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DETECTION OF THE MILK PROTEINS BY RP-HPLC

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Abstract
In this study the bovine κ-, α- and β-caseins, α-lactalbumin and β-lactoglobulin by HPLC-UV using an RP column was detected by developed a simple and rapid method. Gradient elution was carried out at a 1 ml/min flow rate, and a temperature of 25 °C, using a mixture of two solvents. Solvent A was acetonitrile: water: trifluoroacetic acid (100:900:1) and Solvent B was acetonitrile:water:trifluoroacetic acid (900:100:1). The effluent flow was monitored by a UV detector at 220 nm. Different chromatographic profiles were obtained for bovine milk proteins which was prepared with different methods for RP-HPLC. It was suggested that using of Solvent A and Solvent B during the sample preparation by addition into the sample before injection at given ratio was allowed the separation more clear and effective than the other sample preparation methods. By the current sample preparation technique and elution practice which was occurred in this study, it was achieved detection of bovine milk proteins was being simple, rapid and sensitive provide.

Keywords: Milk, proteins, caseins, whey proteins, RP-HPLC.

SÜT PROTEİNLERİNİN RP-HPLC İLE SAPTANMASI

Özet
Bu çalışmada, κ-, α- and β-kazein ile α-laktalbumin and β-laktoglobulin, bir RP kolonunun kullanıldığı HPLC-UV ile basit ve hızlı bir yöntem geliştirilerek saptanmıştır. Gradient elüsyonu, 1 ml/dk akış hızında ve 25 °C sıcaklıkta iki çözücü karışımı kullanılarak yürütülmüştür. A çözücüsü asetonitril: su:trifloroasetik asit (100:900:1) ve B çözücüsü asetonitril: su: trifloroasetik asit (100:900:1) karışımlarından oluşmaktadır. Akış, bir UV dedektörü kullanılarak 220 nm'de kaydedilmiştir. Farklı yöntemler kullanılarak RP-HPLC analizi için hazırlanan örneklerde, süt proteinlerine ait farklı kromatografik profiller elde edilmiştir. Diğer örnek hazırlama yöntemlerine kıyasla, enjeksiyondan önce belirtilen oranlarda A ve B çözücülerinin kullanıldığı örnek hazırlama yöntemi ile separasyonun daha keskin ve efektif olduğu ortaya konulmuştur. Bu çalışmada ortaya konulan örnek hazırlama tekniği ve elusyon pratiği ile, süt proteinlerinin analizi basit, hızlı ve duyarlı bir şekilde gerçekleştirilmiştir.

Anahtar kelimeler: Süt, proteinler, kazeinler, serum proteinleri, RP-HPLC.

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INTRODUCTION

Milk is a complex food, constituted mainly of water, proteins, lactose, fat and inorganic compounds. Bovine milk typically consist of 3.0-3.5% (w:w) proteins. According to its solubility at pH 4.6 (20 °C), the protein fraction can be separated into caseins and whey proteins. 80% of milk proteins that precipitated at pH 4.6, is caseins. This protein complex, presents at micelle form in normal milk, consists of α_{s1}-, α_{s2}-, β- and κ-casein, and they occur in the ratio of 4:1:4:1, respectively. The whey proteins, which are soluble at pH 4.6, consist mainly of β-lactoglobulin (β-lg) and α-lactalbumin (α-la) in a ratio of 3:1. Immunoglobulins (Ig) and bovine serum albumin (BSA) are also categorized in the whey proteins (1).

The determination of milk proteins has been studied by several chromatographic and electrophoretic techniques, but none of them gave optimum separation of all the major bovine milk proteins, in particular of the whey proteins (2-10).

Simultaneous separation of the caseins and whey proteins has been reported by capillary electrophoresis, isoelectric focusing and high-performance liquid chromatography (HPLC) over the past few years. Methods using HPLC did not separate α-lactalbumin (α-La) and β-caseinB (β-CNB) (6), α-La and β-lactoglobulin (β-lg) (11-13), or κ- casein (κ-CN) and α_{s2}-casein (α_{s2}-CN) (8, 14).

