DEVELOPMENT OF FOOD IMPROVING CALCIUM-ENRICHED BIOACTIVE AGENTS PRODUCED FROM CHITINOUS WASTES GENERATED IN THE PROCESS OF AQUATIC ANIMAL PROCESSING

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Abstract
The CHIZITEL chitin-and-mineral complex is obtained from crustacean wastes. The BAA formula helping to facilitate calcium, phosphorus and magnesium fixation in the human body was found to be the result of this study. This multifunctional food improving bioactive agent - CALCIUM-D3-CHIZITEL, as a valuable polysaccharide of chitin, contains D3 vitamin and citric acid and can be used as a supplementary source of natural calcium. It is efficient in eliminating calcium deficiency in humans and preventing nutritional diseases.

Key words: *wastes from crustacean processing, calcium, chitin-and-mineral complex, CALCIUM- D3- CHIZITEL bioactive food improving agent (BAA CHIZITEL).*

1. Introduction

It is known [1] that the yield of wastes after shellfish processing for food purposes exceeds 50 – 80%. The wastes contain large amounts of chitin, calcium, magnesium and phosphorus. Presently, the majority of populations worldwide experience a shortage of vitamins, mineral substances, dietary fibres and naturally occurring bioactive agents in daily rations. Calcium deficiency is one of the most widespread and dangerous eating disorders. The average daily consumption of calcium in Russia is 200 – 530 mg per day [2, 3], compared to the recommended daily intake of 1250 mg [4]; hence, it is 2.3 – 6.3 times lower than the optimal physiological need.

Calcium is the main construction material for bones in the human body and it also performs many other functions: it affects muscle tone, nerve conduction and blood coagulation. Calcium deficiency may often result in osteoporosis, frequent bone fractures, rickets, muscle cramps, thrombosis, hypertensive disease, allergy, rectal cancer, decayed teeth and brittle nails, fatigue, decreased mental alertness and senility [2, 3, 5].

Lack of dietary fibres (i.e. chitin) in a food ration may cause gastrointestinal and cardiovascular diseases and provoke the accumulation of heavy and transition metal ions and radionuclides in the human body.

For this reason, the development of food improving biologically active agents (BAA) and functional food products with high content of the above-mentioned microelements is extremely vital. Their regular use in daily rations is one of the ways to solve this problem [2, 3, 6].

The OAO Giprorybflot (St. Petersburg) has developed an electrochemical method for processing crustaceous wastes and obtained, as a result, a chitin-and-mineral complex CHIZITEL, which is a new product with unique properties [7].

2. Materials and methods

The following raw materials were used: crusts of sea and fresh water shellfish (gammarus), vitamin D_3, citric acid (see Process Specification), and vitamin C (***Table 1***).

The CHIZITEL was obtained from electrochemically-treated gammarus crusts (42, 43, 44).

Total nitrogen was determined according to the Kjeldahl method. A 0.2 - 0.3 g sample was weighed with an absolute error of 0.0005 g into a tube made of filter paper or tinfoil that was closed at one end. The tube was placed into a 100 cm^3 combustion flask, where several small cupric sulphate crystals (0.2 - 0.3 g) and 10 - 20 cm^3 of sulphuric acid with a density of 1840 kg/m^3 were added.

The flask was placed in the draught cupboard for heating; special care was taken to prevent splashing of the liquid. The flask and its contents were heated to achieve a homoge-

neous mixture; this was cooled and 0.5 g of potassium sulphate was added, and the content was heated again until the liquid became transparent, greenish-blue with no brown shades.

Upon completion of combustion, the flask contents were cooled and quantitatively transferred into the 500 - 750 cm^3 stripping flask. The combustion flask was rinsed with water and checked for complete washing out by adding one or two drops of methyl red solution.

A 250 - 300 cm^3 conical flask was used as a receiver and 25 - 30 cm^3 of sulphuric acid solution with a density of 0.05 mol per dm^3 was poured from a measuring burette into the flask. The end of the condenser tube was submerged in sulphuric acid solution.

Approximately 50 - 70 cm^3 of 330 g per dm^3 sodium hydroxide solution was poured slowly down one side of the stripping flask to avoid the mixing of liquids; a strip of litmus paper was thrown into the flask, which was quickly closed with a cap connected through a liquid trap with the condenser. The stripping flask content was carefully stirred and heated. The reaction of liquid inside the flask should be sharply alkaline.

