# Food supplements and products for external use based on bioactive principles from whey, egg and bee by-products

# Suplimente alimentare și produse de uz extern pe bază de principii bioactive din zer, ou și produse ale stupului

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**Key-words:** *food supplements, bioactive principles, whey, egg, bee by-products*

**Abstract**

**Bioactive principles extracted from natural sources are of great interest to medical and pharmaceutical researchers, especially those that may result from residual products remaining after industrial processing. Thus, new methods are being sought to capitalize the active compounds in the by-products obtained from the processing of various products, such as whey proteins, organic calcium extracted from eggshells and propolis extracts. The present research started from the individual benefits of these products for human health, resulting in their use as raw materials for the formulation of main pharmaceutical preparations, namely, food supplements and products for external use.**

**Rezumat**

**Principiile bioactive extrase din surse naturale sunt de mare interes pentru cercetătorii din domeniul medical și farmaceutic, mai ales cele ce pot rezulta din produsele de reziduu ce rămân în urma procesării industriale. Astfel, se caută noi metode de a valorifica compușii activi din derivatele secundare obținute în urma procesării diferitelor produse, precum proteinele din zerul bovin, calciul organic extras din cojile ouălor și extractele de propolis. Prezenta cercetare a pornit de la beneficiile individuale ale acestor produse pentru sănătatea umană, având ca rezultat folosirea lor ca materii prime pentru formularea de preparate farmaceutice, și anume, suplimente alimentare și produse de uz extern.**

## Introduction

The interest of medical and pharmaceutical specialists is increasingly directed towards new methods of extracting bioactive principles from natural sources, especially from unvalued by-products and derivatives resulting from the current processing of milk, eggs and beekeeping products, with the aim of replacing synthetic substances as much as possible.

Whey, a liquid by-product in the cheese industry, is known for its content in many valuable constituents. These include proteins that possess important nutritional and biological properties - especially in terms of health promotion and disease prevention. The main fractions are ß-lactoglobulin, α-lactalbumin, bovine serum albumin (BSA), immunoglobulin (Ig) and minor proteins are lactoferrin, glycomacropeptide, lactoperoxidase (Acharya, 2010). The composition of whey is presented in Fig.1.

Whey proteins are pure, natural and high-quality proteins.

They are considered the best proteins for human food use, due to their balanced amino acid profile and superior digestibility. Whey proteins contain all the essential amino acids and are therefore a "complete source" of high-quality proteins. (Jelicic *et al.,* 2008).

The egg is the most complete food, containing all the substances necessary for the body. It is rich in proteins with high biological value, fats, vitamins and minerals. It has both nutritional and therapeutic properties due to the components present in yolk and white: ovotransferin, ovomucin, ovalbumin, lysozyme and bird immunoglobulins (IgY). A graphic representation of the composition of the egg is found in Fig.2.

The content of the hyperimmune egg is a valuable source of antibodies, with many advantages such as the mechanism of rapid support of passive immunity, especially in patients with deficiencies of various immune components.

Immunoglobulin Y (IgY) is the main antibody synthesized by chickens (*Gallus domesticus*). It is continuously synthesized on a large scale, secreted into the blood and transferred to the egg yolk, where it accumulates (Young *et al*., 2007).

Avian IgY is the evolutionary ancestor of mammalian IgG and represents the main defense mechanism against systemic infections (de Paula *et al.,* 2011).

The IgY transfer from chicken to its poult takes place in two stages; IgY is initially transferred from the chicken's blood to the ovarian follicle (egg yolk) and then to the embryo (Hamal *et al.,* 2006).

To obtain IgY antibodies with specificity against pathogenic strains, laying hens are inoculated with the antigen of interest; antibodies are purified from egg yolks. This non-invasive method of obtaining antibodies is one of the advantages of using IgY in various therapy regimens (Kovacs-Nolan *et al.,* 2012).

Egg yolk contains 8-10 mg IgY/mL and from a single egg about 100-200 mg of total IgY can be extracted, of which 2-10% is antigen-specific (Davison *et al*., 2008).

Specific avian antibodies have been successfully synthesized against a wide variety of antigens, including proteins, peptides, lipid hormones, and carbohydrates from a wide range of species such as viruses, bacteria, fungi, plants and animals (Hamal *et al.,* 2006, Spillner *et al*., 2012).

Propolis is a resinous, brown matter collected by bees from the leaf buds of tree species such as birch, poplar, pine, alder, willow and palm and transported to hives where it is transformed with the help of enzymes from bee glands.

Propolis possesses antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antitumor and immunomodulatory properties; due to these effects it has been used as a remedy in traditional medicine, in apitherapy, in cosmetics and pharmacy since ancient times (Kim *et al*., 2008).

Chemically propolis is composed of about 180 different chemicals and tests have shown more than 300 different components (Chan *et al*., 2013; Drescher *et al*., 2017; Sforcin, 2016).

