# Oldentification of Milk and Milk Product Adulteration by Molecular Methods / Original article

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# الكشف عن غش الحليب ومنتجاته بالطرق الجزيئية

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## Abstract

The polyacrylamide gel electrophoresis sodium dodecylsulfate (SDS-PAGE) has been extensively used to separate native and denaturalized proteins. In various countries this has been useful to detect milk adulteration with whey, which is a fraud according to the current legal standards. The presence of glycomacropeptide (GMP) constitutes a marker of adulteration. It is released to the serum due to the hydrolysis of –casein peptide catalyzed by rennin incheese elaboration. In this work, the GMP detection was standardized as a pasteurized milk adulteration index, by means of GMP isolation with sequential precipitation in trichloroacetic acid at 24% and 50%, treatment with ethanol-ether and resuspention in buffer Tris-HCl 0.05 M, EDTA 1 mM, from sweet whey, acid whey, recently drawn raw milk, mixtures of whey milk; pasteurized milk and powder milk locally commercialized. Precipitates were analyzed by SDS-PAGE and GMP was evidenced as a trimer of 20.8 kDa in samples of sweet whey, and mixtures of whey: milk (1, 5, 10 and 50%), but absent in samples of acid whey and drawn raw milk.. The results obtained demonstrate that investigation of GMP by SDS-PAGE in milk, constitute a sensible and specific parameter to detect milk adulteration with whey, to levels as low as 1%, something that can not be revealed only with the evaluation of the physical-chemical parameters of milk.

**Keywords: Casein, GMP, Milk adulteration, SDS-PAGE, Whey.**

## المستخلص

تم استخدام دوديسيل كبريتات الصوديوم (SDS- PAGE) على نطاق واسع لفصل البروتينات الأصلية وغير الطبيعية. كان هذا في بلدان مختلفة. مفيد للكشف عن غش الحليب بالشرش ، وهي عملية احتيال وفقًا للمعايير القانونية الحالية. يشكل وجود (glycomacropeptide,GMP) علامة على الغش. يتم إطلاقه في المصل بسبب التحلل المائي لببتيد الكازين المحفز بواسطة وضع الرينين. في هذا العمل ، تم توحيد كشف GMP كمؤشر غش الحليب المبستر ، عن طريق عزل GMP مع الترسيب المتسلسل في حمض ثلاثي كلورو أسيتيك بنسبة ٪24 و ٪50 ، والمعالجة بإيثانول إيثر وإعادة التعليق في داريء Tris-HCl -EDTA، من الشرش الحلو ، والشرش الحمضي ، والحليب الخام المسحوب حديثًا ، وخليط شرش الحليب ؛ الحليب المبستر. تم تحليل الرواسب بواسطة SDS-PAGE و GMP تم إثباته على أنه 20.8 كيلو دالتون في عينات الشرش الحلو ، وخليط شرش الحليب (1 ، 5 ، 10 و ٪50) ، ولكنها غائبة في عينات شرش الحليب الحمضي والمسحوب الخام. حليب. توضح النتائج التي تم الحصول عليها أن فحص GMP بواسطة SDS-PAGE في الحليب ، مهم للكشف عن غش الحليب مع شرش الحليب ، إلى مستويات منخفضة تصل إلى 1 ٪ ، وهو أمر لا يمكن الكشف عنه إلا من خلال تقييم الحالة الفيزيائية الكيميائية للحليب.


**الكلمات المفتاحية: الكاسيين، GMP ، غش الحليب، الرحلان الكهربائي للهلام بولي أكريلاميد، الشرش.**

## Introduction

Milk is a foodstuff of great importance for its nutritional properties and high biological value. for its nutritional properties and high biological value. Approximately 1,400,000,000 litres of milk are produced each year. milk per year, which are used in the production of different dairy different dairy products, including pasteurized milk, powdered milk, cheese and other dairy products that are part of the daily diet. dairy products that form part of the Venezuelan diet (Briñez et al., [1]; Neelima et al.,[2]). The most important constituent elements in the milk of the different mammalian species are proteins, which are present in 3.2% of this food. mammals are proteins, which are present in 3.2% of this foodstuff. Of these proteins, 80% are caseins and the remaining 20% is represented by serum proteins - lactoglobulin, -lactalbumin, bovine serum albumin, euglactosomal albumin, euglactosomal serum albumin, bovine serum albumin, euglobulin, pseudoglobulin, mucins, globulin membrane proteins, and proteins, fat globule membrane proteins, other albumins, as well as numerous enzymes (Hua et al., [3]).

