AUXILIARY DISCIPLINES

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PROSPECTS FOR EVALUATING THE *IN VITRO* BIOAVAILABILITY OF PROTECTED VITAMIN A IN BIORELEVANT MEDIA OF FARM ANIMALS

Research article

Abstract

Bioavailability is one of the most important indicators in assessing the absorption of vitamins by a living organism. Bioavailability is the assessment of the amount of nutrients absorbed from food/feed products through the gastrointestinal tract of an organism. Vitamin A is said to be bioavailable when it is converted to retinol and retinoic acid; absorbed by the intestinal epithelium. This study followed a new approach as an addendum to the conventional in vitro digestion models using microencapsulation to look into parameters that affect the bioavailability of vitamin A using in vitro models of pigs and cattle; to evaluate the bioavailability of a protected vitamin A in biorelevant environments of the gastrointestinal tract of farm animals. Encapsulated samples of vitamin A (retinol acetate) were obtained from Arnika LLC (Vladivostok, Russia) at different concentrations and divided into six groups. Parameters affecting the release of vitamin A from the microcapsules were evaluated through a spectrophotometric method by measuring the optical density of the biorelevant medium at a wavelength of 850 nm in an RTS-1 bioreactor. The result showed that the solubility kinetics of microencapsulated vitamin A samples varied with time and depended on the biorelevant media of pigs and cattle. It was also observed that the simulated gastrointestinal fluid (GIF) media at the intestinal stage showed solubility of microencapsulated vitamin A. Thus, it can be concluded that the components of the microcapsule shell are most soluble in the biorelevant environment of the intestine. It is also clear that microencapsulation technology can increase vitamin A bioavailability. Our findings unequivocally demonstrated that to enhance the bioavailability of vitamins; there is a need to incorporate modern technologies like microencapsulation for better delivery of the vitamins and other macromolecules.

Keywords: bioavailability in vitro, biorelevant media, feed vitamin A, feed additives, retinol acetate, farm animals, encapsulated vitamin A, bioreactor, capsule solubility, microcapsule.

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ПЕРСПЕКТИВЫ ОЦЕНКИ БИОДОСТУПНОСТИ ЗАЩИЩЕННОГО ВИТАМИНА А *IN VITRO* НА БИОРЕЛЕВАНТНЫХ СРЕДАХ СЕЛЬСКОХОЗЯЙСТВЕННЫХ ЖИВОТНЫХ

Научная статья

Аннотация

Биодоступность является одним из важных показателей при изучении усвояемости витаминов живым организмом. Биодоступность – это оценка количества питательных веществ, абсорбированных из пищевых продуктов/кормовых продуктов через желудочно-кишечный тракт организма. Считается, что витамин А биодоступен, когда он превращается в ретинол и ретиноевую кислоту; поглощается кишечным эпителием. В этом исследовании использовался новый подход в качестве дополнения к традиционным моделям пищеварения *in vitro* с использованием микрокапсулирования для изучения параметров, влияющих на биодоступность витамина А, с использованием моделей свиней и крупного рогатого скота *in vitro*; оценить биодоступность защищенного витамина А в биорелевантных средах желудочно-кишечного тракта сельскохозяйственных животных. Инкапсулированные образцы витамина А (ретинола ацетата) были получены от ООО «Арника» (Владивосток, Россия) в различных концентрациях

и разделены на шесть групп. Параметры, влияющие на высвобождение витамина А из микрокапсул, оценивали спектрофотометрическим методом путем измерения оптической плотности биорелевантной среды при длине волны 850 нм в биореакторе PTC-1. Результат показал, что кинетика растворимости образцов и биодоступность микрокапсулированного витамина А менялась со временем и зависела от биорелевантных сред свиней и крупного рогатого скота. Также было замечено, что среда, имитирующая желудочно-кишечный флюид, на стадии кишечника показала растворимость микрокапсулированного витамина А. Таким образом, можно сделать вывод, что компоненты оболочки микрокапсулы наиболее растворимы в биорелевантной среде кишечника. Также ясно, что технология микрокапсулирования может увеличить биодоступность витамина А. Полученные результаты продемонстрировали, что для повышения биодоступности витаминов; необходимо использовать современные технологии, такие как микрокапсулирование, для таргетной доставки витаминов и других макромолекул.

Ключевые слова: биодоступность *in vitro*, биорелевантные среды, кормовой витамин А, кормовые добавки, ретинола ацетат, сельскохозяйственные животные, инкапсулированный витамин А, биореактор, растворимость капсул, микрокапсула.

