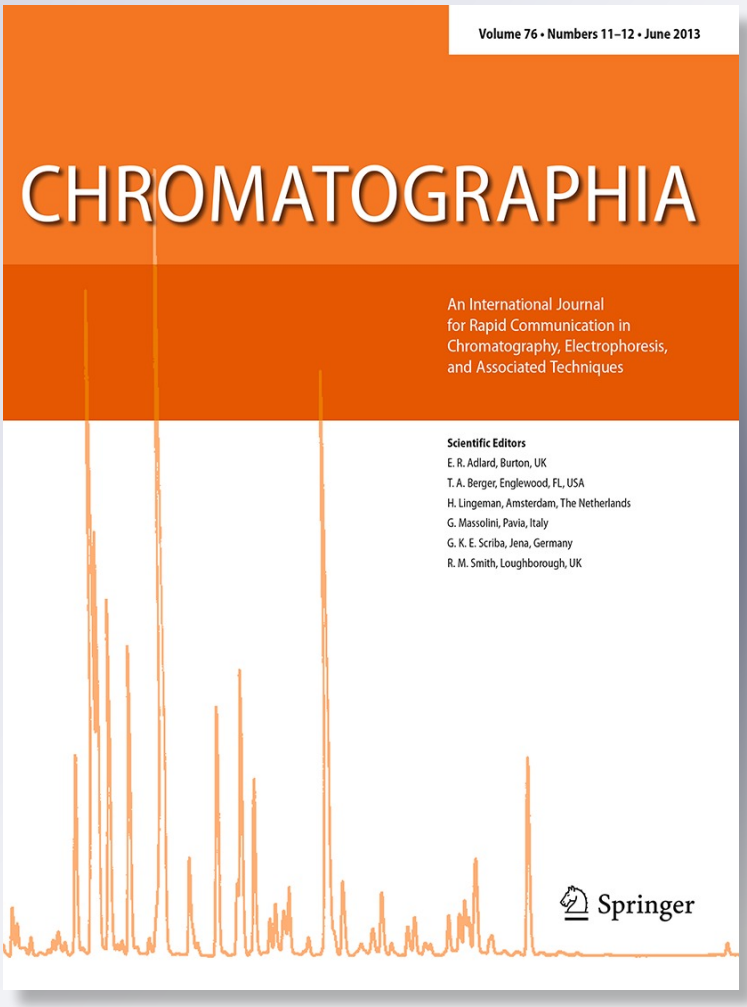

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Separation of Lactoferrin from Human Saliva Using Monolithic Disc

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Abstract In this study, we optimized method for the isolation and detection of lactoferrin from human saliva using 3 mm short monolithic disc. We optimized the conditions for separation as flow rate 4 mL min^{-1} and ionic strength of effluent as 2 M·NaCl. We estimated limit of detection of whole method, which was hyphenated to the Bradford's assay, down to 100 ng mL^{-1}. The purity of the isolated fractions was verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and recovery of isolation was found to be 51 % using minimally processed sample of saliva. Further, we tested the optimized method on group of healthy volunteers ($n = 7$). We were able to distinguish between the healthy subjects and subject suffering from celiac disease, which reported at least 2.5× higher level of lactoferrin in comparison to healthy ones. The results were correlated with standard enzyme-linked immunosorbent assay (ELISA) kit with obtained correlation coefficient $R^2 = 0.8446$. Analysis of lactoferrin in saliva by monolithic disc and subsequent offline photometric detection is faster and cheaper method compared to ELISA commercial kit. The total analysis of one sample takes <20 min.

Keywords Liquid chromatography · Monolithic disc · Spectrophotometry · Enzyme-linked immunosorbent assay · Immunodiagnostics · Human saliva · Lactoferrin

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Introduction

Lactoferrin is the member of the transferrin gene family and its molecular mass is ~80 kDa consisting of 692 aminoacids [1, 2]. Lactoferrin is a net positively charged protein with the isoelectric point (pI) as 8–8.5 [3, 4]. Structure of lactoferrin is single peptidic chain arranged into two domains. Each of these two domains are able to bind an atom of mostly divalent metals as Fe^{2+} or sometimes Cu^{2+}, Zn^{2+} and Mn^{2+} [3, 4]. Lactoferrin is important compound of non-specific immune system [5] and plays a role in a defence against bacterial infection. The bacteriostatic and bactericidal activity of this molecule against Gram-positive and Gram-negative pathogens was found [6]. The fungicidal activity against *Candida* species was also described [7]. Furthermore, the antiviral activity against wide of range human RNA and DNA viruses was revealed [8].