When the liquid started to boil, the receiver was lowered in such a way that the condenser's tube was positioned at a certain distance from the solution surface. The stripping process lasted until 2/3 of the liquid had evaporated.

The end of stripping was detected by the use of red litmus paper. The process is considered complete when a strip of red litmus paper does not turn blue. The stripping was stopped when boiling shocks appeared.

Subsequent to the termination of the stripping process, the end of the condenser tube was washed with water into a receiving flask and an excess amount of sulphuric acid was titrated by means of hydroxide sodium solution with a density of 0.1 мol/per dm^3 in the presence of methyl red or a dual level indicator.

Determination of water retention value (WRV) – The chitosan samples were soaked in distilled water for 20 hours at ambient temperature. Excess water was removed by filtration and the residue was spun in a bench-top centrifuge for 10 min at 4000 min^{-1}. The samples were dried to a constant weight at 105 °C and the WRV was calculated based upon the weight differences of dry and wet samples according to the equation:

$$WRV = (m_1 - m_0)/m_1 \times 100\%$$

where m_1 - weight of the sample after centrifugation, and m_0 - weight of the sample after drying.

Vitamin D content was determined according to the method of normal-phase high-performance liquid chromatography. A 10 g premix sample weight with an absolute error of 0.0005 g was placed into a 250 cm^3 conical flask with a reflux condenser. An oxidation retarder (0.1 g of hydroquinone) and 50 cm^3 of aqueous-alcohol solution of alkali were added

to the flask and its content was subjected to saponification in a water bath at 82 - 85 °C within 30 min.

After saponification, the flask content was cooled to ambient temperature and 50 cm^3 of distilled water and 50 cm^3 of hexane were added; the mixture was thoroughly stirred and settled. As soon as the mixture stratified, the hexane layer was poured carefully into a 500 cm^3 separating funnel.

The hexane extraction was repeated twice using 50 cm^3 volumes; the last fraction was placed into a separating funnel by filtering the flask content through a gauze covered funnel. The residue left on the gauze filter was squeezed out using a glass rod and the rest was thrown away. The funnel was rinsed with distilled water (approx. 50 cm^3) into a stripping flask, where hexane extractions were collected. The rinsed extract was transferred into a dry distilling flask by filtering it through an ashless filter filled with anhydrous sodium sulphate (approx. 50 g). The stripping flask and filtration material were rinsed with 40 cm^3 of hexane. The mixture was then dehexanised using a rotary evaporator, with a water bath temperature of 70 °C. The dry residue was dissolved in 3 cm^3 of hexane, which was added in small volumes; after that, this was relocated into a preliminary calibrated test-tube with ground cover.

The extract obtained was used for chromatographic analyses.

A hexane and ethanol mixture in the ratio of 99.5:0.5 was used as a mobile phase. The elution rate corresponding to the column performance for certain types of premixes was set within the range from 0.5 to 2 cm^3 per min. A colour filter with a wavelength of 254 nm was used to detect vitamin D. The speed of registration was 0.3 - 0.6 cm per min.

The method of comparing against the standard was used for chromatographic measurements. The chromatographic peaks of the analysed sample and reference standard sample were compared.

Lipid content was defined by the gravimetric determination of total fat according to the Soxhlet chloroform/methanol fat extraction procedure. The 5 - 10 g weight sample (with an absolute error of 0.01 g) was placed into a porcelain jar. Anhydrous sodium sulphate (or sodium phosphate) was added in the amount twice or thrice as much as the sample weight and the mixture was thoroughly mixed with a pestle.

The dehydrated product was quantitatively transferred into a packet or a special casing (see i. 3.7.2.3). The porcelain jar was wiped with an ether-wetted cotton wool pellet, which was further added to the dried sample and placed inside the Soxhlet extraction apparatus. The extractor was connected to a preliminary flask that was dried at 105 °C and weighed. Ether was poured with consideration that its amount should be half as much as the extractor's volume. The extractor was connected by means of a ground cap with the condenser. Water was poured through the apparatus' condenser and the flask was slightly heated in a water bath.

The fat extraction lasted for 10 - 12 hours. The heating rate should be intensive enough to ensure that ether is drained not less than 5 - 6 and not more than 8 - 10 times per hour. Fat recovery rate was checked by applying a drop of the solution running from the extractor on a watch glass. The watch glass should be free of a fat stain after the drop of solution has evaporated.