1. Introduction

1.1 Background

The effectiveness of feed additives is an important indicator for ensuring the health of farm animals, including for ensuring profit in farms. For efficient feeds, the amount of both macro and micronutrients must tally with the recommended values of daily intake for the animals. Apart from carbohydrates, proteins, fats and minerals, there is a need to attain the daily requirement of vitamins in feed for pigs and cattle. For instance, the daily requirement intake (DRI) of vitamin A for cattle and pigs is equivalent to 0.30 mcg of retinol or 0.344 mcg of retinyl acetate [21], [22].

Cattle and pigs' feeds usually are based on grains, forages and silage. These products are lacking in vitamins and other essential nutrients. Therefore, there is a need for the feeds to be fortified to increase their nutritional content, digestibility and bioavailability [29].

1.2 Vitamin A

Vitamins are biologically active substances that stimulate metabolic processes in the body, and not only people need them. Vitamins are of great importance in reproduction, the absence of various kinds of diseases and the growth of animals.

The value of retinol in animal nutrition is very high: it is necessary for normal growth and reproduction, as well as to increase the body's resistance to pathogens of various diseases. The main biological role of vitamin A in animals is that it takes part in the synthesis of visual pigment (rhodopsin), which is a protein compound with vitamin A, and maintains normal mucous membranes. However, retinol stimulates the growth of young animals.

Vitamin A is the name of a group of fat-soluble retinoids, primarily retinol and retinyl esters [7]. Vitamins are present in different feed sources, because of their essential metabolic processes within the animal. Animal feeds like human food contain two sources of vitamin A: preformed vitamin A (retinol and retinyl esters, Fig. 1) and provitamin A (carotenoids) as outlined by [21]. Preformed vitamin A is not available for many animals including cattle and pigs as it is mostly derived from animal sources. Unlike the former, provitamin A carotenoids are found in plants' pigment that the human and animal body converts into vitamin A in the intestine [18], [27]. The main provitamin A carotenoids in the human diet and animal feeds are beta-carotene, alpha-carotene, and beta-cryptoxanthin [25]. Vitamin A is crucial for vision, reproduction, growth, immunity and maintenance of tissues in the body [32].

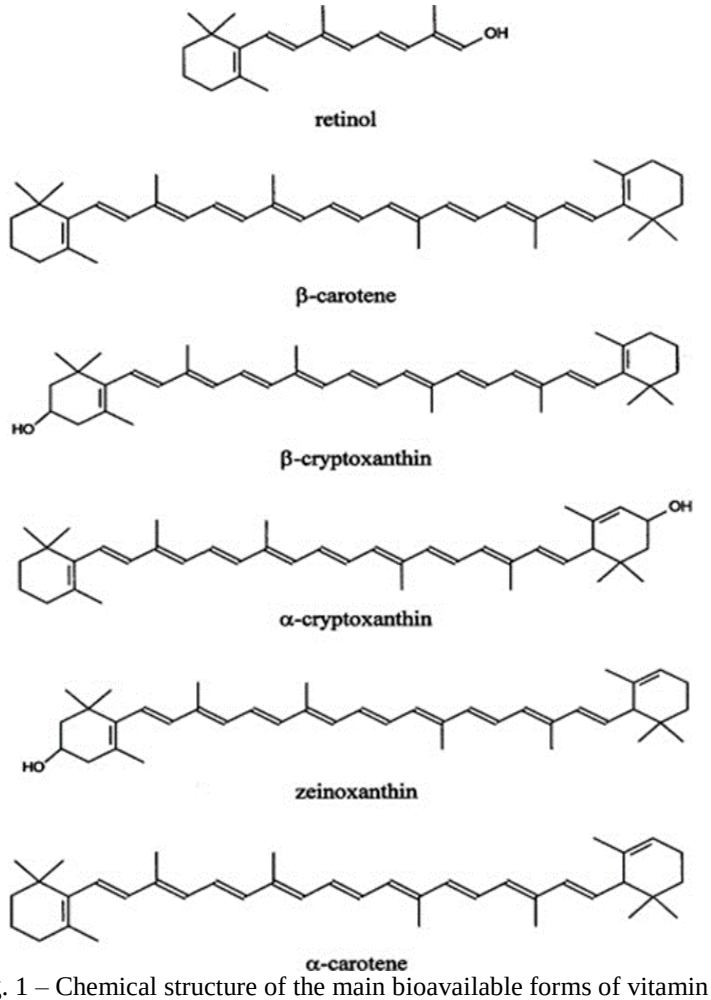

Fig. 1 – Chemical structure of the main bioavailable forms of vitamin A
Note: source [2]

The use of fortified forms of vitamin A is common in manufactured feeds which are usually used for vitamin supplements. Foods containing natural vitamin A (usually cod liver oil) are mostly used to fortify animal feeds. Plant sources like green forage, dehydrated alfalfa, and high-quality legume hays are also good sources of β-carotene. Both natural vitamin A and synthetic β-carotene are easily destroyed by air, light, and high temperatures [5]. For these reasons, it is very difficult to rely solely on natural feedstuffs as sources of vitamin A [21]. That is why most often animal and human diets are fortified by vitamin A. Fortification of animal feeds by vitamin A additive is regulated by the European Food Safety Authority Regulation of European Commission (EC) No 1831/2003 of maximum levels of vitamin A in feed intended for the main food-producing animals - pigs, cattle and poultry [7]. As vitamin A is usually found as the precursor of carotene; after consumption, carotene is converted to vitamin A from beta carotene, found in feeds like green forages and yellow corn in the small intestine [21]. In this regard, it is necessary to study aspects of the bioavailability of carotene concerning cattle and pigs.