The occurrence of this protein was detected in the secrets of mucus membranes, i.e., this protein can be found in mammal milk, tears, blood plasma, sweat, sperm, vagina effusion or saliva [3]. Saliva as a body fluid is a mixture of biologically important minerals, peptides hormones such as insulin, leptin [9]. Saliva proteins and glycoproteins have

protective effect, especially lysozyme, cystatins, immunoglobulin's, lactoferrin, histatins [10]. The enzymes such as α-amylase or salivary lipase participates in food digestion and are involved in hormonal metabolism [11]. All the organic compounds of saliva are dissolved in water with other low molecular inorganic substances making electrolyte [12]. The amount of water in saliva varies according to physiological state of an organism [12]. Properties of saliva like nonspecific immunity, antimicrobial, antifungal activities are associated with lactoferrin because its ability to bind physiologically active metal ions, which many of bacteria species requires for its own growth [13]. Therefore, the increasing of level of lactoferrin in blood is connected to the inflammation processes in the body [14]. Saliva has great potential to be one of the diagnostic fluids for many illnesses, which relates to immune system disorders, cancer, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, systemic, psychiatric and neurological diseases [15].

For isolation of lactoferrin from saliva it is possible to use protocols based on different pI of this protein, which is above the value of the rest of proteins in the matrix. Therefore, methods based on ion exchange chromatographic separation are mostly applied for this purpose [16–18]. Immobilized single stranded DNA chromatography [19], affinity membrane chromatography [20] and pseudo-affinity chromatography [21] belong to other methods used for isolation or purification of lactoferrin. For quantitative determination there are common used enzyme-linked immunosorbent assay (ELISA) [22, 23], radioimmunoassay (RIA) [24, 25] or luminescence-based immunoanalysis (LSA) [26]. The limit of detection for these methods is moving in the broad range from 10 ng mL^{-1} to 0.2 mg mL^{-1}. There has been also published determination of lactoferrin using other methods like high pressure liquid chromatography connected to the diode array detector with limits of detection varying from 4.5 μg mL^{-1} to 3 mg mL^{-1} [27–29].

In this study, the isolation of lactoferrin from human saliva was optimized using ion-exchange monolithic disc. Monolithic discs and columns are very suitable tool for fast, robust and careful separation of proteins especially thanks to the pore size, which is suitable to large molecules. The CIM disc has only 3 mm thickness but the wide of the disc is almost 12 mm. Monolithic column is thanks to its construction very gentle to the structure of proteins and thus separation can be done under higher flow rate and, at the same time, under lower pressure without damaging of proteins compared to standard columns [30]. Monolithic column also affords great separation properties and high binding capacities [31]. Moreover, monolithic columns can be modified to enhance capacity and separation efficiency. Epoxide groups with diethylamine were used for determination of oligonucleotides [32]. Phosphoric acid modified methacrylate monolithic column shows good results mainly for dynamic binding capacities and permeabilities, and low back pressures for separation of peptides and proteins [33]. Methacrylate monolithic column bearing 3-*N*,*N*-diethylamino-2-hydroxypropyl (DEAE) and quaternary amine (QA) are very stable in harsh condition e.g. alkali condition [34, 35]. The fabrication of monoliths using UV-initiated grafting for attaching of the monolith layer to the plastic wall of the microfluidic chip was described [36] or preparation by single-step UV photocopolymerization [37]. The maximal effort during production of monoliths is focused on the normalized size of inner pore diameter, which must have accurate size. Uniform porous size ~1,500 nm was found as good compromise for good separation of DNA [38, 39] or for whole viral particles [40] or microorganisms such as *Staphylococcus aureus* [41]. The optimal condition for protein separations can be carried out using CIM disc, which can be used for 2D separation using one chromatographic column (conjoint liquid chromatography) [42].

We used the monolithic disc modified by sulfhydryl groups for our purposes. The determination of lactoferrin in the collected fractions was done using photometric Bradford's method and related to the concentration of total proteins. The purity of fractions was estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The optimized method was further employed for determination of lactoferrin in healthy subjects and celiac subject. The results were correlated with ELISA.

Experimental Section

Chemicals and pH Measurement

Standard of bovine lactoferrin was purchased from Sigma Aldrich (St. Louis, MA, USA). HPLC-grade methanol (>99.9 %; *v*/*v*) was from Merck (Dortmund, Germany). Other chemicals were purchased from Sigma-Aldrich in ACS purity unless noted otherwise. Stock standard solutions of lactoferrin (1 mg mL^{-1}) was prepared with ACS water (Sigma-Aldrich) and stored in dark at −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions by mobile phase A. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by software MultiLab Pilot (Weilheim). The pH-electrode (SenTix H, pH 0.0.14/0.0.100 °C/3 mol l^{-1} KCl) was regularly calibrated by set of WTW buffers (Weilheim). Deionised water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified

using Millipore RG (Millipore Corp., Billerica, MA, USA, 18 MΩ)—Mili-Q water.