During breaks in working hours, the level of ether inside the extractor should be sufficient to keep the sample weight submerged, and fat extraction should continue by infusing throughout the whole idle period.

Following fat extraction the flask was de-ethered and placed inside a drying cupboard where its content was dried at 100 - 105 °C until the fixed mass was achieved, cooled in a desiccator and weighed with an absolute error of 0.001 g.

Fluorine content was defined by spectroscopic methods in the near-infrared region according to a standard procedure.

Calcium content was defined according to the complexometric method.

Approximately 0.2 - 0.3 g of the studied sample was weighed into a combustion flask or a test tube, two cubic centimetres of hydrogen peroxide was added and within 1.5 - 2 min, three cubic centimetres of selenium-containing sulphuric acid was poured into the flask; after that, the flask was shaken slightly. The flasks (test tubes) were heated at 340 - 380 °C until complete de-colourisation of the solutions occurred. In cases where no de-colourisation of solutions occurred within 30 min in flasks or within 1.5 hours in test tubes, the samples were cooled to 60 - 80 °C, one cubic centimetre of hydrogen peroxide was added and the samples were heated again for 30 min. After being de-colourised, the solutions were cooled, quantitatively transferred to volumetric flasks (or test tubes), made up to 100 cubic centimetres by the addition of distilled water, and all of the components were thoroughly stirred.

Simultaneously, a blank determination was performed, repeating all of the stages of the analysis except the weight sampling of the studied material. A conical flask with a volume of 250 cubic centimetres was filled with the studied solution. The amount of solution varied from 5 to 50 cubic centimetres depending on its calcium content. Distilled water was added to make the volume of the solution up to 100 cubic centimetres (this was achieved by using graduated flasks and pouring water not using a measuring cylinder). The following ingredients were then added in small volumes, with swirling performed after each addition: a pinch of sodium citrate (approx. 30 mg), a pinch of hydroxylamine, 10 cubic centimetres of 20% solution of potassium hydroxide (pH of the studied solution should be not lower than 12.5 - 13.5), and approximately 30 mg of one of the indicators. Titration was started no later than 10 minutes after the addition of Trilon B solution, at a concentration of 0.01 mol per cubic decimetre and in the presence of a "witness" (a reference solution). Titration lasted until the colour changed from red-and-pink to blue in the case of eriochrome blue-black P(R), from yellow-green to pink in the case of calcein, and from violet to dark-blue in the case of chrome dark-blue acid.

The following ingredients were used to prepare the reference solution: 100 cm^3 of distilled water, equal amounts of the above-mentioned reagents and several drops of Trilon B.

Magnesium content was defined by the flow-through injection method with photo-colorimetric determination using a FIAstar 5010 Analyser from Tecator.

The method is based on the injection of a preliminary prepared (cinerated at 45 °C, dissolved in a weak solution of HCl) sample into the carrier flow generated in the fluid communications of a chromatograph, and on further subsequent injection of reagents used to carry out an analytical reaction inside the device. The concentration of the studied components was determined according to a detector signal. A photo-colorimetric sensor was used to detect the extinction of the solution, according to which the concentration of ions in solution could be determined using the calibration schedule.

3. Results and discussions

It is known that high absorption properties are usually shown by naturally occurring sources of calcium with easily digestible formula and consisting of complex conglomerates of macro- and micro-elements that are extremely important for metabolic processes in the human body. Mineral substances after shellfish processing are an excellent raw material for producing medicated products used to compensate for mineral losses in humans: i.e. deficiencies of calcium, iodine, selenium, molybdenum and other chemical elements that play an important role in human vital activity [11].

Chitinous wastes contain not only mineral substances but chitin itself, which also has a number of unique properties (lipotropic, sorption, bacteriostatic, immune modulating, regenerative and others), and can be determined as a parapharmaceutical preparation that is recommended for the treatment of obesity, atherosclerosis and high cholesterol levels in blood [12].

The following important biogenic elements comprise the CHIZITEL: calcium – 22 - 28%, magnesium – 3 - 4%, phosphorus – 1.3 - 1.5%, sodium – 1.26%, potassium – 0.25%, ferrum – 0.12%, zinc – 0.09%, as well as cuprum, chrome and others.