Caseins are a group of proteins that contain phosphorus and are specific to milk, where they are almost always found as micelles and precipitate at pH almost always in the form of micelles and precipitate at a pH of 4.6. Six types of caseins are known: s1 (alpha s1), s2 (alpha s2), (beta), (gamma), (kappa) and (lambda) (Hua et al., [3]). Casein has unique physico-chemical properties, when compared with other milk proteins. Some of these properties are insensitivity to precipitation by calcium ions, so it remains soluble in calcium solutions (Hua et al., [3]).With concentrations that precipitate all other caseins; stabilising effect on the other caseins when calcium is present in the suspension; undergoes hydrolysis of calcium ions in

the suspension; and in the suspension; undergoes hydrolysis by chymosin in a specific peptide bond, which resulting in destabilization of micelles and formation of micelles precipitates; furthermore, it is the only one of the casein that contains cysteine and carbohydrate residues in its structure (Valbuena et al., [4]; Faria and Bocan, [5]:).

Due to its status as a perishable foodstuff and the high risk of economic loss that this entails, the quality of milk is affected in certain aspects, such as microbiological, due to lack of hygiene, and physicochemical, due to adulterations, with the consequent nutritional, economic and legal consequences ( B (Briñez et al.,[ 1]; O'Riordan et al., [6]). Among the most frequent adulterations that are made to milk are milk include: the addition of chemical substances not permitted by legislation, such as the addition of water and chlorides; the addition of preservatives or neutralizers; inadequate heat treatments that alter the native conformation of proteins; the addition of sweet whey, among others (Kavanaugh *et al*., [7]). These adulterations occur at any stage of production, such as during transport or processing, and are intended to increase milk volumes in order to increase yields.

Sweet whey is the milk by-product that is separated from the curd during the cheese-making process. It is obtained by enzymatic coagulation of the milk, by the action of the enzyme chymosin, which hydrolyses the kappa λ -casein in milk at the Phe-Met bond (105-106), producing two fragments: a hydrophilic, acidic portion, consisting of 64 amino acids, called glycomacropeptide (GMP), which remains in solution in the serum, and the other portion is hydrophobic, basic in character, consisting of 105 amino acids , called Para casein, which is retained in the clot ( Pinto, [8]; Hua et al., [3]).

Several studies worldwide(Rosas, [9]; Chen et al., [10]), have shown the presence of GMP in milk samples. This is an indicator of adulteration of raw milk for commercial purposes, representing a fraud for the consumer or the industry, but it means an increase in the volumes of profit for those who practice it, since this for the consumer or industrialist, but means an increase in profit volumes for the practitioner, as this whey has a cost very low compared to the price of milk. This practice is made possible because whey is a natural component of milk and therefore its colligative properties are similar, which makes its presence almost imperceptible when it has been added to milk.So far, several methods have been developed to to detect adulteration of milk by whey addition, differing in their sensitivity and versatility of the equipment used in the analysis (Chen et al., [2012] ).The isolation of GMP by trichloroacetic acid precipitation (TCA) followed by polyacrylamide (SDS-PAGE) gel electrophoresis has been widely used to detect the presence of GMP in processed milks for human consumption (Chow and Harper, [11]). In order to detect the presence of GMP as a marker of sweet whey milk adulteration, in the present work, it was proposed to standardize extraction and detection of this peptide by precipitation with TCA and SDS-PAGE respectively.

## Materials and Methods

### Sample Collection

Freshly milked raw milk (fresh raw milk) free of whey as a negative control, liquid whey was included as a positive control. Acidic whey was used as a negative control, the which was obtained by acid coagulation of fresh raw milk with 30% acetic acid Sweet whey-milk and sweet whey-water mixtures were prepared in the following proportions: 1: 5: 10 and 80 (%V/V).

The whey used to prepare the mixtures with milk was previously heated at 80°C for 10 minutes to remove any residual chymosin activity in the whey (Chow and Harper, [11]; Souza et al., [ 12]).