Propolis contains polyphenols (flavonoids, phenolic acids and esters), phenolic aldehydes and ketones, benzaldehyde derivatives, carbohydrates and vitamins (B1, B2 complex, B6, C, E), amino acids and esters, enzymes and minerals (Cd, Sn, Pb, Ti, Ag, Co, Mo, Al, Si, V, Ni, Mn, Cr Na, Mg, Cu, Ca, Zn, Fe, K) (Pasupuleti *et al.,* 2017).

A graphic representation of the composition of the propolis sample is found in Fig.3 below.

Zeolite is considered worldwide as one of the best substances for purifying and detoxifying the human body.

Due to its honeycomb structure, it attracts and absorbs heavy metals and positively charged toxins, without removing beneficial and vital electrolytes. It is used as a dietary supplement having multiple benefits in boosting the immune system.

Eggshell accounts for about 10% of the total mass. Eggshell is rich in natural calcium, easy to digest and absorb, also representing a balanced form of microelements.

From the shell of an egg of medium size, about a teaspoon of powder is obtained, which contains about 750-800 mg of elemental calcium, plus 27 other microelements, such as magnesium, boron, copper, iron, manganese, sulfur, zinc, etc. (Nakano *et al.*, 2003).

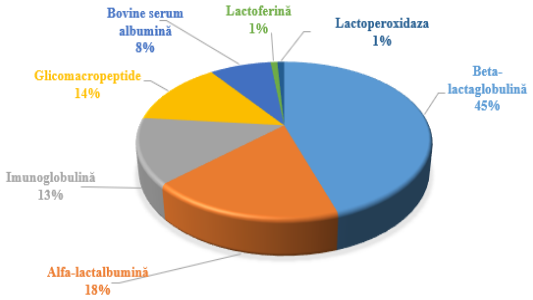



**Figure 1.** Composition of whey

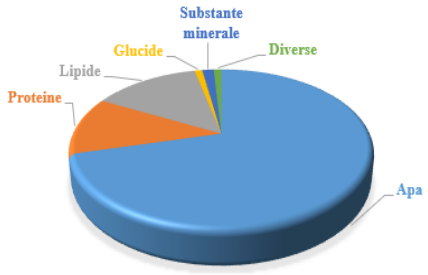



**Figure 2.** Composition of whole egg

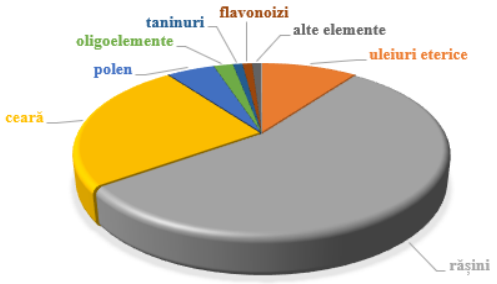



**Figure 3.** Composition of propolis

This study aimed to exploit by-products from the milk processing, egg and beekeeping industries to obtain functional ingredients with health benefits, such as whey protein and protein fractions, Y immunoglobulins and lyophilized hyperimmun egg, propolis tincture and organic calcium citrate, with important nutritional and functional attributes.

Using these natural principles, new dietary supplements have been formulated, which promote health by providing increased protein intake and preparations for external use, with a role of sanitizing the skin.

## 1. Material and Method

### 1.1. Materials

This study was carried out within the Immunoinstant research and Development Department belonging to Romvac Company S. A. (Ilfov, Romania).

Samples of bovine whey were provided by the cow farm in Călărași county and were processed in the form of 10 kDa retentate at the Biotechnology Department of Romvac.

The solution of CPZ 10 kDa was lyophilized and a powder was obtained that was stabilized with silicon dioxide (aerosil) and corn starch (Fig.4).

The hyperimmune egg was obtained from chickens inoculated with an antigen complex.

The polyvalent antigen contains a mixture of bacterial and fungal strains, such as:

- *Pseudomonas aeruginosa,*
- *Klebsiella pneumoniae,*
- *Salmonella spp.,*
- *Escherichia coli,*
- *Enterococcus faecalis,*
- *Salmonella enteritidis,*
- *Salmonella typhimurium,*
- *Streptococcus mutans,*
- *Staphylococcus aureus,*
- *Streptococcus grup B,*
- *Proteus mirabilis,*
- *Acinetobacter baumannii,*
- *Helicobacter pylori,*
- *Clostridium difficile*,
- *Candida albicans,*
- *Candida glabrata,*
- *Candida krusei.*

The antigen was administered by intramuscular injection of 0.5 mL at four different points in the chest muscles in conventional laying hens or SPF laying hens, every 14 days, three times.

Egg collection was done 14 days after the third antigen administration when the antibody titer in serum and yolk is high.

The antibody titer was periodically assessed from egg yolk from hens immunised with the given antigen by ELISA tests. Whole egg powder (Fig.5) was obtained by lyophilizing the contents of the entire hyperimmune egg within the Drug Production Department - Romvac Company. It contains antibodies against 18 bacterial and fungal pathogenic strains present in the antigen used in the inoculation of chickens. The powder was kept at 4 $^0$C until use.