Liquid Chromatography with Monolithic Disc

Liquid chromatography system Biologic DuoFlow (Biorad, Philadelphia, PA, USA) consists of two chromatographic pumps for carrying of elution buffers, monolithic disc consisted of one CIM disc (3 mm length, 12 mm diameter) modified by $-SO^{3-}$ functional groups (Bia Separations, Ajdovscina, Slovenia), injection valve with 2 mL injection loop, UV–VIS detector and fraction collector (Biorad). Sample fractions were collected in volume of 1 mL prior to elution time of standard peak (lactoferin 50 μg mL^{-1}) into the 2 mL test tube (Eppendorf, Hamburg, Germany).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was used for determination proteins in saliva and of isolated lactoferrin. The 7.5 % separation gel was used while the concentration of focussing gel was 5 %. The apparatus Maxigel (Biometra, Goettingen, Germany) was used. Separation was conducted under 150 V, until forefront in the gel did not reach down end (~1 h). During separation the whole space was cooled by water. The gel was silver stained according to Krizkova et al. [43]. After the incubation of gel for 1 h in solution No. 1 (1.14 % acetic acid, 6.4 % methanol, 0.1 % formaldehyde) with three times washing for 15 min in solution No. 2 [methanol/Mili-Q 1:1 (*v*/*v*)] the gel was incubated 1 min in solution No. 3 (0.02 % sodium thiosulfate) and washed two times per 20 s with distilled water. This was followed by 20 min long incubation in solution No. 4 (0.02 % $AgNO_3$, 0.076 % formaldehyde) and washed 20 s with distilled water. Finally the gel was incubated in solution No. 5 (6 % Na_2CO_3, 0.0004 % $Na_2S_2O_3$, 0.05 % formaldehyde) till appearing of observable colouring. While colouring was optimal (~3 min) gel was immediately washed 2 min × 2 min with distilled water and fixated in solution No. 6 (6.4 % methanol, 1.14 % acetic acid).

Offline Photometric Determination

The automatic spectrometer BS-200 (Mindray, Shenzhen, China) was used for offline spectrophotometric analysis. The device is consisted of space for cuvette (tempered at 37 ± 0.1 °C), regent space equipped by carousel for reagents and samples (cooled on 4 ± 1 °C) and optical detector [44]. For lactoferrin and total protein concentration determination the Bradford′s method was used [45]. Briefly, 10 μl of a sample was added to 190 μl of reagent (0.01 % Coomassie brilliant blue G-250, 4.7 % ethanol, 8.5 % phosphoric acid in distilled water) according to Zor et al. [46]. Detection was carried out at 590 nm and data were collected within 10 min time period.

Determination of Lactoferrin by ELISA

Assay was carried out using commercial ELISA kit (AssayMax Human Lactoferrin ELISA kit, AssayPro, St. Charles, MO, USA). Standard or diluted sample (1,000×) were adsorbed on polystyrene 96 well microplate and incubated for 2 h at 25 °C. After five-times repeated washing of wells with wash buffer, biotinylated lactoferrin antibody was added to each well and incubated for 1 h. After washing the microplate, 50 μl of streptavidin-peroxidase conjugate was added per well and incubated for 30 min. Subsequently, the third washing was applied. For detection 50 μl chromogenic substrate was used per well and incubated for 15 min. Finally, 50 μl of stop solution was added. Absorbance was read on a microplate reader (TECAN INFINITE M200PRO, TECAN, Männedorf, Switzerland) at 450 nm immediately.

Real Samples of Saliva

For the experiment seven persons aged 23–28 years (four women, two men) and one celiac person (sample No. 4, woman) was chosen. Saliva samples were collected using Salivette tubes (Sarstedt, Germany). Attached cellulose was swallowed by subject within 2 min and then centrifuged in Salivette tube under 3,000 rpm for 5 min (Universal 320, Hettich Zentrifugen, Tuttlingen, Germany). Clear solution of saliva was than diluted in ratio 1:1 by 25 mM Tris–HCl (pH = 7) and subsequently filtered using microfilter (microStar 0.45 μm CA, Costar Cambridge, MA, USA). 200 μL were used for protein assay and 2 mL of obtained sample was used for liquid chromatographic analyses.

Descriptive Statistics

Data were processed using MICROSOFT EXCEL® (Redmond, WA, USA) and STATISTICA.CZ Version 8.0 (Prague, Czech Republic). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). The detection limits (three signal/noise, *S*/*N*) were calculated according to Long and Winefordner [47], whereas *N* was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

Recovery of lactoferrin was evaluated with homogenates spiked with standard. Before the extraction, 100 μL of lactoferrin standard and 100 μL of water were added to saliva samples. Homogenates were assayed blindly and

lactoferrin concentrations were derived from the calibration curves. The spiking of lactoferrin was determined as a standard measured without presence of real sample. Calculation of recovery was carried out as indicated by Causon [48] and Bugianesi et al. [49].

