Study of Different Types of Milk Protein Analysis Using Reverse Phase Chromatography RP-HPLC

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Abstract
Milk proteins have been extensively studied for over 90 years. Proteomics and associated technologies have the potential to facilitate an advanced knowledge of the proteins in milk. Proteomics allows detection, identification and characterization of milk proteins. In the present paper a study of milk proteins, using different analytical techniques - methods of dosing photometric and chromatographic analysis. With these techniques we want to reveal differences that occur at the protein level, between different animal species. As biological material was examined 4 different types of milk used for human consumption – cow's milk, buffalo, sheep goats respectively.

Keywords: chromatography RP-HPLC, milk proteins

1. Introduction

Milk proteins have been extensively studied for over 90 years. Proteomics and associated technologies have the potential to facilitate an advanced knowledge of the proteins in milk. Proteomics allows detection, identification and characterization of proteins in milk. Cow's milk is considered a staple food in many diets, is very rich in various essential nutrients. With modern analytical methods, biochemistry and cell biology has been demonstrated also the presence of other components (minor) that have biological activity. The achievements obtained in the dairy industry and enzyme separation techniques have allowed the possibility to isolate, concentrate and modify these compounds so that it became possible to apply them in functional foods, dietary supplements and food product.
Cow's milk proteins can be classified broadly in whey casein and protein (whey). Caseins are represented by several families of molecules (β, αs1, αs2, k), that shows genetic polymorphism and post-translational modifications represented phosphorilation and / or glycosylation. The main whey proteins are β-lactoglobuline and α-lactalbumin for which genetic variants are also known.
The fraction of whey also contains substantial amounts of immunoglobulin and serum albumin, which passes the mammary gland and are secreted in breast milk. Proteins lactoferrin and lactoperoxidase is best known from the list of minor proteins present in milk [1].

2. Materials and methods

The biochemical analysis performed has left the determination of proteins present in milk, using Bradfort. To determine the concentration of various types of proteins present in milk have made two different dosing: assays of total protein (whole milk) and protein determination whey (obtained by precipitation of casein in acid medium at pH isoelectric specific). The difference

was calculated with the concentration of casein present in milk.[2] The next step was also one of dosage, this time realizing is the concentration of casein present in milk, using a conventional method, volumetric [3].

As biological material we decided to analyze the 4 different types of milk used for human consumption – cow, buffalo, sheep respectively goats milk.

To achieve effective separation, in the short time analysis was used technique called chromatography HPLC - (High performance pressure liquid chromatography). [4]

For the analysis of milk protein was used in gradient separation, separating the mobile phase changes during the analysis.

For separation it has been used a gradient system where solvent A (solvent fixation) was an aqueous solution 0.01% TFA and solvent B (solvent elution) was 90% acetonitrile (v / v) and 0.01% TFA in water [5]. Separation of proteins in milk samples was performed in gradient whey the following conditions: - 31% solvent B for 5 minutes - linear increase from 31% to 46% solvent B for 40 minutes - 100% solvent B for 5 minutes - 100% solvent A for 10 minutes - a rate of 1ml/minute.

Detection was performed at two different wavelengths: 220 nm (specific absorption wavelength Peptide bond) and 280 nm (specific absorption wavelength nuclei of aromatic amino acids).

Before injection, samples (skim milk and whey) were diluted in a clarifying solution prepared in Bis-Tris buffer 0.1 M at pH 8 and containing urea (8 M), the trisodium citrate (1.3%) and Dithiothreitol (DTT) 0.3%. The solution is to destabilize the structure of casein micelles in milk (clarification) and to dissociate proteins that are thus maintained in a reduced state. For separation were injected and 20 µ l skimmed milk respectively clarified and whey to optimize the separation column was maintained at a temperature of 40° C.

3. Results and discussion

To determine total protein concentration present in milk that soluble protein (from whey) method was used Bradfort first step in the analysis of achieving the standard curve, utilizing standard solution of bovine serum albumin [2].

In Figure 1 is shown the standard curve obtained and the equation it (the equation used to calculate concentration after taking).

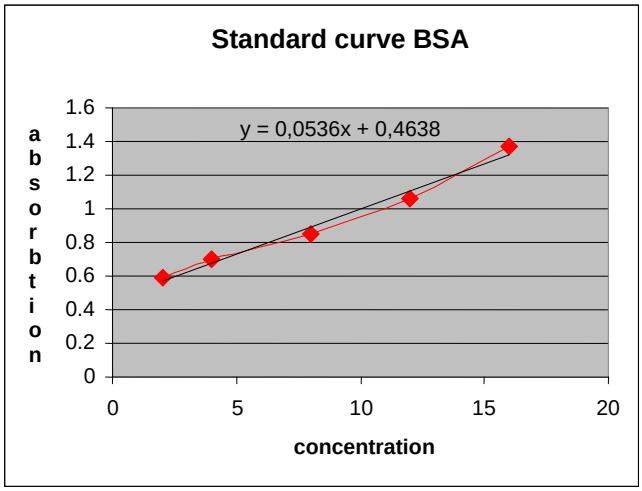


Figure 1: Standard curve obtained with standard solution of BSA

Table 1: Total protein concentration (mg / ml milk) in skim milk derived from 4 different species