Due to its natural origin, CHIZITEL, like any other N-acetyl-β-D-glucosamine, is non-toxic (LD_{50} = 15 g/kg, 4 toxicity class), biocompatible with human tissues, biodegradable and biologically active.

Tests on pets (*in vivo*) performed by the Russian Federation Health Ministry Institute of Toxicology (St. Petersburg) demonstrated that the CHIZITEL is a multi-functional, comprehensive medication that has not only parapharmaceutical (similar to chitosan) but also neutroceutical properties as well.

It was found that the CHIZITEL as a parapharmacuetical is able to:

– normalise lipid metabolism by acting as a lipotropic and anti-sclerotic agent, lowering the level of general lipids, triglycerides, low-density lipoproteins and atherogenic index;

– detoxify heavy metals in the case of intoxication in the human body (it binds toxic elements and lowers their level in blood and bones);
– normalise the gastrointestinal microbial population.

Calcium deficiency was simulated by the surgical removal of parathyroid glands from rats and, as a result, the level of calcium in their blood was reduced to half the normal level; clonicotonic convulsions developed and the animals died within 5 - 6 days. Administration of CHIZITEL helped to stop the convulsions and prolong the life of rats that had undergone parathyroid gland removal for a certain period of time. The level of calcium in blood also normalised.

Consequently, CHIZITEL was proven to be efficient in replenishing calcium in the human body. It demonstrated high bioavailability of calcium as a component of the medication and showed that CHIZITEL could be successfully used as a nutriceutical.

CHIZITEL is not accumulated in the human body, nor does it cause any astringent taste in the mouth. CHIZITEL is not dissolved in the gastrointestinal tract (GIT) (unlike chitozan), so there is no risk of penetration through the intestinal walls; it normalises hyperacidity in the stomach and has no negative effects on digestion.

Sanitary measurements of CHIZITEL comply with requirements of the current Sanitary Regulations and Standards (SanPin 2.3.2.1078-01). The shelf-life of CHIZITEL, with all of its properties (i.e. organoleptic, microbiological, functional), is 3 years. The Russian Federation Public Health Authority has issued a sanitary and epidemiology certificate for CHIZITEL.

It was previously proven [13 - 15 that the rate of calcium absorption by the intestinal tract largely depends on whether a human is properly supplied with vitamin D_3, which is converted by the kidneys into calcitriol (1.25-dihydroxyvitamin D_3). This hormone is necessary for efficient calcium transportation through the intestine. In its absence, no more than 10% of all of the calcium taken into the human body can be absorbed. Vitamin D_3 also activates calcipexy in bones and dentine by inhibiting parathyroid hormone (PTH) which, in turn, initiates calcium resorption (transfer of calcium from bones to blood).

The efficiency of calcium-based medications depends on the nature of the salt anions and the gastric acidity level. Citric acid ions facilitate the absorption of Ca^{2+} by humans [13 - 15].

It is worth mentioning, however, that CHIZITEL, being a water-insoluble flake powder with low apparent density, is not fit for human consumption.

The experimental procedure of the present study was aimed at the development of the CHIZITEL-based BAA formula and the method of production. The main purpose was to provide conditions for the efficient absorption of CHIZITEL powder in the form of easy-to-administer tablets and the fixation of its calcium component.

To enhance the efficiency of CHIZITEL as a nutriceutical and a rich source of calcium, it was necessary to develop compositions consisting of ingredients that are able to boost the absorption and fixation of this microelement.

The production process included the treatment of crustaceous raw material inside the electrolyser (no corrosives used), which made it possible to preserve the native structure of the chitin-and-mineral complex. Based on the product obtained, the authors developed a production process for the CALCIUM-D_3-CHIZITEL bioactive agent, mostly consisting of CHIZITEL, vitamin D_3 and citric acid.

The substantiation of vitamin D_3 proportion in CALCIUM-D_3-CHIZITEL was based upon the analytical estimation performed in accordance with the recommended daily consumption rate of the vitamin (200 ME) [17], taking into consideration the fact that a certain amount of vitamin D_3 can be administered in the human body together with some other food products, and certain inevitable losses of the vitamin during the manufacturing process were considered as well. BAA was prepared with a 15000 ME/g vitamin D_3 water solution in the following proportions: 0.5 ml per 100 g of the CHIZITEL.