Results and Discussion

In this study, the strong cation exchanger column with $-SO_3$ groups was used because the separation has to be driven by pI of lactoferrin (pI > 8), which is higher than rest of matrix proteins (pI < 8). Therefore, $-SO_3$ CIM disc is promising for the separation purposes. Due to its dimensions (12 mm × 3 mm) and low height equivalent of theoretical plate (HETP) value in isocratic mode we applied gradient elution, where we attempted to obtain optimal separation conditions.

Gradient Settings

To isolate protein of interest from human saliva sample and to achieve higher resolution using proper fraction collecting, we had to optimize various experimental conditions compared to our previously published paper [50], where we eluted lactoferrin form pre-treated milk sample by step elution's only. As mobile phase A we used 25 mM Tris–HCl (pH 7), which had minimal impact on the elution of lactoferrin from the CIM column during sample injection but provides good efficiency for eluting of redundant saliva proteins. As mobile phase B we used gradient of NaCl (in 25 mM Tris–HCl) which concentration was optimized. To obtain effective separation of lactoferrin from other proteins it was necessary to optimize linearly increasing gradient (Fig. 1a). Optimal gradient was considered by determination of peak height and width. Standard of lactoferrin (50 μg mL^{-1}) was eluted by five variants of gradient with subsequent linear final hold and ending re-equilibration. As it is shown in Fig. 1b, peak high maximum was in 65 mAU when gradient iii was used. Afterwards peaks height had decreasing trend. As the most effective gradient we have chosen linear increasing gradient iii: 0 → 6 mL (0 % B), 6 → 12 mL (100 % B), 12 → 16 mL (100 % B), 16 → 17 mL (0 % B), 17 → 21 mL (0 % B). In consensus of preliminary experiment we estimated the flow rate was 4 mL min^{-1} and concentration of NaCl in mobile phase B 2 M. UV detector for setting of collection window where fraction was collected was set on 280 nm according to [50].

Influence of Flow Rate on Separation

The optimal flow rate was tested by determination of peak width and peak height using standard of lactoferrin detected by UV detector in concentration 50 μg mL^{-1}. In consensus of preliminary experiment the 2 M NaCl in mobile phase was used. The maximal effort was to obtain such conditions to elute the lactoferrin in as small volume as possible and thus to reach of its minimal dilution. Flow rate was tested using values of 2, 2.5, 3, 3.5, 4, 4.5 and 5 mL min^{-1}. It clearly follows from the obtained results that higher flow rate caused narrowing of the peak and increasing of the peak height up to flow rate of 4 mL min^{-1} (Fig. 2a), which was app. five times higher than we used in our previous paper for milk sample [50]. Under 4 mL min^{-1} flow rate, peak was the highest and narrowest as it is clearly shown in Fig. 2b. In addition, this also contributes to higher speed of the isolation and

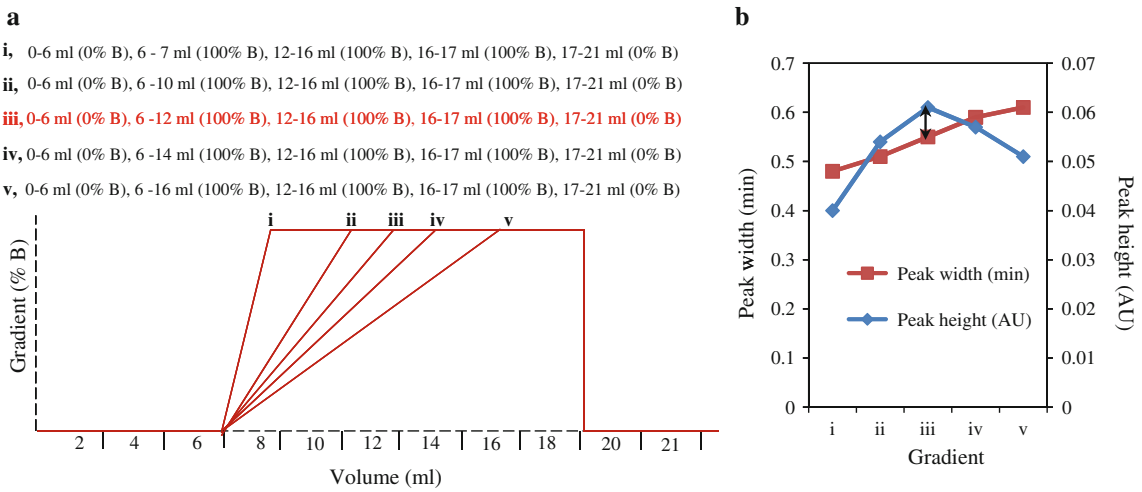

Fig. 1 **a** Tested increasing linear gradients marked as *i*, *ii*, *iii*, *iv* and *v*. **b** Dependence of peak height and peak width on gradient. For testing 2 M NaCl in mobile phase B and flow rate 4 mL min^{-1} was used