Samples of raw propolis (Fig.6) were collected from bee colonies (Apis mellifera) and obtained from the Apicultural Institute, stored in polyethylene bags, in a dry space, at 4 $^0$C, until use.

Alcoholic tincture of propolis (Fig.7) was obtained within the Phytotherapy Compartment-Romvac Company.

Zeolite powder (Fig.8) was made available by the Phytotherapeutic Compartment.

Organic calcium citrate (Fig.9) was obtained from conventional eggshells, by a method based on the chemical reaction between citric acid and calcium carbonate, within the National Research and Development Institute for Chemistry and Petrochemistry-ICECHIM.

**Figure 4.** Freeze dried powder of CPZ 10 kDa + aerosil+starch

**Figure 5.** Lyophilized whole hyperimmune egg powder

**Figure 6.** Raw and ground propolis sample

**Figure 7.** Propolis tincture

**Figure 8.** Zeolite powder

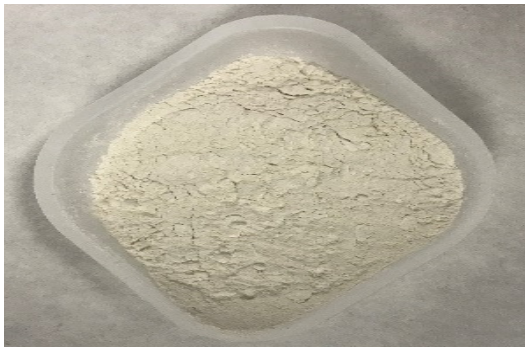


**Figura 9.** Organic calcium citrate

### 1.2. Identification and characterisation of the purity of protein fractions from bovine whey samples by SDS-PAGE

To identify the protein profile and confirm the presence of proteins in whey protein concentrate samples, the dodecyl sulphate-polyacrylamide gel electrophoresis separation method (SDS – PAGE) was used.

**Samples used:**

P1-Retentate 5 kDa, Mw=5-1000 kDa

P2-Retentate 10 kDa, Mw=10-1000 kDa

- samples were properly diluted directly in loading buffer, with mercaptoethanol followed by incubation for 10 minutes at approximately 90$^0$C. Samples were diluted in multiple dilutions
- each sample was charged in electrophoresis in the amount of 5 µL
- the samples were maintained 80 min. at an intensity of 90 mA and 200 V for migration
- the colouring of the samples was done overnight
- discoloration was carried out with discoloration solution no. 1 (acetic acid, ethanol), for 2h, then at room temperature, static with discoloration solution no. 2 (7% acetic acid) for 24h

### 1.3. Chromatographic analysis of propolis extract

After studying the literature data, a gradient method was chosen, which uses 5% acetonitrile and acetic acid as the mobile phase. The determinations were made on a Hitachi Chromaster HPLC and a Hypersil BDS C18 Thermo Scientific column was used. The chromatographic separation conditions are shown in Table 1.

The samples were prepared by diluting 0.5 mL propolis extract, to 5 mL, with acetonitrile-acetic acid mixture 5% (30/70, v/v). After ultrasonication and filtration by 0.45 µm PVDF filter, 20 µL were injected into the chromatographic system.

**Table 1**
Chromatographic separation conditions

| Chromatographic column | Hypersil BDS C18 Thermo Scientific, L = 250 mm, ID=4.6 mm, 5 µm | | | |
|---|---|---|---|---|
| Composition of the mobile phase | Solvent a: acetonitrile<br>Solvent B: acetic acid 5% | | | |
| Mobile phase | Time (min) | % solvent A | % solvent B | Flow rate (mL/min) |
| | 0 | 8 | 92 | 1 |
| | 30 | 91 | 9 | |
| | 35 | 8 | 92 | |
| | 40 | 8 | 92 | |
| Wavelength | 280 nm | | | |
| Column temperature | 25 $^0$C | | | |
| Autosempler temperature | 25 $^0$C | | | |
| Injection volume | 20 µL | | | |

Standard stock solutions of caffeic acid, syringic acid, rutin trihydrate, coumaric acid, ferulic acid, benzoic acid, salicylic acid, mandelic acid and quercetin, were prepared by dissolving 10 mg substance in 10 mL ethanol, and dilution for the working solution was made in acetonitrile-acetic acid 5% mixture (30/70, v/v).

### 1.1. Formulation of preparations

#### 1.1.1. IMUNOZEOMILK, protein powder for oral suspension

The protein powder was prepared by mixing freeze-dried bovine whey powder (10 kDa) stabilized with silicon dioxide (aerosil) and maize starch with freeze-dried whole hyperimmun egg powder, calcium citrate

and zeolite in the proportions shown in Table 2 below.