Adding citric acid to CHIZITEL tablets can result in its additional demineralisation, which may occur during storage; this, in turn, will lead to complete distortion of the chitin-and-mineral structure and, consequently, to the loss of sorption properties. The optimal amount of citric acid to be added to CALCIUM-D_3-CHIZITEL was defined as 460 mg per 100 g of CHIZITEL, taking into consideration the minimal effect it could have on the mineral component of CHIZITEL during its shelf-life.

Apart from the functional components of the BAA, it was necessary to define proper additives, ensuring the satisfactory moulding and compacting properties of tablets formed from CHIZITEL.

Compacting of CHIZITEL powder, even though the particle diameter was ≤1.0 mm (optimal size for any compression process), showed no compliance of the tablet's strength with the requirements specified by the State Pharmacopeia [16]. Also, due to the low apparent density of CHIZITEL powder, its thickness was less than 2.0 – 2.5 mm.

The following process approaches were used to solve these problems:

– tablets were ground and compacted anew, and, as a result, CHIZITEL powder with 3mm particles and better tap density was prepared. This allowed the mass and thickness of one tablet to be increased up to 1 g and 3.0 - 3.5 mm accordingly (sample № 1);
– special wetting substances were added to CHIZITEL powder with the purpose of preparing powder with increased apparent density:
  1) 32% sugar syrup (sample № 2);
  2) 10% lactose solution (sample № 3);
  3) 3% starch paste (sample № 4).

The wetting reagents were mixed with CHIZITEL in the ratio of 1.4:1.0. The mixture was stirred for 20 – 30 min until all of its components were distributed evenly and it became

homogenous. The mixture was dried at 50 – 60 °C (moisture content being no more than 8%), and dry powder was calibrated through the 2.0 mm diameter sieves of an oscillating granulator.

The organoleptic and strength tests of tablets showed that samples №1, № 3 and № 4 had unsatisfactory mouldability. The most optimal strength properties complying with the State Pharm Authority [16] were shown by sugar syrup-treated tablets. The only disadvantage of 20 mm tablets was their relatively low mass of 0.98 g.

Consequently, it was decided to increase the tablets' mass and the powder was additionally fractionated to remove large fractions that negatively affected the proper shaping of tablets. For this purpose, sample №2 was sieved through the granulators with sieve diameters of 1.5 and 0.8 mm, which made it possible to increase the mass of the tablets without changing their diameter: a 20 mm tablet weighed 2.00 g and a 2.01 mm tablet weighed 2.12 g.

The tablets had satisfactory organoleptic properties: light-beige colour, no odour, slight mouthfeel of cooked shellfish.

The dissolution kinetics was tested as follows: one CHIZITEL tablet prepared with the use of sugar syrup was placed into a glass of warm water (36 °C) with a 1:2 mesh ratio and left unstirred. The tablet disintegrated completely into CHIZITEL particles within 60 minutes. The dissolving could be sped up by stirring the mixture slowly for 10 sec.

Consequently, a 32% sugar solution was chosen finally as a wetting reagent to support the strong structural properties of CHIZITEL tablets. This improved the flavour of CHIZITEL and its processing behaviour without affecting its efficiency as a parapharmaceutical.

A flow-chart of the CALCIUM- D_3- CHIZITEL bioactive agent production process is provided in ***Figure 1*** (see page 62). Ground CHIZITEL 1.0 mm-size particles were mixed with citric acid and vitamin D_3. The prepared mixture was placed into a granulator, and a 32% sugar solution was added continuously to the mixture in small portions until 0.8 mm sized granules were formed.

The granules were dried in the oven at temperatures no higher than 60°C until the moisture content was as low as 8%; then, the granules were loaded into the mixer and calcium stearate was added (2% by mass of granules) and the mixture was carefully stirred to avoid coalescence.

The granules, treated in this manner, were sent to the rotary tabletising press. The tablets made had the following properties: mass – 1 g, diameter – 20 mm, height – 3.00 mm (***Figure 2***). CALCIUM- D_3- CHIZITEL BAA in the form of tablets was packed into jars containing 30, 60, 90, 120, 150 or 180 pcs.

State registration certificate № 77.99.23.3.У.1024.2.06 was issued for the CALCIUM- D_3- CHIZITEL bioactive agent.