## Glycomacropeptide extraction

GMP extraction was performed following the methodology of (Alemka et al., [13]) with some modifications: A 50 mL of sample (fresh raw milk, sweet whey, acid whey, whey-milk and whey-water mixtures), were added slowly and under constant agitation for 2 minutes,25mL of a 24% TCA solution was added slowly and under constant stirring for 2 minutes. This mixture was allowed to stand at room temperature for 60 minutes. After this time, the precipitate (caseins) was separated from the supernatant (whey proteins) by filtration using Whatman 42 paper, of 50% TCA was added to 30 mL of the filtrate, and allowed to stand under refrigeration (4-6°C (for 60 minutes. The resulting suspension was centrifuged in at 7000 rpm at 4°C for 10 minutes. The supernatant obtained was discarded and the precipitate was washed twice with 10 mL of a 1:1 ethanol-ether mixture and then centrifuged under the conditions of the previous centrifugation. The supernatant was discarded and the tubes were drained on absorbent paper to remove excess wash mixture. The final pellet was resuspended in 300 µL of Tris-HCl 0.05 M Buffer; EDTA 1mM, pH 7.2 and stored under freezing at -20°C until use (Laemmli,[14 ]).

## Polyacrylamid gel electrophoresis (SDS-PAGE)

Samples resulting from precipitation with Trichloroacetic acid (TCA) were analyzed by SDS-PAGE, using the Laemmli discontinuous buffer system (Laemmli, [14]). In this system the proteins are denatured by heating in

buffer containing SDS and β-mercaptoethanol as reducing agent (McKee and McKee, [15] ). Electrophoresis was performed in a Mini- GEL preparation, at a constant voltage of 200 V, with a current ranging between 85 and 35 mA, using 4% and 15% acrylamide stacking and separation gels, respectively. The run was performed using tris-glycine buffer pH 8.3 for 60 to 120 minutes. A broad molecular mass range marker Bio-labs (New-England) was included in all runs. Visualization of the protein bands was performed by staining the gels with Coomasie blue R-250, 0.2% in methanol: acetic acid: water (25: 10: 85) at room temperature for a minimum time of 6 hours followed by decolourisation in a solution of methanol: acetic acid: water (85: 10:50).

## Results and Discussion

### Detection of GMP in SDS-PAGE

In order to recognize the band corresponding to GMP, the electrophoretic profile of fresh raw milk, acid whey (negative control) and sweet whey (positive control) before and after treatment with trichloroacetic acid were analyzed on the same gel (Figure1). The protein profile of authentic milk, acid whey and sweet whey, respectively, prior to treatment with TCA is shown in lines (3,5 and 7). In the milk sample, a highly coloured and extensive area between 30 and 40 kDa, which corresponds to the casein zone, whereas in the whey samples, the protein bands in the serum samples are 19 kDa and 13.5 kDa, corresponding to -lactoglobulin and -lactoalbumin monomer, respectively. Lines 4, 6 and 8 show the same samples after TCA treatment. The obvious difference between the protein profile in these three lines is given by a band with an apparent molecular mass of 20.9 kDa present in the line 8 which is also present in the sweet serum (line 7). The TCA treatment

allows the precipitation of GMP, therefore, in the PAGE-SDS of raw milk and acid whey which not containing the glycomacropeptide treated with this treated with this acid, as expected, no band corresponding to GMP is observed in the PAGE-SDS of raw milk and acid whey. On the other hand, if we compare the protein profile of acid whey (line 5) and sweet whey (line 7) we can see that they are practically identical except for the 20.9 kDa band. This observation suggests that this band corresponds to the GMP.

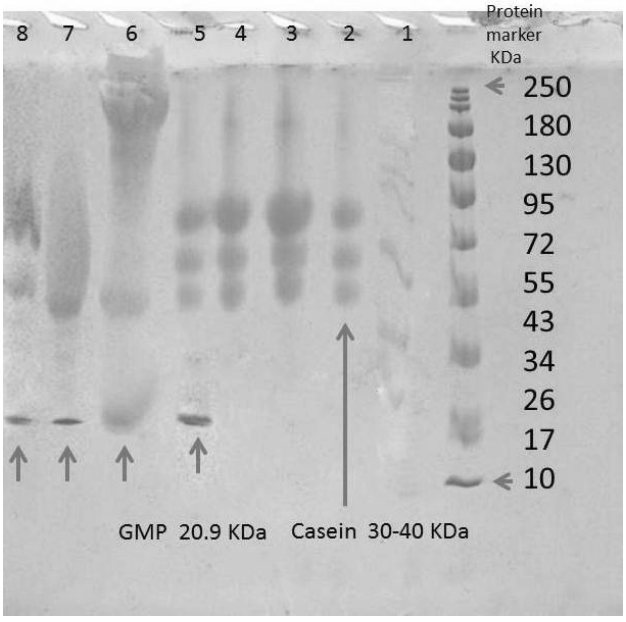


**Figure 1. Protein profile of milk, acid whey and sweet whey by (SDS-PAGE)**

It has been reported that the monomer of GMP has a molecular mass of molecular mass of 6.8 kDa without considering the glycidic portion; It has also been reported that it forms aggregates at neutral pH and that the molecular mass of the aggregate depends on the pH at which it is found (Kawasaki et al., [16]). GMP present in bovine whey has been observed in its aggregated form when analyzed by gel chromatography (Alemka et al., [13]) or electrophoresis

