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Identification of glycomacropeptide as indicator of milk and dairy drinks adulteration with whey by immunochromatographic assay

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Abstract

The present study aimed at the detection of fraudulent manipulation of milk with the low-cost component, whey, by immunochromatographic determination of glycomacropeptide. Ten commercial milk/dairy beverages samples of different brands from the national market were analyzed (unprocessed and processed milk, condensed milk, powdered milk, and dairy beverages of cappuccino, chocolate, banana and strawberry flavors). The results obtained showed additional whey (1-2%) in 70% of the selected samples after casein removal by precipitation with 20% trichloracetic acid. The samples found positive with glycomacropeptide were fresh milk, two processed milk and four samples of milk drinks.

Keywords**:** whey proteins, glycomacropeptide, adulteration, immunochromatography

Introduction

Adulteration of food products continues to represent a major concern not only for consumers but also for food manufacturers. Foods or ingredients most susceptible to fraudulent practices are those with high-value and which undergo several processing steps. Milk is one of the products which can be adulterated in many ways affecting the quality of further dairy products. Extension of milk with a low value ingredient (watering of milk, milk of different species, addition of whey, etc.) also known as "economic adulteration" has been often practiced.

Different methods were developed to detect fraudulent addition to milk of rennet whey, a by-product of the dairy industry. Assays developed for the analysis of casein/whey protein ratio included indirect methods, based on determination of certain protein fractions, and direct methods, which separate protein mixtures into components. Indirect determination of whey protein to casein protein ratio included polarographic [1] and second and fourth derivative spectroscopy methods [2, 3]. Direct determination of protein fractions is based on effective but laborious electrophoretic, chromatographic and immunoturbidimetric methods [4, 5, 6, 7].

Recently, detection of fraudulent manipulation of milk with whey has been focused on the analysis of glycomacropeptide (GMP) also known as caseinomacropeptide (CMP) [8, 9]. It is a bioactive 64 amino acids residues glycopeptide released enzimatically in whey from *k*-casein by the action of chymosin during cheese making [10]. GMP structure lacks aromatic

amino acids and retains a net negative charge, even at pH 3. Quantitative determination of GMP includes chromatographic methods, *e.g.* RP-HPLC and combined LC-MS [11], and electrophoretic methods, *e.g.* SDS-PAGE and capillary electrophoresis techniques [12, 13]. For the analysis of powder milk adulterated with whey, European Commission adopted two methods of gel-filtration HPLC for GMP detection (Commission Regulation EC No. 213/2001).

New strategies based on immunochemical assays have been also developed to analyze GMP. ELISA assay targeting bovine GMP has been successfully employed [14]. The company Operon has developed a rapid immunochromatographic test (Immunostick c-GMP) based on monoclonal antibodies against GMP.

GMP identification has been used to detect fraudulent addition of whey to milk powders [9] and UHT milks [12] as well as to monitor the renneting process [13, 15]. Despite the fact that generally, whey addition in milk does not represent a health hazard it was shown that supplementation of infant formula with GMP enhance the absorption of trace minerals [16]. Consequently, reduction of trace minerals in formulas is needed in order to avoid possible adverse effects of excess dietary intake because of supplemental GMP.

The aim of the present paper was to select a simple, rapid and with minimal sample preparation technique in order to detect GMP in different processed and unprocessed milk samples from the Romanian market. We have extended our investigation also on dairy beverages which become very attractive to consumers. As chemical methods of GMP analysis are effective but time consuming or have some limits regarding insufficient selectivity/sensitivity, we have applied the prospective immunochromatographic assay.

Materials and Methods

Milk and milk drinks samples

Six commercial bovine milk samples from the national market were used (fresh, pasteurized, UHT, omega-3 UHT, condensed and milk powder) and four dairy beverage samples of cappuccino, chocolate, banana and strawberry flavors. Fresh milk sample was obtained from a farm in the neighborhood of Sibiu and the other milk/milk drinks samples were obtained from commercial sources (brands A, B, C, D, E, F and G). Nutritional values of the samples were obtained from the information given by the manufacturer. The milk powder sample was reconstituted with deionized water following the instruction given by the manufacturer.

Where necessary, total protein content was determined from nitrogen content by Kjeldahl method (crude protein: N x 6.38).

Sample preparation

The procedure consists of a pre-treatment of samples with trichloroacetic acid (TCA) to precipitate casein and major whey proteins [17]. Precipitation was done with 20% TCA for 10 minutes at r.t. The precipitate was removed by centrifugation at 5000 *g* for 10 minutes at r.t. The supernatant was filtered on 5 μm low protein adsorption filter. Three dilutions were obtained (1/10, 1/100 and 1/1000 respectively) using the Tris buffer pH 7.2 with 0.1% sodium azide. All the reagents were of analytical grade purity.