**Table 2**
Composition of Imunozeomilk powder

| | | |
|---|---|---|
| Lyophilised powder of bovine whey protein concentrate (stabilised) | Whey protein concentrate 10 kDa<br>Aerosil (silicon dioxide)<br>Maize starch | 40% |
| Lyophilized whole hyperimmune egg powder | | 40% |
| Zeolite powder | | 11,7% |
| Calcium citrate | | 8,3% |

### 1.1.2. PROPOL FRESH, in the form of a solution and gel for cleaning and moisturizing hands, with propolis

The preparations were formulated as hydroalcoholic solutions using absolute ethyl alcohol and propolis tincture, to which glycerin, panthenol and aloe vera were associated for the moisturizing effect they give. Hydroxyethylcellulose was used to gel the hydroalcoholic solution to obtain the preparation as a gel. Table 3 below shows the composition of each preparation.

**Table 3**
Composition of Propol Fresh solution and gel preparations

| Propol fresh solution | | Propol fresh gel | |
|---|---|---|---|
| Ethanol 96$^0$ | 65% | Ethanol 96$^0$ | 65% |
| Propolis tincture | 0,5% | Propolis tincture | 0,5% |
| Glycerin | 2,5% | Glycerin | 2,5% |
| Aloe vera gel | 3% | Aloe vera gel | 3% |
| Panthenol | 1% | Panthenol | 1% |
| Deionised water | 28% | Hydroxyethylcellulose | 2% |
| | | Deionised water | 26% |

## 1.1. Analysis of physico-chemical parameters

For the analysis of the protein powder we analized the concentration of immunoglobulin Y, carried out by the ”in house" ELISA technique, in the Immunoinstant Research and Development Department and the protein content determined by the Kjeldahl method, in the Physico-Chemical Control Laboratory.

### 1.1.1. Quantitative determination of immunoglobulin Y (IgY) by direct ELISA test

The ELISA test is prepared in house especially for each test. The ELISA test can detect immunoglobulins at very high dilutions. The minimum amount of IgY detected is 10 nanograms in the test material.

Due to the specificity and reproducibility of the enzyme immunoassay reaction, the ELISA test is used in the production process of IgY, in production phases, in qualitative and quantitative control.

**Working technique:**

- prepare standard IgY in successive dilutions in the range of 60 ng/mL - 1 ng/mL and the sample in dilution 1:10$^5$;
- 100 µl /well of the diluted suspension shall be allocated according to the plate configuration;
- the plate is left to cover for 60 minutes at +37 $^0$C;
- the plate is washed 3 times with washing solution PBS-Tween 20 (0.05%).
- the reaction is blocked with a buffer, 300 µl/well and incubated for 30 minutes at room temperature;
- the blocking fluid is removed;
- distribute 100 µl of 1:10,000 diluted bird anti-IgG conjugate into each well using the conjugate dilution buffer;
- incubate for 2h at +37$^0$C;
- wash 4 times with PBS-Tween 20 (2%) washing solution;
- add 100 µl TMB and leave at room temperature 5-15 min;
- add 100 µl stop solution;
- read the absorbance at 450 nm.

### 1.1.2. Determination of protein content by Kjeldahl method

The procedure establishes the methodology for determining the protein content of biological products.

**Working technique:**

- samples undergoing analysis must be passed into the solution with the help of purified water and prepared in triplicate
- in each sample falcon tube 6-7 mL of 40-50% trichloroacetic acid solution is added, after which the falcon tubes are left in the stand, at a temperature of maximum 20 $^0$C, for a period of minimum 12 hours, then centrifuged at 1700-1800 rpm for 10-15 min.
- the precipitate is dissolved with the help of a solution of sulfuric acid 45-55%
- the contents of the falcon tube are quantitatively passed into a mineralization test tube and one pill of mineralization catalyst (Kjeltabs or Missouri) and 20 mL 98% sulfuric acid are added for nitrogen determination
- the last two mineralization tubes are the blank sample tubes
- start the Mineralizer Speeddigester K-425,
- the KjelFlex K-360 distiller will start simultaneously with the start of the G10S titrator
- at the end of the distillation procedure, the titrator will start and perform the titration of the resulting mixture, displaying the volume of sulfuric acid 0,05 M consumed
- after the end of distillation and titration of the 2 blank samples, the average titration volumes can be made
- continue the distillation and titration of the samples themselves, noting the titration volumes

The protein content is calculated according to the formula:

$$\frac{(\text{Vsample} - \text{Vblank}) \times z \times M \times f \times 14{,}0067}{\text{m(v)}} (\times 6{,}25)$$

**where**:

$V_{sample}$ is the volume of 0.05M sulfuric acid consumed for sample titration

$V_{blank}$ is the average volume of 0.05M sulfuric acid consumed in the titration of blank samples

z is the number of hydrogen atoms in the acid with which it is titrated (in the case of sulfuric acid z=2)

M is the molarity of the acid with which it is titrated (in this case M = 0.05)

f is the volumetric correction factor of the acid with which it is titrated (in this case f=10000) 14.0067 and 6.25 are correspondence constants

### 1.2. Determination of antimicrobial activity of Propol Fresh products

To carry out this screening on 5 representative microbial strains, we used a method derived from a compendial technique, namely, the determination of bacteriostatic/bactericidal, fungistatic/fungicidal activity by direct contact between microbial suspensions at different concentrations and the product to be tested.The test strains come from the ATCC collection, in the form of a lyophilized pellet. Before being placed in direct contact with the samples, microbial suspensions are made in sterile saline with a titre of $10^8$ UFC/mL.