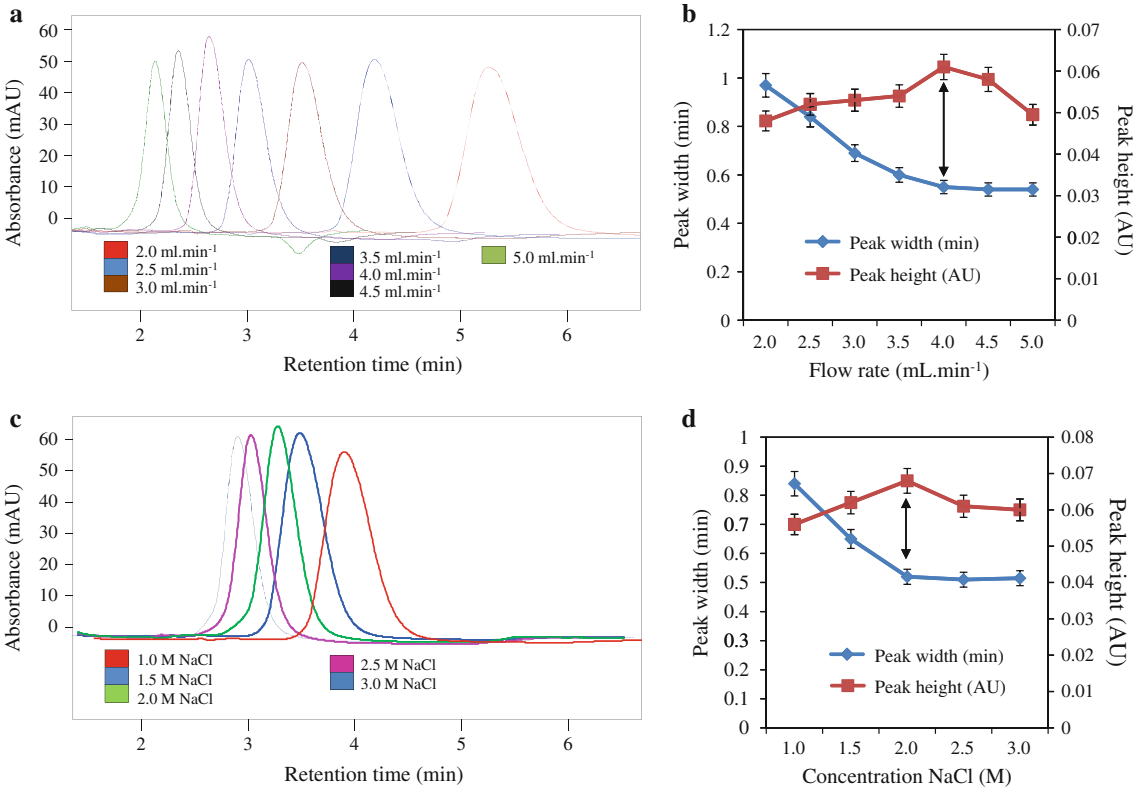

Fig. 2 **a** Overlay of chromatograms of standard of lactoferrin (50 μg mL^{-1}) measured under various flow rate (2–5 mL min^{-1}) of mobile phase. **b** Dependence of peak height and width on flow rate. **c** Overlay of chromatograms of standard of lactoferrin (50 μg mL^{-1}) eluted by different tested ionic strength ranging from 1 to 3 M NaCl in mobile phase B. **d** Dependence of peak height and width on ionic strength of mobile phase B. Other experimental details see in "Liquid Chromatography with Monolithic Disc". For testing it was used 2 M NaCl in mobile phase B and gradient iii (as mentioned above)

decreases time of analysis to less than 4 min compared to paper by Adam et al. [50], where time of a measurement was 25 min Decreasing of the peak height beyond the value of 4 mL min^{-1} is probably caused by the decreasing of numbers of ionic interactions during passing of injected sample through the column and thus less pre-concentration of lactoferrin on the column limited by higher mass transfer.

Influence of Ionic Strength on Separation

Next to the flow rate the concentration of NaCl in the eluent was the crucial factor. We applied concentrations of NaCl 1.5, 2, 2.5 and 3 M in 25 mM Tris–HCl (pH = 7). It clearly follows from the results obtained that higher concentration of NaCl facilitates the elution of lactoferrin as is demonstrated on the overlay of chromatograms in Fig. 2c. Peak width became to be minimal after reaching the concentration of 2 M and the peak height was slightly higher compared to other applied concentrations of NaCl. The most effective elution was reached under the concentration of 2 M NaCl (Fig. 2d). In consequence of concentration of salts in collected fraction it is better to hold this value as low as possible due to known influence on destabilisation or denaturation of structure of proteins [51].