Studies using reverse-phase high-performance liquid chromatography (RP-HPLC) have shown that the β-CN peak area decreases as the time interval increases between sample preparation and injection, when the samples were prepared with urea (13). Also, the type of reducing agent used in the sample preparation affects the separation of α-La and β-Lg. When reducing agent was not used, α-La eluted between α_{s1}-CN and β-CN (6, 14).

Recently the simultaneous separation methods were developed and reported for six major bovine milk proteins (15-18). All of these methods use complex sample preparation and RP-HPLC tools. And they also need to evaluate the caseins and whey proteins separately, before injection.

Present chromatographic methods for milk proteins lack the capacity to user-friendly separation of the major bovine milk proteins. In the field of bovine milk quality, it is of particular interest caused by social and economic importance of the dairy products. Therefore, it is necessary to have sensitive and simple analytical methods available for the determination of all milk components, especially milk proteins. The detection of the simple manipulation during the sample preparation requires and a clear analytical characterisation of each major bovine milk proteins constitutes the goal of this work.

The objective of the current study is to develop a simple and accurate RP-HPLC method for separation of the major bovine milk proteins. Also the method is allowing to a user-friendly sample preparation procedure and use less time interval between injections on the subsequent separation of milk proteins by RP-HPLC.

MATERIALS AND METHODS

Samples, standards and reagents

Raw and pasteurized bovine milk samples were obtained from a local dairy (A.O.Ç. Dairy Plant, Ankara, Turkey). All milk samples were skimmed at 5000 rpm and 7 °C for 10 minutes. Sodium azide solution was added to the skim milk samples at 1:10000 (w/v) to prevent microbial growth. The samples were stored at 7 °C in refrigerator.

For identification of milk proteins, several standards were prepared with purified bovine milk proteins (κ-CN, α_s-CN, β-CN, α-la, β-lg) which were purchased from Sigma (Witten, Germany).

Acetonitrile and trifluoroacetic acid and ultra-pure water were of HPLC grade. All other chemicals were of analytical grade.

Sample preparation

Purified proteins

Purchased standards were dissolved in 50 mM phosphate buffer (pH 6.8), the final concentrations were being as follows; β-CN 62.5 mg/mL, κ-CN 25 mg/mL, α-CN 62.5 mg/mL, α-la 50 mg/mL, β-lg 125 mg/mL. And then individual standards were diluted 50 mM phosphate buffer (pH 6.8) and solvent A and solvent B mixture (70:30), seperately. The individual standards which were diluted in phosphate buffer and the solvents were mixed

same ratio. 100 µl of sample were diluted with 3.7 ml of the buffer or solvents and then injected into the column (C-18 RP-HPLC column). At the same time another purified protein mixture was prepared as that of milk protein ratio (80% caseins and 20% whey proteins).

Skim milk samples

Raw and pasteurized skim milk samples were diluted with two different solutions as mentioned above; 50 mM phosphate buffer (pH 6.8) and solvent A and solvent B mixture (70:30) and then filtered through 0.45 µm cellulose acetate filter (EG0492-1) before injection.

Caseins and whey proteins (acid precipitation method)

2 ml of skim milk sample was diluted with 14 ml distilled water in a test tube. pH values of the samples were adjusted to pH 4.3 with 200 µl acetic acid solution (10 %, v/v) and then 200 µl of 1 M sodium-acetate solution was added. The volume of the aliquot was up to 20 ml with distilled water. The aliquot was centrifuged at 3000 g and 20 °C for 10 minutes. The volume of supernatant (sn) was recorded (approximately 18.5 ml of sn). The resultant precipitate (ppt) was acidified with 1 mM sodium-acetate buffer (pH 4.3) at the same volume of sn, precipitated again and then it was centrifuged at the same conditions. The second sn was poured and 620 µl of phophate buffer was added into the resultant ppt. The mixture was dissolved in a sonicator. The dissolved casein fractions of the milk samples (ppt) was diluted with three different solutions above mentioned. The solutions were 50 mM phosphate buffer (pH 6.8) , the phosphate buffer contained 0.1% TFA and a mixture of solvent A and solvent B (70:30). The aliquots were filtered through 0.45 µm cellulose acetate filter before injection. The first obtained sn, which was consisted of whey proteins, was also examined with RP-HPLC.

RP-HPLC

A modified method proposed by several authors (9, 15-18) was used to separate the major proteins of bovine milk (caseins, β-Lg and α-La) in a single run.