**Test-microorganisms:**

- *Pseudomonas aeruginosa* ATCC 9027 (Gram-negative bacteria)
- *Staphylococcus aureus* ATCC 6538 (Gram-positive bacteria)
- *Bacillus subtilis* ATCC 6633 (sporulated bacteria)
- *Candida albicans* ATCC 10231(yeast)
- *Aspergillus brasiliensis* ATCC 16404 (filamentous fungi)

The $10^8$ CFU/mL suspension of each test microorganism is diluted to $10^6$ CFU/mL and then decimal dilutions are made in sterile saline for all microorganisms to the lowest dilution (10 CFU/mL). Each dilution of each test microorganism shall be put in direct contact with the test sample in a ratio of 1:1, using 1 mL of each (microbial suspension:test product).

The microorganisms diluted at the 6 concentrations are left in contact with the sample for 5 minutes, then 1 mL of suspension is taken, which is distributed on the surface of a Petri dish with solid culture medium (tryptic Soy agar – bacteria;

Sabouraud agar - filamentous fungi and yeasts). Both the Petri dishes and the microorganism+sample test tubes were thermostated as follows: test tubes and Petri dishes containing bacteria are incubated at 35 $^0$C for 24 hours, while test tubes and Petri dishes containing fungi are incubated at 25 $^0$C for 48 hours. Control Petri dishes are also prepared and incubated in the same way as sample plates.

At the end of the incubation period, the colonies developed on the Petri dishes that have been inoculated with suspension after a contact time of 5 minutes will be counted. 0.1 mL of suspension shall be taken from each test tube that has been placed at the thermostat and distributed over the surface of other Petri dishes with Tryptic Soy agar and Sabouraud agar to determine possible antimicrobial activity after a contact time of 24 hours. Petri dishes inoculated with bacteria will be incubated at 35 ℃ for 24 hours, while Petri dishes inoculated with fungi will be incubated at 25 ℃ for 2-3 days. After the end of the incubation period, the plates are visualized and the possible developed colonies are counted.

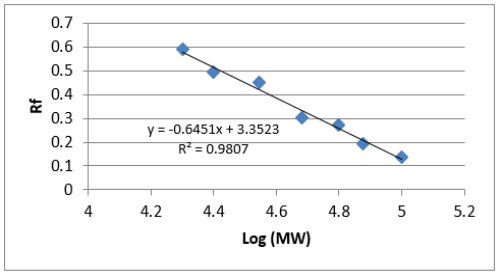
### 1.1. Quality control of prepared formulations

a) Organoleptic control (appearance, color, smell) - was carried out according to the provisions of the Romanian Pharmacopoeia ed. X (FRX) and the literature.
b) Pharmacotechnical control - PH determination - was performed by Potentiometric method according to the provisions of the Romanian Pharmacopoeia Ed. X and the literature.

## 2. Results and Discussions

### 1.2. Identification and characterisation of the purity of protein fractions from bovine whey samples by SDS-PAGE

To calculate the masses of fractions expressed as a result of electrophoretic migration, a mass curve is made based on the migration model of the molecular marker (Fig. 10). Based on the equation of the straight line, the molecular mass of separate fractions for the samples taken in the analysis is calculated.




From the analysis of the protein profile of the whey concentrate samples in Fig. 11 it can be seen that the most pronounced bands are located in the region with molecular mass between 12 and 20 kDa, representing the apparent molecular masses for the α-lactalbumin fraction and the β-lactoglobulin monomer, respectively.

The corresponding bands for immunoglobulins, lactoferrin and BSA are more pronounced in the case of sample P2 on lines 9-12. The calculated molecular masses and probable protein fractions in the samples analysed are presented in Table 4.