(Nakano and Ozimek, [17]). The mechanism of aggregation is unknown, and it is also unclear why the GMP aggregate does not dissociate on gels in the presence of SDS. It has been reported that SDS binds to the peptide moiety of a GMP molecule and avoids hydrophobic interactions with other GMP molecules. However, the glycine chains of the different GMP molecules can interact with each other through hydrogen bonding, which resulting in molecular masses greater than 18,000 Da,for this peptide in PAGE-SDS. Furthermore, the affinity of SDS for GMP is too weak to dissociate the GMP trimer, which may contribute to the predominance of the aggregated forms in the presence of SDS (Oria, [18]).

It has also been pointed out that at the level of the milk, -lactoglobulin and casein-, can form bonds due to the effect of heat treatment and this may eventually block the binding of the peptide portion of GMP and SDS (Neelima et al., [2]).The 20.9 kDa in the TCA precipitate of the sweet whey, probably represents an aggregated form (trimer) of this peptide, which corresponds to that reported in previous research.on GMP where its monomeric form has rarely been observed. . In this sense, several researchers have analyzed the structure of GMP, using various extraction, purification and characterisation methods, finding that in SDS-PAGE, sweet whey GMP appears as 2 bands with molecular masses of 20.740 Da (26.1%) and 18.380 Da (73.9%) (Bottacini et al., [19] ). The structural heterogeneity of GMP, due to the variation in its glycidic composition, determines that the reported molecular mass values for GMP depend in part on its depend on the type of extraction and the method used to detect it (Chen et al., [10]). In order to observe a gradual increase in the 20.9 kDa band, corresponding to GMP, as a function of the degree of systematic adulteration of fresh raw milk with sweet whey, electrophoretic runs were carried out on mixtures of whey: milk. Figure 2, shows the protein pattern of whey: milk mixtures treated with TCA to extract GMP, It can

be seen in this gel that there is a gradual increase in the 20.9 kDa band of GMP, proportional to the concentration of whey in each mixture. In this sense, if lines 4, 5, 6, and 7 of this figure are compared, it can be observed that the intensity of this band increases as the proportion of whey in each mixture increases. The results of this gel show that the 20.9 kDa band corresponds to GMP as it is absent in line 3 where fresh raw milk treated with ATC while in lines 8 and 9 it is clearly present in samples of sweet serum treated with ATC.

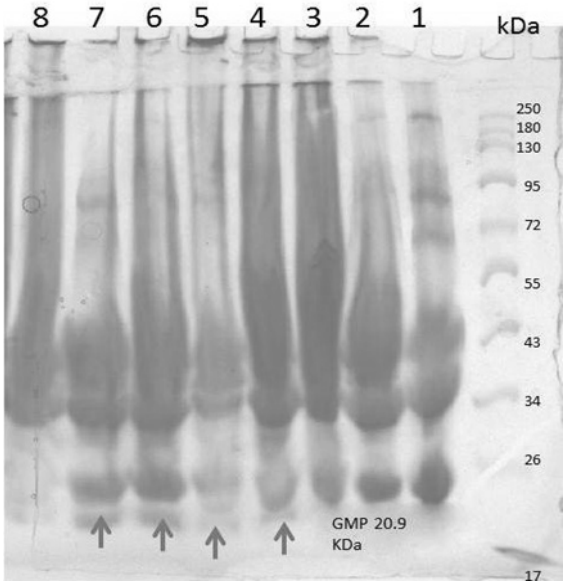


**Figure 2. Protein profile in whey (milk mixtures), by SDS-PAGE**

## GMP as an index of milk adulteration

Fifteen (15) samples of pasteurized milk were classified to (A, B, C, D and E) according to the regions from which the samples were collected, were subjected to TCA treatment and analyzed by SDS-PAGE in order to evaluate the efficiency of the method in the detection of sweet whey adulteration of milk. (Table 1 )

summarizes the results obtained with pasteurized milk type A, B, C and E, while class D milk did not show the presence of GMP. Based on the results as described in (Figure 2) the percentage of adulteration in milk samples where adulteration in the milk samples where GMP was found to be present was estimated presence of GMP was evidenced. In that sense, milk B corresponded to with 1%; brands A and C with 10% and brand E showed a little more than 50% adulteration (Table 1).