Determination of Efficiency of the Column

For the interpretation of efficiency of the CIM disk column simplified van Deemter plot has been used [$H = L/N$; $N = 16 \times (t_r/W_{base})^2$]. The analysis of the column efficiency was done using different linear flow rates but under the optimal gradient elution conditions described above. The concentration of lactoferrin 50 μg mL^{-1} was used. It clearly follows based on the results obtained that optimal HETP value from van Deemter plotting has been reached between 4 and 5 mL min^{-1} (Fig. 3).

Isolation of Lactoferrin from Saliva

The above optimized method has been used for separation and isolation of lactoferrin from human saliva. The resulting optimal approach for the isolation of lactoferrin from the saliva is shown as scheme in Fig. 4. Samples, which have been prepared according the "Real Samples of Saliva", were separated and 1 mL fractions has been

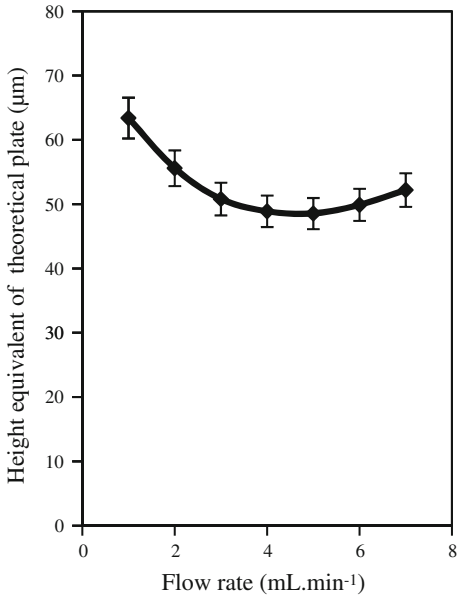

Fig. 3 Dependence of height equivalent of theoretical plate (HETP) on linear flow rate of mobile phase for CIM monolithic disc assembled on the instrument. Other details see in "Liquid Chromatography with Monolithic Disc" and "Determination of Efficiency of the Column"

collected in the time of separation 2.66–3.1 min as it is shown in Fig. 5a. From the obtained chromatogram it is obvious that the majority of the saliva protein matrix with lower pI was eluted in earlier time than lactoferrin. Protein composition of whole saliva and isolated lactoferrin has been verified by SDS-PAGE, which was carried out according the "Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis". Electrophoretogram of real samples of saliva without isolation is shown in Fig. 5b, where lot of other bands which represents the saliva matrix have been visualised. On the s electrophoretogram (Fig. 5c) the same samples after isolation on monolithic disc are shown. There is well observable only one band, which fits to the ladder in position about 77 kDa, which belongs to the lactoferrin according to the comparison of distance of bend from the forehead of the electrophoretogram. The intensity of bands of the isolated lactoferrin corresponded with determined concentration by this method. None of any other proteins acting as impurities were detected.

Collected fraction as well as the original sample of saliva have been subsequently analysed by automatic photometer wherein for purpose of total protein determination the Bradford's method has been implemented ("Offline Photometric Determination"). The complete ion exchange liquid chromatography method, which was hyphenated to offline photometric determination using Bradford's method, was than calibrated on lactoferrin within the range from 0.1 to 62 μg mL^{-1} with good linearity $R^2 = 0.992$ (Fig. 6a) and limit of detection (3 *S/N*) as 10 ng mL^{-1}. Other analytical parameters of determination are shown in Table 1.

Thanks to the fact that the saliva is complex of number of substances, which might interfere and data might be thus misinterpreted we estimated the recovery of the isolation. We added the known concentration of lactoferrin into three various isolated real samples, which has been diluted (1:1) using standard preparation procedure for estimation of recovery mentioned in "Descriptive Statistics". By this