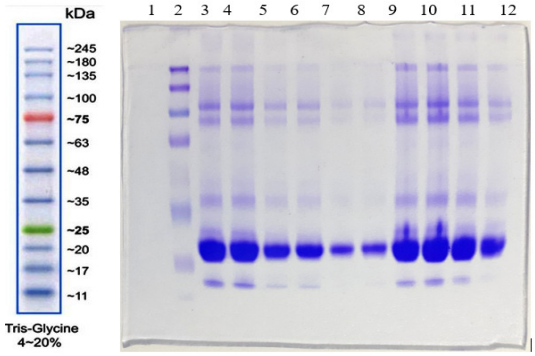



**Figure 11.** Polyacrylamide gel electrophoresis (SDS-PAGE) of protein fractions in bovine whey protein concentrate samples

SDS-PAGE 12% , 5 µl sample/well
Marker protein VI, Applichem

| | | | |
|---|---|---|---|
| 1. | Free | 7. | P1 dilution 1: 50 |
| 2. | Marker | 8. | P1 dilution 1: 50 |
| 3. | P1 dilution 1: 10 | 9. | P2 dilution 1:50 |
| 4. | P1 dilution 1: 10 | 10. | P2 dilution 1:50 |
| 5. | P1 dilution 1: 20 | 11. | P2 dilution 1: 100 |
| 6. | P1 dilution 1: 20 | 12. | P2 dilution 1: 200 |

**Table 4**

Calculated molecular weights and probable proteins identified in the whey protein concentrate samples

| MW (kDa) | Probable proteins |
|---|---|
| 135.7857 | ≈ Immunoglobulins (150,000) |
| 101.2616 | |
| 79.29945 | ≈ Lactoferrin (80,000) |
| 65.21246 | ≈ BSA (69,000), ≈Lactoperoxidase (70,000) |
| 53.62793 | |
| 41.99683 | ≈β-lactoglobulin (36,000) |
| 27.04597 | |
| 22.24144 | ≈ β-lactoglobulin monomer (18,000) |
| 15.79496 | |
| 12.9891 | ≈ α-lactalbumin (14,000) |
| 8.784154 | ≈ Glycomacropeptide (6,700) |

## 2.1. Chromatographic analysis of propolis extract

The specific chromatogram of propolis extract is shown below (Fig.12).

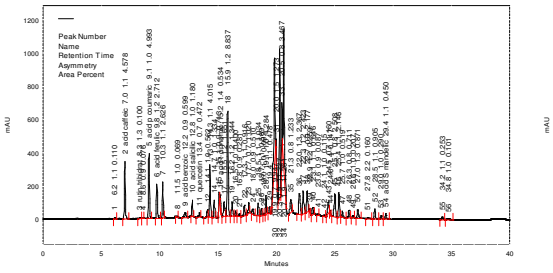



**Figure 12.** Propolis extract specific chromatogram

## 2.1. Formulation of preparations

### 2.1.1. IMUNOZEOMILK, protein powder for oral suspension

A homogeneous, fine powder of beige-gray color, characteristic of the components, was obtained.

Protein powder IMUNOZEOMILK (Fig.13) is a dietary supplement for oral administration that can be used by people with special dietary needs, of all ages.

In its composition, lyophilized powders of bovine whey concentrate and whole hyperimmune egg are combined with zeolite and calcium citrate.

The product provides a major protein intake in conditions of body weakness, in anorexia, in convalescence, gives extra energy, helps reduce physical fatigue and improves the immune system.

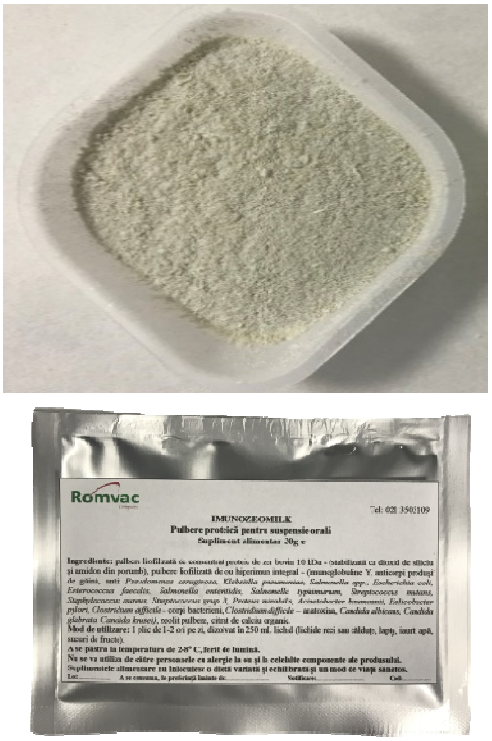



**Figura 13.** IMUNOZEOMILK, protein powder for oral suspension

IMUNOZEOMILK protein powder is conditioned in aluminized sachets with a powder content of 30g (for single-dose administration).

The product is taken orally, dissolved in cold or lukewarm liquids, milk, yogurt, water, fruit juices, 1 sachet 1-2 times a day. It is recommended to brew in 250 mL of liquid (equivalent to one glass).

### 2.1.2. PROPOL FRESH Solution and purifying Gel for cleansing and moisturizing hands, with propolis

Two formulations for external use were obtained and are presented as follows:

- Propol Fresh purifying gel in the form of a translucent gel, yellowish in color and characteristic smell of propolis, conditioned in 50 mL vials provided with a dropper stopper (Fig.14).