Samples	Absorption at λmax = 595nm	Total Protein mg / ml skimmed milk
Skimmed cow milk	0.5932	28.97
	0.6269	36.50
	0.7084	54.71
Media - cow milk		**40.03**
Buffalo skimmed milk	0.6988	52.61
	0.700	52.88
	0.7409	62.03
Media – buffalo milk		**55.84**
Goat skimmed milk	0.7300	59.50
	0.5398	17.01
	0.7491	63.87
Media – goat milk		**46.79**

	0.7684	68.10
Sheep skimmed milk	0.7351	60.73
	0.7717	68.93
Media – sheep milk		**65.92**

Milk proteins are composed of two different classes - specific soluble whey proteins and insoluble protein, called casein. Soluble proteins present in whey are those that remain in milk after they were removed and lipids after Isoelectric precipitated caseins at pH of around 4.6. The determination of the whey proteins was achieved, and the results are given in Table 2.

Table 2: Total protein concentration (mg / ml milk) in whey originating from 4 different species.

Samples	Absorption at λmax = 595nm	Whey protein mg / ml
Cow		
	0.5461	9.21
	0.5733	12.25
	0.5882	13.92
Media - whey cow		**11.79**
Buffalo		
	0.5873	13.82
	0.5870	13.79
	0.5961	14.8
Media - whey buffalo		**14.13**
Goat		
	0.6274	18.31
	0.6230	17.82
	0.6156	16.99
Media - whey goat		**17.7**
Sheep		
	0.7126	27.85
	0.6927	25.62
	0.6123	16.62
Media - whey sheep		**23.36**

In terms of soluble protein from whey, the highest values occurring in sheep milk.

Table 3: Concentration of total protein (mg / ml milk), soluble proteins and caseins in skim milk derived from 4 different species

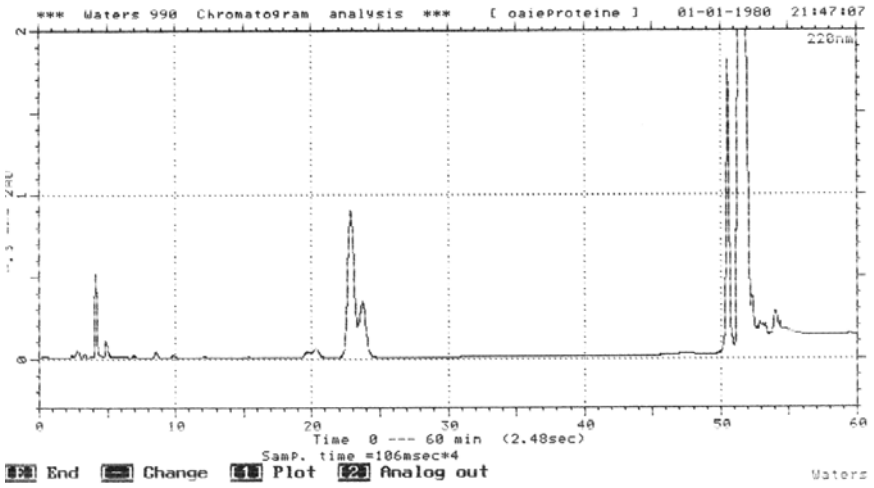
Samples	Total proteine mg/ml	Casein mg/ml	Whey proteine mg/ml
Cow			
	28.97	19.76	9.21
	36.50	24.25	12.25
	54.71	40.79	13.92
Media - cow	**40.03**	**28.26**	**11.79**
buffalo			
	52.61	38.79	13.82
	52.88	39.09	13.79
	62.03	47.23	14.8
Media - buffalo	**55.84**	**41.7**	**14.13**
Goat			
	59.50	41.19	18.31
	17.01	-	17.82
	63.87	46.88	16.99
Media - goat	**46.79**	**44.03**	**17.7**
Sheep			
	68.10	40.25	27.85
	60.73	35.11	25.62
	68.93	52.31	16.62
Media - sheep	**65.92**	**42.55**	**23.36**

To determine the differences in concentration of casein in skimmed milk compared to normal milk, was achieved one nine dosing casein in normal milk, this time using a conventional method, volume, the results are presented in Table 4.