**Table 1. Glycomarcopeptide (GMP) levels in five types of pasteurized milk**

| Type | Evidence of GMP in Adulterated Samples as a Percentage | Estimated percent of whey added |
|---|---|---|
| A | 100 | 5 |
| B | 66,6 | 10 |
| C | 66,6 | 10 |
| D | 0 | 0 |
| E | 100 | 50 |

## Physiochemical parameters of the analyzed milk

Table 2 shows the average values of the physiochemical parameters of the pasteurized milks A, B, C, D and E analyzed in this study and fresh raw milk, compared with the values established by current legal regulations, for pasteurized milk. It can be seen in this table that the milk of brand E, which showed the highest degree of adulteration, has values for total protein, fat, total solids and other physicochemical parameters, lower than the rest of the brands studied and lower than the value established in the value established in the standard, which was the product of an evident dilution of the milk as its lowering of critical point was observed. These results correlate with those observed by (Briñez et al., [1]; Léonil andMollé, [20]). that milks whey adulterated with cheese whey in

percentages of 10 and 15%, showed lower total protein values (2.95 and 2.85%, respectively) than those established by the established in the current legal regulations. On the other hand, milk class E also exhibits relatively low percentage of caseins (1.6%) compared to the other commercial brands (around 2.5%); similar to what was found by (Faría and Bocan,[5]) who describe values of 1.99 and 1.85% casein for the samples previously adulterated with 10 and 15% serum, respectively; this is probably a consequence of the dilution of the caseins of the caseins with the addition of whey. The percentage of whey protein, obtained by difference between total proteins and caseins, were similar (about 0.8%) for brands A, B, C, and D, while brand E milk, showed a relatively lower value (0.55%) compared to the resulting value in fresh raw milk (1.25%). This corresponds to the low value obtained for total protein for this brand of milk.

**Table 2. Physiochemical average values of pasteurized milk**

| Parameters | Type of pasteurized milk | | | | | Raw milk |
|---|---|---|---|---|---|---|
| | A | B | C | D | E | |
| Total protein | 3,36 | 3,36 | 3,50 | 3,25 | 2,15 | 3,75 |
| Caseine % | 2,50 | 2,50 | 2,70 | 2,50 | 1,6 | 2,50 |
| Serum protein % | 0,86 | 0,86 | 0,80 | 0,754 | 0,55 | 1,25 |
| Fat % | 3,60 | 3,70 | 3,40 | 3,10 | 2,30 | 4,00 |
| Total solids % | 12,0 | 12,0 | 12,6 | 11,9 | 9,7 | 13,0 |
| Acidity | 17,0 | 18,0 | 15,0 | 18,0 | 15,0 | 19,0 |
| Freezing point | 0,545 | 0,525 | 0,535 | 0538, | 0,513 | 0,531 |

These findings, in correlation with the results of (Faría and Bocan, [5]), milk samples adulterated with whey between 1 and 10% cannot be detected

by evaluating only the physico-chemical parameters of the samples. parameters alone. Thus, milks A, B and C were shown to be chemically adequate when comparing their values with those of the the standard and numerically they do not differ from those of brand D milk, this suggests that the detection of GMP by SDS-PAGE is a specific and sensitive parameter as a marker of whey milk adulteration.

## Conclusion

The extraction and run conditions in PAGE-SDS used in this work indicate that GMP is evident as a band of 20.9 KDa which probably represents a trimeric form of this peptide. The results obtained indicate that the investigation of GMP in milk, represents a specific and sensitive specific and sensitive parameter to detect adulteration levels of milk with whey, from 1%: what cannot be prevented. The presence of GMP in four brands of pasteurized milk analyzed in this study reveals adulteration by whey; however, this was only a descriptive study which needs to be carried out in a more complete study in future research, to carry out a more complete study to evaluate the quality of pasteurized milk sold in the region.

### Recommendation

The presence of GMP in the signal of interest collected and analysis by HPLC and mass spectrometry of adulterated samples comparison of the chromatographic run with a commercial GMP standard (retention time), and by the recognition of GMP-specific monoclonal antibodies on an immunostick strip. The presented method is easy to implement and develop in the laboratory; it can be applied to routine tests of milk arriving at the dairy

plant. It can also be used in finished products by small or large milk processing industries, distributors, and even government regulatory entities to promote the quality and protection of the authenticity of milk, as a product for daily consumption, which is considered a basic in the family diet.

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