The Agilent 1100 series HPLC system consisted of a quarternary pump (Agilent, G1311A), a manual injection block (Agilent, G1328B), a variable wavelength UV-detector (Agilent, G1314A), a column thermostate (Agilent, G1316A) and degasser (Agilent, G1379A). The equipment was controlled by a software (Agilent ChemStation) that controls the solvent gradient, data acquisition and data processing. A silica-based C-18 RP-HPLC column (250 mm length x 4.6 mm i.d., Agilent Zorbax 300SB-C18, particle size 5 µm, pore size 30 nm) was used for protein separation. All solutions were filtered through a nylon filter (47 mm, 0.45 µm, EG0492-1).

Chromatographic conditions were as follows; *Solvents.* A: Acetonitrile, water and trifluoroacetic acid in a ratio of 100:900:1 (v/v/v). B: Acetonitrile, water and trifluoroacetic acid in a ratio of 900:100:1 (v/v/v). *Total run time*; 30 minutes. *Column temperature*; 25 °C. *Flow rate*; 1.0 mL min^{-1}. *Detection wavelength*; 220 nm. *Injection volume of final sample solution*; 20 µL.

A solvent gradient programme started at 20% of solvent B, and it was generated immediately after sample injection by increasing the proportion of solvent B to 46% at the end of the run. And then it was returned to the started conditions in 2.4 minutes.

RESULTS AND DISCUSSION

In this study the RP-HPLC conditions were optimised for mobile phase, gradient, operating temperature, flow rate and detection wavelength.

The aim of the study was to develop a sample preparation for HPLC procedure which was rapid and simple. That's why it was started to examine the separation of caseins (CN) and whey proteins (WP) from raw and pasteurized milk individually. The obtained CN precipitates were diluted with phosphate buffer and mixture of solvents, in raw and pasteurized milk.

It is shown from the Figure 1 that the CN peaks were obtained sharper and more clear in the samples prepared with mixture of solvents than that of phosphate buffer which was used widely in the former studies. Also the supernatants of the raw and pasteurized skim milk samples obtained from acid precipitation were analysed (Figure 2).

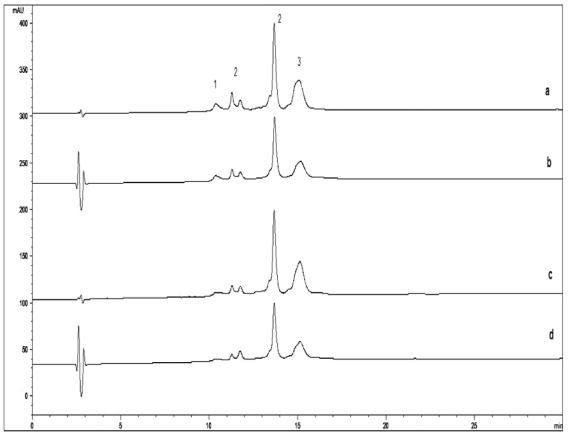

Figure 1. Separation of bovine caseins, obtained from raw and pasteurized skim milk by acid-precipitation sample preparation procedure, by RP-HPLC using different sample dilution solutions. Details of the sample preparation and separation conditions are described in Material and Methods. Chromatograms of raw bovine skim milk caseins diluted with solvent A: solvent B (70:30) mixture (a), diluted with phosphate buffer (b), pasteurized bovine skim milk caseins diluted with solvent A: solvent B (70:30) mixture (c), diluted with phosphate buffer (d). Peak identifications are shown; 1= κ-casein, 2= α-caseins, 3= β-casein.