- Propol Fresh purifying solution in the form of a hydroalcoholic solution, clear, yellowish in color and characteristic smell of propolis, conditioned in 100 mL vials equipped with spray pump (Fig.15).

PROPOL FRESH products have purifying, cleansing and moisturizing action of the skin, ensuring full hygiene of the hands.

The high concentration of ethyl alcohol in the composition has a degreasing role, provides effective hand sanitization, without disturbing the natural balance of the skin, significantly reducing the spread of microbes.

Propolis tincture is known for its powerful disinfectant and anti-inflammatory action on the skin and additionally impresses a pleasant, subtle smell of the preparations.


**Figure 14** Propol Fresh purifying gel


**Figure 15** Propol Fresh purifying solution

Propolis has a proven beneficial effect on human health and is used for various medical and pharmaceutical purposes due to its properties:

- Antibacterial – bacteriostatic and bactericidal action against Gram-positive and Gram-negative bacteria. The mechanism of action involves stopping cell division and protein synthesis and destroying the cell wall (Salomao, K., *et al.*, 2008);
- Antiviral action - by destroying viral replication and inhibiting the entry of viruses into cells (Kuropatnicki, A.K., *et al.*, 2013).

PROPOL FRESH products not only cleanse, but also maintain and nourish the skin thanks to the content of glycerin, aloe vera gel and panthenol that have a satinizing and moisturizing role of the skin, precisely to prevent drying and cracking of the hands, which occurs following the use of products with a high alcohol content.

PROPOL FRESH sanitizing products are practical and easy to use in any situation. They clean the hands without soap or water and moisturize them. The quick-drying formula does not leave a sticky feeling. Ideal for travel or situations where hand washing is not possible.

## 2.2. Analysis of physico-chemical parameters

Fig.16 and Fig.17 below show the plate configuration for the ELISA test for quantitative determination of the concentration of IgY in the Imunozeomilk product. The sample analyzed is in position no.3.

| | Martor | Standard IgV | Standard IgV | Probe | Probe | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Blank | 60 ng/mL | 60 ng/mL | P1- $10^5$ | P1- $10^5$ | | | | | | | |
| B | SN1:1000 | 30 ng/ml. | 30 ng/ml. | P2- $10^5$ | P2- $10^5$ | | | | | | | |
| C | SN1:1000 | 15 ng/ml. | 15 ng/ml. | P3- $10^5$ | P3- $10^5$ | | | | | | | |
| D | SN1:1000 | 10 ng/mL | 10 ng/mL | P4- $10^5$ | P4- $10^5$ | | | | | | | |
| E | SP 30 ng/mL | 5ng/mL | 5ng/mL | P5- $10^5$ | P5- $10^5$ | | | | | | | |
| F | SP 30 ng/mL | 2.5ng/mL | 2.5ng/mL | | | | | | | | | |
| G | SP 30 ng/ml. | 1 ng/ml. | 1 ng/ml. | | | | | | | | | |
| H | Blank | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |

**Fig.16** Configuration for the ELISA plate

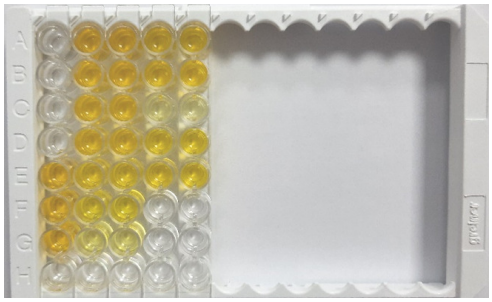


**Fig.17** ELISA plate image - determination of IgY content from the product Immunozeomilk

The results are processed based on the calibration curve performed for the standard IgY. Using the equation of the resulting line and taking into account the dilution factor and the method of processing the sample, the total IgY content of the analyzed sample is determined.

Table 5 shows the results obtained for the analysis of total nitrogen, protein content and immunoglobulin Y concentration for the lyophilized powders and for the product Immunoseomilk, respectively.

**Table 5**
The results obtained for the analysis of physico - chemical parameters

| Sample | Analite | Value | Method of analysis |
|---|---|---|---|
| Lyophilized hyperimmune egg powder | Total nitrogen | 86.4405 mg/g | Kjeldahl Method |
| | Total protein | 540.2531 mg/g | Result obtained by calculation (protein nitrogen x 6,25) |
| | IgY Concentration | 1,277 g/100g | Quantitative determination by direct ELISA |
| Whey protein concentrate 10 kDa (lyophilisate) | Total nitrogen | 55.5765 mg/g | Kjeldahl Method |
| | Total protein | 347.3531 mg/g | Result obtained by calculation (protein nitrogen x 6,25) |
| Imunozeomilk powder | Total nitrogen | 43.193 mg/g | Kjeldahl Method |
| | Total protein | 269.956 mg/g | Result obtained by calculation (protein nitrogen x 6,25) |
| | IgY Concentration | 0.5108 g/100g | Quantitative determination by direct ELISA |

## 2.1. Determination of antimicrobial activity of Propol Fresh products

The results of antimicrobial activity are presented in Table 6 below.