Table 4: Casein concentration (mg / ml) in whole milk and skim milk derived from 4 different species and concentration of literature data

Samples	Whole milk casein mg / ml	skimmed milk Casein mg / ml	Literature data [FAO. 1998]
Cow			
	27	19.76	
	23.8	24.25	26
	24	40.79	
Media – cow	**24.9**	**28.26**	
Buffalo			
	29	38.79	
	30.6	39.09	35
	31	47.23	
Media – buffalo	**30.2**	**41.7**	
Goat			
	45	41.19	
	46.4	-	46
	44	46.88	
Media – goat	**45.13**	**44.03**	
Sheep			
	31.5	40.25	
	31.4	35.11	34
	32.2	52.31	
Media - sheep	**31.7**	**42.55**	

The milk protein analysis, separation and quantification of casein whey protein that was used chromatography RP - HPLC. Figure 2 shows the chromatograms obtained in the separation of milk proteins in three species taken in the analysis - sheep, cows and buffaloes. Protein profile obtained for goat milk (Figure 3) was similar to that obtained for the other three species examined.



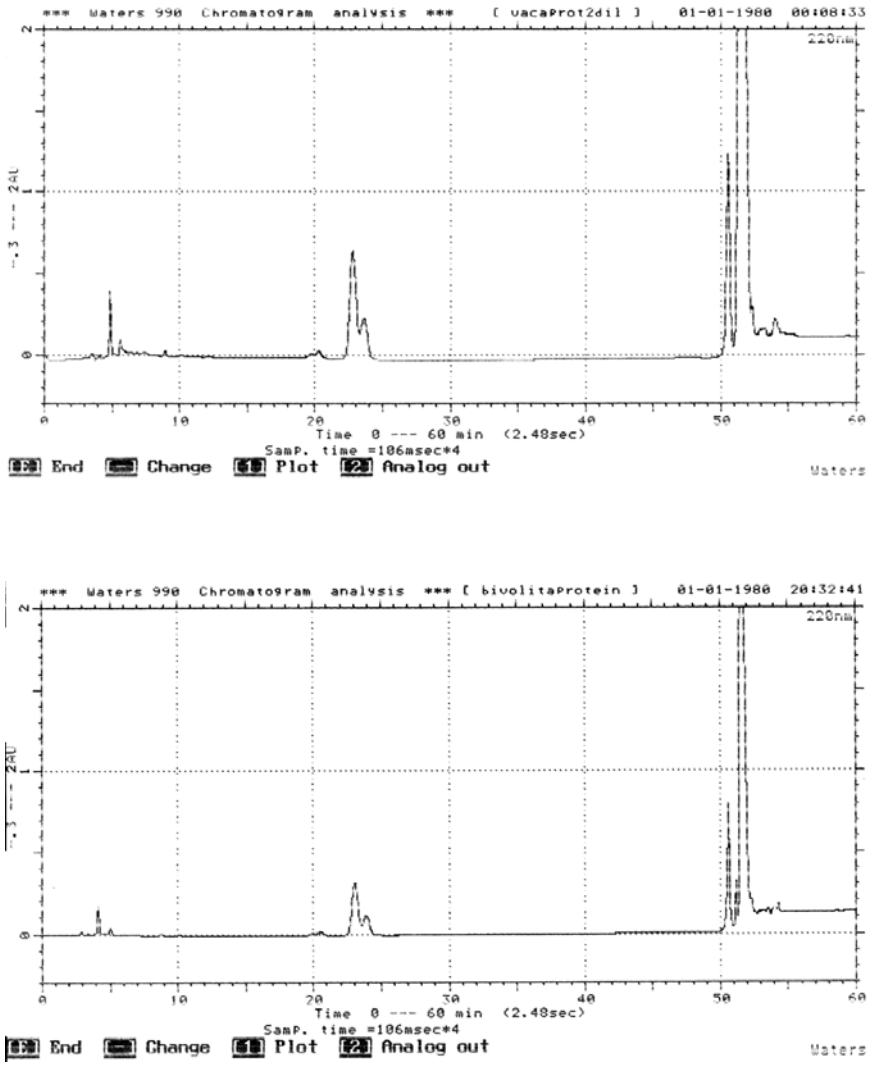
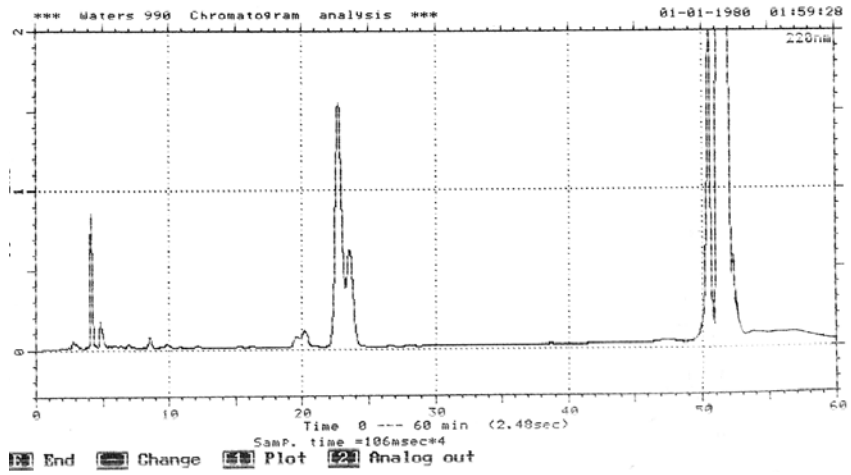