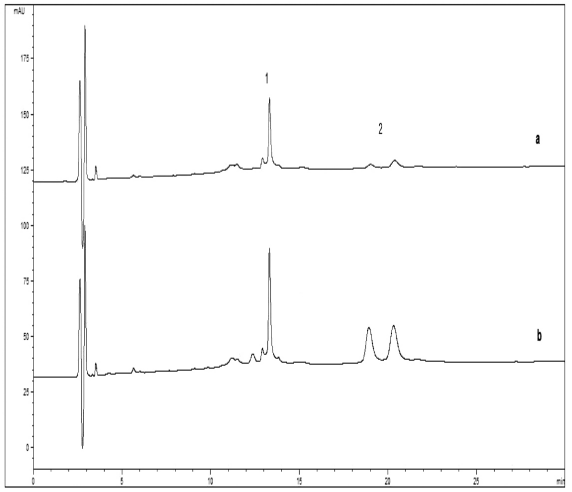

Figure 2. Separation of bovine whey proteins obtained from raw and pasteurized skim milk by acid-precipitation sample preparation procedure, by RP-HPLC. Chromatograms of pasteurized skim milk whey proteins injected as such (without dilution) (a), raw skim milk whey proteins injected as such (b). Peak identifications are shown; 1= α-lactalbumin, 2= β-lactoglobulin.

For examine the apparent effect solvent mixtures during the sample preparation on whey protein separation, the supernatant which was obtained from acid precipitation of the milk samples was diluted with mixture of solvents at the ratio of 1:1. However, the area of whey protein peaks were found lower in that diluted samples caused by lower protein concentration than that of supernatant as predicted. This result shows that the supernatant (whey protein fraction) has to be injected without any dilution instead of diluting with mixture of solvents if acid precipitation method is used for identification of milk proteins with HPLC (Figure 3).

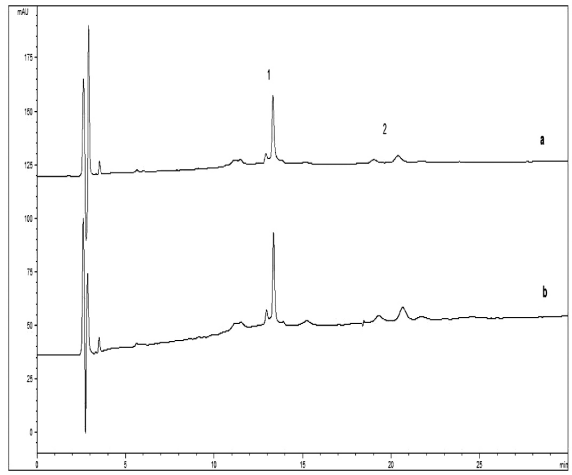

Figure 3. Separation of bovine whey proteins obtained from pasteurized skimmilk by acid-precipitation sample preparation procedure, by RP-HPLC diluted with solvent A: solvent B (70:30) mixture (a), and without dilution (as such) (b). Peak identifications are shown; 1= α-lactalbumin, 2= β-lactoglobulin.

On the other hand modifiers are substances added to the mobile phase, usually in relatively low concentration that interact with both of the stationary phase and sample constituents to alter retention. Interaction with sample components vary with the type and concentration of modifier. Trifluoroacetic acid (TFA) is a modifier used most frequently for peptide and protein separations in RP-HPLC and it plays an ion-pairing agent role. At the same time phosphate buffer or acetate buffer which dissolves the protein easily at given pH degrees are commonly used for protein separation and they may offer improved resolution or peak shape (15, 18).

For this reason CN fractions (ppt obtained from the acid precipitation method) which was obtained from pasteurized milk for RP-HPLC were diluted with three different solutions;

- CN precipitate which was diluted with phosphate buffer (50 mM, pH 6.8)

- CN precipitate which was diluted with the phosphate buffer contained 0.1% TFA
- CN precipitate which was diluted with mixture of solvents

As shown from the Figure 4, the most clear and sharp peaks were obtained from the sample diluted with mixture of solvents before injection. It is suggested that it is necessary to use not only TFA but also acetonitrile during the preparation of bovine milk samples for the best separation of major proteins in RP-HPLC methods. It was also found that the CN fractions which were prepared in mixture of solvents contained both of TFA and acetonitrile gave better separated peaks during the RP-HPLC.