GMP detection

Qualitative determination of GMP in selected samples was performed using the Immunostick c-GMP visual assay. Immunochromatographic sticks (purchased from OPERON S.A., Spain) which contain monoclonal antibodies specific for GMP and anti-GMP antibodies were dipped into solution samples diluted 1/1000. Development of a red band in addition to the control blue band on the reactive strips after 5 minutes was considered a positive result.

Results and Discussion

A modern rapid antibody-based analytical method was applied to detect fraudulent addition of sweet whey in ten commercial milk and milk beverages samples of different brands from the national market, as follow: fresh milk, pasteurized milk, UHT milk, UHT milk fortified with omega-3 fatty acids, condensed milk, powdered milk, cappuccino-, chocolate-, banana- and strawberry flavor milk drinks.

GMP was isolated from k-casein and other non-GMP compounds by deproteinization with 20% TCA and then 1/1000 diluted samples were analyzed by colorimetric development on immunosticks with monoclonal antibodies highly specific for GMP/k-casein. As the activity of certain bacterial proteinases present in milk might give false positive results for GMP detection [18], only samples properly kept at low temperature and with excellent microbial quality were considered.

The obtained results are given in table 1.

Table 1. Identification of GMP after TCA precipitation in various milk and dairy beverages samples (dilution 1/1000).

Products	Protein content (% w/v)	GMP test results
Fresh milk	3.4	+
Pasteurized milk (3.5% fat) brand A	3.0	+
UHT milk (2.8% fat) brand B	3.0	+
Omega-3 UHT milk (1.5% fat) brand C	3.5	-
Condensed milk brand D	-	-
Whole milk powder brand E	31-37*	-
Cappuccino milk drink brand F	3.0	+
Chocolate milk drink brand F	3.2	+
Banana milk drink brand G	2.6	+
Strawberry milk drink brand G	2.6	+

Symbols: *milk protein content expressed as % w/w; „+“ positive result on developed immunochromatogram with blue control band and red band: „-“ negative result with only blue control band

Strips after performance of chromatographic process are presented in figure 1.

Figure 1. Immunochromatograms of samples positive **(a)** and negative **(b)** for GMP.

As specified by the immunochromatographic sticks manufacturer, at dilution 1/1000 the test can detect 15-30 μg/ml of sweet whey added to milk (1-2%). No interfering results from other proteins (BSA, bovine antibodies, α-, β- and γ-lactalbumins) affect the qualitative determination by using these immunochromatographic test-strip readers.

The results revealed the presence of GMP in 70% of the investigated samples. As shown in table 1, the unprocessed sample and two processed commercial milk samples by pasteurization and UHT treatment, were found positive with GMP, resulting in sweet whey addition of 1-2% at the dilution 1/1000 in 50% of milk samples. All the selected four flavored milk drinks samples were found adulterated with sweet whey.

Several assessments of UHT milks adulterated with whey have been done, all the methods quantifying whey protein from total protein. For example, UHT milk samples from different Spanish geographic area were analyzed by determining whey protein to total protein ratio using 4^{th} derivative spectroscopy which detected UHT milk adulterated with whey up from 5% [19]. Little reports are on GMP determination [11].

Adulteration of powdered milks with whey was studied by Ferreira and coworkers who validated a method of GMP analysis by a HPLC/UV procedure with a detection limit of 2 μg/ml [9]. They found one of the selected four milk powder samples adulterated with rennet whey.

De Souza and coworkers [8] reported studies on complex dairy mixtures adulterated with whey by analyzing not GMP but the correlation between the percentage of casein/α-lactoalbumin/β-lactoalbumin and the percentage of added whey using the SDS-PAGE and densitometric method with a sensitivity of 5% of additional whey. They found that 29% and 49% of two different types of dairy beverages samples distributed in a School Meals Program were adulterated with whey.

For the detection of whey added to margarine, Nakano and coworkers [20] proposed a SDS-polyacrylamide gels or cellulose acetate strips technique to detect sialylated phosphorylated GMP.

The results of the present study showed that screening procedures are recommended for authentication of milk and dairy products in order to detect milk adulteration with whey committed for economical reasons and moreover, that additional whey should be specified on label information of the product. The method used in the present investigation is very simple to handle and to interpret and give good accuracy and precision. Therefore this assay can become a screening test for detection of GMP in milk and dairy products.

Conclusions

For detecting the addition of whey in milk, several analytical approaches based on determination of whey protein to casein protein ratio and/or GMP analysis has been described. In the present investigation the immunochromatographic technique was used to

identify GMP as adulterant in ten commercial milk/dairy beverages samples. GMP was detected in 70% of the selected samples. Fresh milk sample, two processed milk samples of brands A and E and four milk drink samples of cappuccino, chocolate, banana and strawberry flavors of brands F and G showed 1-2% addition of whey.

The results showed that milk adulteration continues to be a concern and efforts should be made by authorities and producers to protect product authenticity.

The described procedure is a useful tool for routine detection of fraudulent manipulation of milk and dairy products with whey.

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