Figure 2: The chromatograms obtained from whole milk protein separation at three different species (sheep, cow, and buffalo)



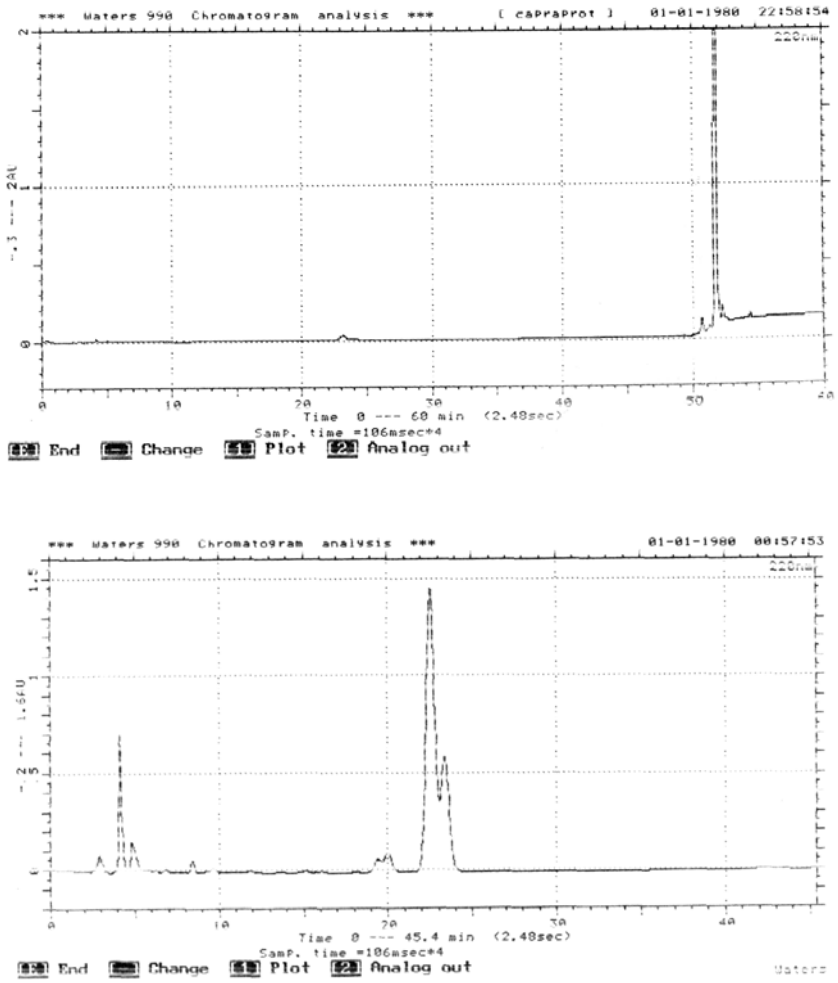


Figure 3: The chromatograms obtained from proteins separation of whole milk, whey and the casein fraction from one species (goat).

4. Conclusions

Determination of protein present in milk requires the use of photometric methods of analysis, the more sensitive the Bradford method.

Highest content in total protein occurs when sheep milk (average of 65.92 mg / ml skimmed milk) and the fell for cow milk (average of 40.03 mg / ml skimmed milk), intermediate values are recorded in goat milk (48.8 mg / ml skimmed milk) followed by buffaloes (55.8 mg / ml skimmed milk).

The total concentration of casein differs from one species to another; reported differences between various species in the milk proteins are due to genetic polymorphism.

Lowest casein content (28.26 mg / ml) appears in the milk of cows while the highest content appearing in goat milk (44.03 mg / ml).

Content of the caseins have values close to buffalo milk, goat sheep respectively, the concentration of 40 mg / ml milk.

Content of soluble protein (whey) varies according to species, the highest values occurring in sheep milk (23.3 mg / ml milk) and lowest in cows (11.8 mg / ml milk).

Separation and identification of proteins present in milk was achieved by reversed phase chromatography RP-HPLC

By comparing chromatograms obtained from all milk samples from 4 different species we can say that the same protein profile, observing the differences in the relative proportion of various caseins, which can be explained by genetic polymorphism.

By chromatographic analysis, in parallel, the various fractions of Milk were able to identify soluble proteins casein and whey.

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