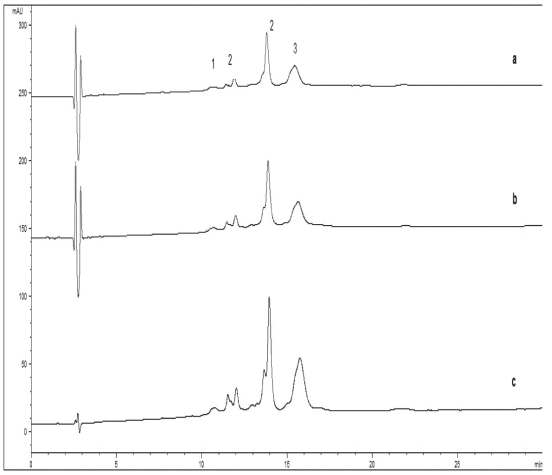

Figure 4. Separation of bovine caseins obtained from pasteurized skimmilk by RP-HPLC using different sample dilution solutions. Chromatograms of casein diluted with phosphate buffer (a), diluted with phosphate buffer contained 0.1 % trifluoroacetic acid (b), diluted with solvent A: solvent B (70:30) mixture (c). Peak identifications are shown; 1= κ-casein, 2= α-caseins, 3= β-casein.

For deciding the reproducibility and accuracy of the current sample preparation method, the external standards of purified major bovine milk proteins were examined individually and in the mixtures.

The β-CN (62.5 mg/mL), κ-CN (25 mg/mL), α-CN (62.5 mg/mL), α-la (50 mg/mL), β-lg (125 mg/mL) standards were diluted with solvent A and solvent B mixture (70:30, v/v) at given ratio. The elution times of the major bovine milk proteins were detected (Figure 5).

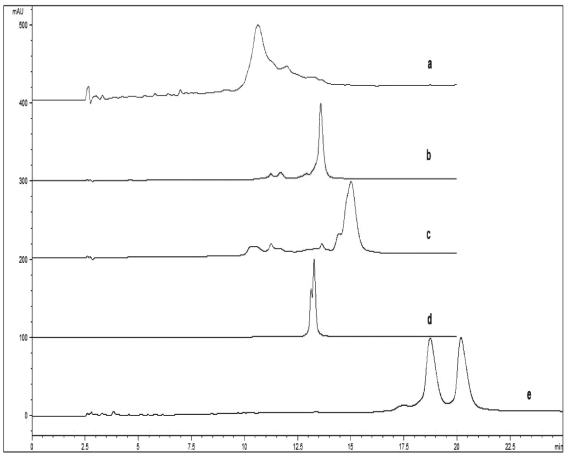

Figure 5. Separation of purified bovine milk protein standards by RP-HPLC. (a): κ-casein, (b): α-caseins, (c): β-casein, (d): α-lactalbumin, (e): β-lactoglobulin.

And then it was determined the more apparent chromatograms in raw and pasteurized skim milk samples at the same dilution ratio with mixture of solvents (Figure 6). Additionally it can be observed the effect of heat treatment on heat-labile bovine milk proteins with this method at the same accuracy.

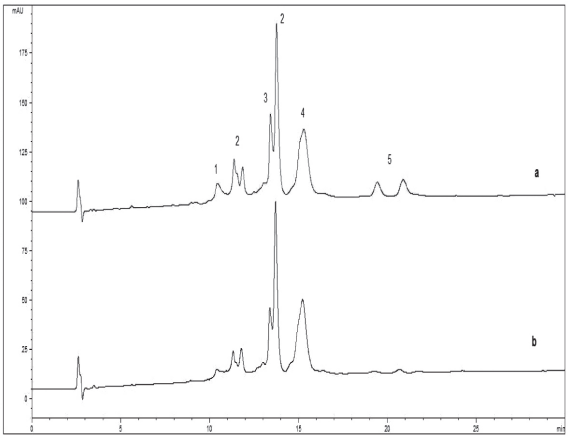

Figure 6. Separation of bovine proteins in raw (a) and pasteurized (b) skimmilk samples diluted with solvent A: solvent B (70:30) mixture by RP-HPLC. Peak identifications are shown; 1= κ-casein, 2= α-caseins, 3= α-lactalbumin, 4= β-casein, 5= β-lactoglobulin.

The current sample preparation procedure was also applied to skim milk samples without isolation of caseins and whey proteins by acid precipitation method. The direct injection of diluted skim milk samples with mixture of solvents gave the same novel chromatograms. It means that it is not nec-

essary to separate the caseins and whey proteins by acid precipitation before RP-HPLC analyses. Shorter preparation and injection time is need with proposed method than that of other methods.

Two different purified protein mixtures were prepared. One of them was included 5 major proteins at the same CN : WP ratio of bovine milk (80% : 20%, respectively). The other one was included the purified stock proteins at the same volume of each other (100 µl from each were mixed). When the standard was prepared as the ratio of protein fractions was same as in milk, the peaks of each protein were found to be similar as obtained from milk samples (Figure 7).