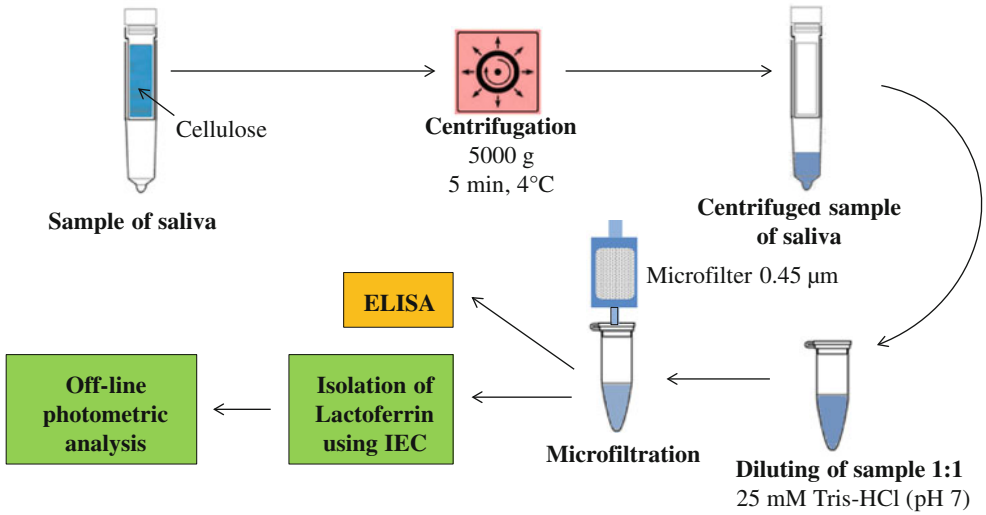

Fig. 4 Scheme of lactoferrin determination from human saliva. The saliva was collected using Salivette tubes and centrifuged. Subsequently the saliva was diluted in 25 mM Tris (pH 7) and filtrated. Prepared saliva was analysed by ELISA and the lactoferrin was isolated using ion exchange liquid chromatography with UV detection. The concentration of lactoferrin was determined using off-line photometric detection with Brdicka's method

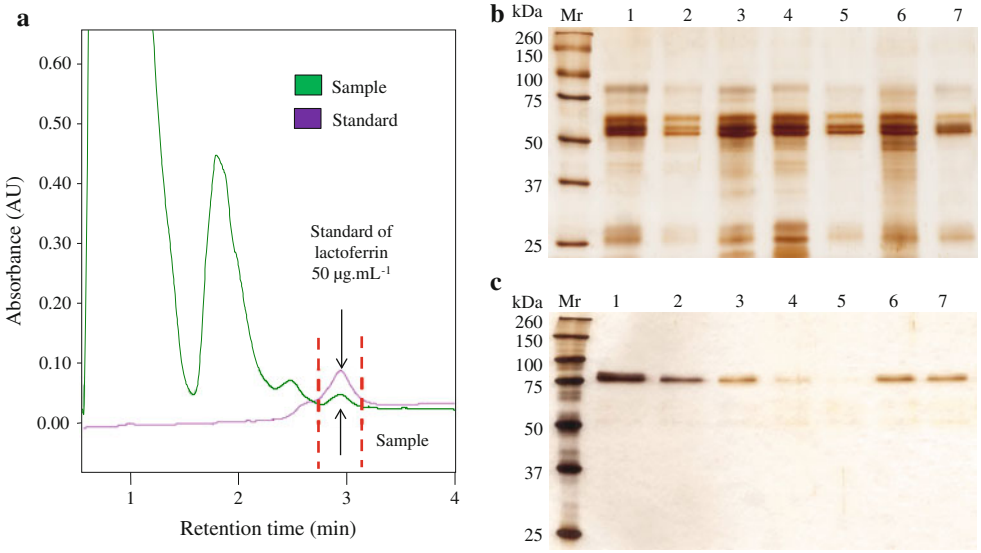

Fig. 5 **a** Overlay of chromatogram of sample and of standard of lactoferrin (50 µg mL^{-1}). The collected fraction is marked by *red dashed line*. **b** Electropherogram of all seven determined samples of whole saliva of studied persons. **c** Electropherogram of isolated fractions from sample of saliva after ion exchange liquid chromatography using CIM monolithic disc assembled on the instrument described in "Liquid Chromatography with Monolithic Disc". All other details concerning SDS-PAGE analysis see in "SDS-PAGE and Real Samples of Saliva"

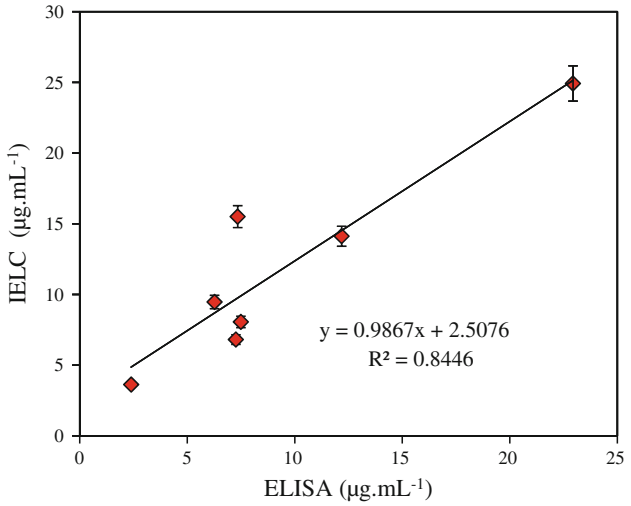

Fig. 6 Correlation curve of determined lactoferrin by ELISA and ion exchange liquid chromatography IEC method ($n = 3$)

standard addition into the real sample we found the recovery was approximately 51 ± 4 % ($n = 3$). This was probably caused by low mass transfer capacity of the CIM disc. On the other hand thanks to the minimal sample pre-treatment requirements and possibility of usage of up to 5 CIM disc in series assembled into the one column holder column length can be increased as well as its separation capacity.