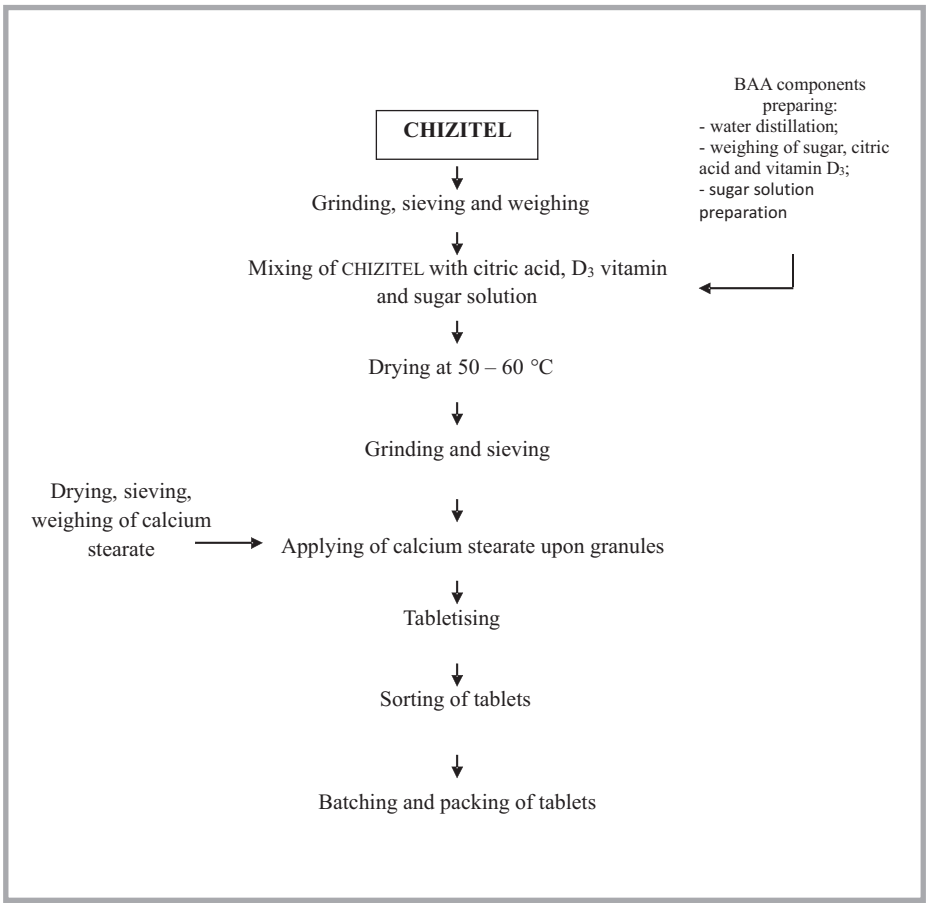

Figure 1. *Chart flow of CALCIUM- D_3- CHIZITEL BAA production process.*

The results of tests performed on the CALCIUM- D_3- CHIZITEL bioactive agent by the Russian Academy of Medical Sciences Institute of Nutrition showed that it is recommended to use BAA as a supplementary source of polysaccharides (chitin), calcium and vitamin D_3. Experts from the Russian Agency for Health and Consumer Rights Protection (Rospotrebnadzor) recommend taking two tablets of the CALCIUM- D_3- CHIZITEL bioactive agent twice a day whilst eating. The optimal duration of the BAA treatment was considered one month.

Two tablets contain at least 200 mg of calcium, 50 ME of vitamin D_3 and no less than 1 mg of polysaccharides.

Administering two tablets of BAA twice a day will enrich a human with at least 400 mg of calcium or 32% of the recommended daily intake (RDI) specified in [17], 100 ME of vitamin D_3 (50% of RDI), and 2 g of polysaccharides (40% of RDI).

4. Conclusions

1. The proposed production method has two advantages: a new BAA-pharmaceutical was obtained and wastes after shellfish processing were utilised efficiently.

2. The use of CALCIUM- D_3- CHIZITEL bioactive agent in the groups of adults over 45 years, teenagers, pregnant and breast-feeding women, patients with calcium fixation pathologies – of all those in need of calcium and bioactive agents support, will eliminate the shortage of calcium and prevent the development of osteoporosis. The BAA will improve food rations and boost the human immune defence system and resistance to adverse environmental impacts.

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