The analysis of the results shows that the product had antimicrobial activity after the first 5 minutes of contact with all concentrations - $10^6$ CFU / mL, for all microorganisms tested, except for *Aspergillus brasiliensis*. In this case, the tested product reduced the concentration of the microorganism by $10^2$ CFU after a contact of 5 minutes. After 24 hours the product had a fungicidal effect.

It can be concluded that Propol Fresh products have bactericidal, yeasticidal and fungistatic effect for the tested microorganisms, at all concentrations, after a contact time of 5 minutes.

**Table 6**
Results of antimicrobial activity-Screening on 5 representative microbial strains

| Test-microorganism | Dilution (CFU/mL) | Results (CFU/0.1 mL/plate) | | |
|---|---|---|---|---|
| | | Contact time 5 minutes | Contact time 24 hours | Control |
| *Pseudomonas aeruginosa* ATCC 9027 | $10^6$ | 0 | 0 | 73 |
| | $10^5$ | 0 | 0 | |
| | $10^4$ | 0 | 0 | |
| | $10^3$ | 0 | 0 | |
| | $10^2$ | 0 | 0 | |
| | 10 | 0 | 0 | |
| *Staphylococcus aureus* ATCC 6538 | $10^6$ | 0 | 0 | 59 |
| | $10^5$ | 0 | 0 | |
| | $10^4$ | 0 | 0 | |
| | $10^3$ | 0 | 0 | |
| | $10^2$ | 0 | 0 | |
| | 10 | 0 | 0 | |
| *Bacillus subtilis* ATCC 6633 | $10^6$ | 1 | 0 | 55 |
| | $10^5$ | 0 | 0 | |
| | $10^4$ | 0 | 0 | |
| | $10^3$ | 0 | 0 | |
| | $10^2$ | 0 | 0 | |
| | 10 | 0 | 0 | |
| *Candida albicans* ATCC 10231 | $10^6$ | 0 | 0 | 48 |
| | $10^5$ | 0 | 0 | |
| | $10^4$ | 0 | 0 | |
| | $10^3$ | 0 | 0 | |
| | $10^2$ | 0 | 0 | |
| | 10 | 0 | 0 | |
| *Aspergillus brasiliensis* ATCC 16404 | $10^6$ | >300 | 0 | 37 |
| | $10^5$ | 74 | 0 | |
| | $10^4$ | 6 | 0 | |
| | $10^3$ | 1 | 0 | |
| | $10^2$ | 0 | 0 | |
| | 10 | 0 | 0 | |

## 2.1. Quality control of prepared formulations

Organoleptic control involved the analysis of the appearance, color and smell of external use preparations and oral powder. the pH was determined for each

sample. The results obtained are presented in Table 7.

**Table 7**
The results obtained for organoleptic and pharmacotechnical control

| | Propol Fresh solution | Propol Fresh gel | Imunozeomilk |
|---|---|---|---|
| **Appearance** | clear solution | translucent gel | fine, homogeneous powder |
| **Color** | yellow | yellow | grey-white |
| **Smell** | characteristic of propolis | characteristic of whey | characteristic of whey |
| **Taste** | - | - | weak-sour |
| **pH** | 5,0 | 5,97 | 5,38 |

## 3. Conclusions

The present study aimed to formulate pharmaceutical preparations using as raw materials by-products resulting from industrial processing of milk, eggs and hive products. As active principles, we used whey from bovine milk, propolis, egg shells to obtain calcium citrate and associated them with lyophilized hyperimmune egg. All these substances are known for their content in valuable principles and include proteins that possess important nutritional and biological properties.

Two types of preparations have been formulated, a dietary supplement in the form of a powder for oral administration and a product for external use, for topical application, in two forms of presentation, a solution and a hydroalcoholic gel for hand sanitization, respectively.

Each component of the products shows specific properties and actions, but their combination in different pharmaceutical preparations leads to a synergism of potentiation of all beneficial effects resulting in complex and complete formulas for the health of the whole organism.

IMMUNOZEOMILK, protein powder for oral suspension is a dietary supplement that can be used by people with special dietary needs, of all ages. It provides a major protein intake in the condition of body weakness, in anorexia, in convalescence, gives extra energy, helps reduce physical fatigue and improves the immune system.

The products for external use, PROPOL FRESH solution and gel have purifying, cleansing and moisturizing action of the skin, ensuring full hygiene of the hands. They contain a high concentration of ethyl alcohol that ensures effective hand sanitization and the combination of propolis tincture contributes to a strong disinfectant action and in addition impresses a pleasant, subtle smell of the preparations. PROPOL FRESH products help to moisturize the skin due to the content of glycerin, aloe vera and panthenol and also prevent the drying of hands following the use of products with high alcohol content.

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