Protein Content Determination of Saliva Samples

We determined the total protein concentration at all studied subjects. Samples have been collected according to "Descriptive Statistics" and analysed by Bradford's assay according to "Offline Photometric Determination". Average concentration of total protein content has been determined as 430 ± 220 µg mL^{-1} ($n = 10$). The obtained concentrations of lactoferrin have been subsequently expressed as µg of lactoferrin per mg of whole proteins determined by studied subjects. The concentrations are shown in Table 2. It is obvious that the obtained results by normal patients showing physiological range between 5 and 25 µg mL^{-1} with approximately 12 µg mL^{-1} and this is in good agreement with previously published results, which reported concentrations from 4.7 µg mL^{-1} [52] to 10.5 µg mL^{-1} [53]. Our results also show that the celiac patient demonstrated markedly higher level of lactoferrin (sample 5). Celiac disease belongs to the category of autoimmune disorder of small intestine which is self-manifesting as elevated immune response to the gluten [54].

Correlation of CIM Column with ELISA

Human saliva samples were also assayed by commercial ELISA kit, which was carried out according the "Determination of Lactoferrin by ELISA". The method was calibrated on lactoferrin within range from 0.625 to 10 ng mL^{-1} with good linearity $R^2 = 0.999$ and limit of detection 0.1 ng mL^{-1}. Other analytical parameters are shown in Table 1. We analysed six healthy persons and one celiac patient by our optimized method using monolithic disc separation and made correlation to the standard ELISA kit. The correlation for the two used methods demonstrated satisfactory regression coefficient $R^2 = 0.8446$ (Fig. 6). Analysis of lactoferrin in saliva by monolithic disc and subsequent offline photometric detection is faster and cheaper method providing compared to ELISA commercial kit. Analysis of one sample takes less than 20 min.

Table 1 Analytical parameters of determination of lactoferrin for both compared methods ELISA and developed method of ion exchange liquid chromatography

Method	Regression equation	Linear dynamic range (ng mL^{-1})	R^2	LOD[a] (ng mL^{-1})	LOQ[b] (ng mL^{-1})	RSD[c] (%)
ELISA	$y = 0.1018x - 0.003$	0.625–10	0.999	0.10	0.3	4
IEC	$y = 2.0274x + 8.4619$	100–62,000	0.992	10	30	3

[a] Limit of detection

[b] Limit of quantification

[c] $n = 3$

Table 2 Concentration of lactoferrin in human saliva samples for both compared methods enzyme-linked immunosorbent assay (ELISA) and developed method of ion exchange liquid chromatography (IEC) convert into whole protein in saliva

Subject	ELISA[a]		IEC[a]	
	μg mL^{-1}	μg mg^{-1} of proteins	μg mL^{-1}	μg mg^{-1} of proteins
1	14 ± 5	39 ± 5	15 ± 4	42 ± 4
2	7 ± 2	15 ± 2	14 ± 3	31 ± 3
3	5 ± 1	9 ± 1	6 ± 1	10 ± 1
4	9 ± 3	23 ± 3	14 ± 4	35 ± 4
5	18 ± 8	89 ± 8	17 ± 8	84 ± 8
6	12 ± 3	36 ± 3	14 ± 4	42 ± 4
7	4 ± 1	9 ± 1	7 ± 2	13 ± 2

[a] $n = 3$

Conclusion

In this study, we developed a fast and robust separation approach for determination of lactoferrin in human saliva sample. Compared to our previous paper Adam et al. [50] where, we isolated the Lactoferrin from the milk by simple peristaltic pump with step gradient, we used and optimized different system for isolation using BioLogic DuoFlow system and linear increasing gradient in this study. From tested parameters during optimization we found the flow rate 4 mL min^{-1}, ionic strength of eluent 2 M NaCl and gradient type of elution as the optimal. Thanks to this improvement we developed the rapid and robust method for lactoferrin isolation from human saliva. The fastness of the separation reported by the optimum of HETP between 4 and 5 mL min^{-1} according Van Deemter theories confirms the time span for one analysis which takes only 4 min. That is comparatively less than is needed for commercial ELISA kit (20 min). We also compared our suggested separation approach using monolithic disc to standard commercial ELISA method. For ELISA the sample had to be 1,000× times diluted first but the achieved correlation coefficient $R^2 = 0.8446$ reports the good agreement in obtained values from seven tested patients. Future perspectives such as easily implementation of the monolithic stationary phase in microfluidic systems can be considered.

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