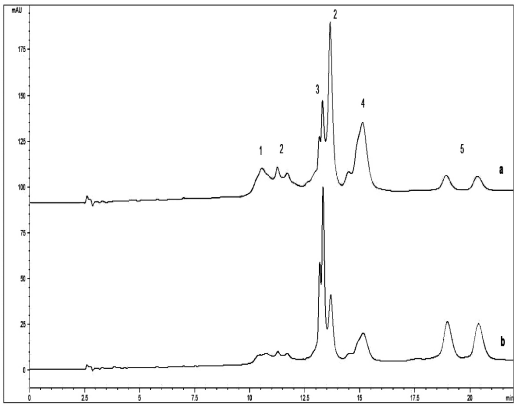

Figure 7. Separation of mixed purified bovine milk protein standards diluted with solvent A: solvent B (70:30) mixture by RP-HPLC. (a) The ratios and concentrations of the individual major proteins were adjusted to that of original skimmilk, (b) the mixture which was prepared by mixing of 1 mL of each 5 stock purified protein standard. Details of the sample preparation and separation conditions are described in Material and Methods. Peak identifications are shown; 1= κ-casein, 2= α-caseins, 3= α-lactalbumin, 4= β-casein, 5= β-lactoglobulin.

On the other hand, if the standard milk proteins were not prepared at the same ratio in milk, the peaks of α-CN and α-La obtained from standard solution would be different, compared to peaks of α-CN and α-La obtained from milk samples (Figure 7). It is also shown that the effect of the difference between the concentrations of α-CN and α-La mixture in Figure 8.

CONCLUSIONS

Despite the effect of phosphate buffer on caseins and whey proteins was known well, it is suggested that the use of an eluent which includes TFA

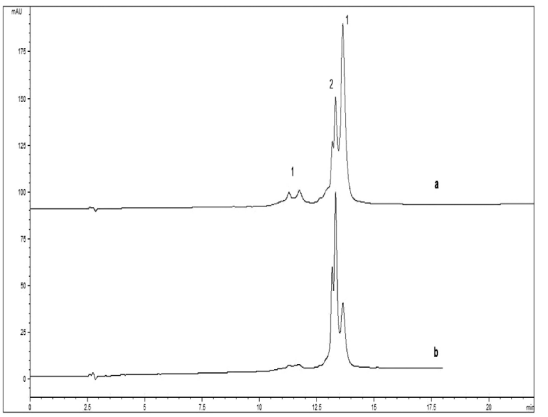

Figure 8. Separation of mixture of purified alfa-caseins and alfa-lactalbumin diluted with solvent A: solvent B (70:30) mixture by RP-HPLC using different ratio of dilution. (a) The ratio and concentrations of the proteins were adjusted to that of original skimmilk, (b) the mixture which was prepared by mixture of 1 mL of each 2 stock prufied protein solutions. Details of the sample preparation and separation conditions are described in Material and Methods. Peak identifications are shown; 1= α-caseins, 2= α-lactalbumin.

and acetonitrile caused more effective results in RP-HPLC. Direct dissolving of proteins in aqueous solutions can be slow. During the separation in RP-HPLC, addition of aqueous portion of solvents (Solvent A) with high proportion, proteins that appeared insoluble will dissolve rapidly. And then increasing of the organic solvent (Solvent B) proportion, the wetting problems of hydrophobic parts are overcome and these parts introduce into the liquid phase. If a mixture of solvents is used during the sample preparation step, the wetting and insolubility problems of hydrophobic proteins and / or hydrophobic parts of the proteins can be overcome easily. Thus, it is also suggested that it causes to sharper and more clear peaks if the HPLC solvents are used in the samples before injection. Furthermore this method provides that time interval decreases between sample preparation and injection compared to other methods in literature. When the mixture of solvents is used for dilution of skim milk samples, it is found that using of a separation method is not necessary (for example, acid precipitation) for the isolation of CN and WP during RP-HPLC analysis of major milk proteins. However, these preliminary results need validation in the future work, it is thought that the results will contribute to the studies on the separation of major milk proteins by RP-HPLC.

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