1.3 Bioavailability

Bioavailability is related to the assessment of the amount of nutrients absorbed from food/feed products through the gastrointestinal tract of an organism [6]. More in-depth, it can be referred to as the portion of eaten food or nutrient that reaches the systemic circulation and the specific body sites (cells and tissues) where it can exert its biological function(s) [9]. Vitamin A is said to be bioavailable when it is converted to retinol and retinoic acid and absorbed by the intestinal epithelium [12].

Upon ingestion, carotenoids are digested and separated from the main feed matrix, emulsified with fat and incorporated into lipid oligomers in the small intestine for absorption by intestinal enterocytes; from there they are taken up exclusively through passive diffusion into the lymphatic system for delivery to the liver facilitated through membrane proteins [20]. This process is affected by many factors some of which are discussed below.

1.4 Factors affecting the bioavailability of vitamin A

The bioavailability of vitamin A depends on heat, processing methods [15]; availability of dietary fat, fiber, and other compounds in the feed that can compete with its absorption and assimilation [26]. For instance, [5] compared the bioavailability of vitamin A *in vitro* using Caco-2 cells and *in vivo* using depletion–repletion rat model. They found that components of food mixture affect the bioavailability of vitamin A both *in vitro* and *in vivo*. This was supported by [27] which found a strong relationship between the bioavailability of vitamin A with food complex matrixes containing sugar and milk fat to influence vitamin A absorption *in vitro*.

It was also found that the bioavailability of vitamin A is affected by longer exposure to higher temperatures, and low-fat contents of food/feed in an *in vitro* digestion and Caco-2 cell models [23]. Due to the fat solubility of the vitamin, it was found that nanocellulose emulsion encapsulation improves the stability and bioavailability of vitamin A *in vitro* [10].

Additionally, apart from the above-mentioned parameters, digestive enzymes also affect the bioavailability of vitamin A. It was found that β-Carotene digested slowly in simulated gastric fluid while digested faster in simulated intestinal fluid *in vitro*. This can be interpreted by a study by [16] which found that liposomes inhibit the digestion of β-Carotene in stomach and therefore the full digestion was fully in the intestine. Apart from that, *In vitro* digestion by [25] revealed that different milk protein complexes protect and inhibit vitamin A digestion under extreme gastric pH.

Bile production is another factor that influences the bioavailability of vitamin A, [31] found that the amount of bile extract present in an *in vitro* digestive medium influenced the estimated bioavailability of vitamin A from cereals. This is because the *in vitro* media used can affect enzyme secretion which in return affects the bioavailability of vitamin A. This is seen in a study by [8] that modified the standard *in vitro* protocol INFOGEST network parameters by increasing the amount of pancreatin, bile, and speed of the stirring machine. They found that those parameters enhanced the bio-accessibility and bioavailability of vitamin A.

1.5 In Vitro Digestion

Digestion is a mechanical and chemical process that breaks larger molecules of foods into smaller and soluble molecules for easy absorption to provide nutrients to the body cells [14].

Digestion can be made *in vitro* by mimicking the *in vivo* gastrointestinal conditions of food/feed. *In vitro* digestion methods mimic physiological conditions *in vivo*, taking advantage of different digestive enzymes, their pH concentrations, digestion time, and salt concentrations [3]. *In vitro* digestion uses models to explain human or animal digestion in relation to digestibility and bioavailability of pharmaceuticals, micro and macronutrients [19].

International Network of excellence on the fate of Food in the Gastrointestinal Tract (INFOGEST) protocol is the frequently used procedure in the study of *in vitro* nutrient digestion [4], [17], [30], [31]. This protocol was created in 2011 by international specialists in the field of digestion from more than 32 countries under the European Cooperation for Science and Technology (COST) action [32]. The network created a simulated digestion model for food [31] which is currently in use across the globe.

In a typical *in vitro* digestion, the mechanical breakdown of food is started by chewing simulation using mortar and pastel or a machine. Then the food bolus is mixed with simulated salivary and gastric fluid (SGF), specific amounts of 0.3 M of $CaCl_2$, and distilled water [30] are added while the pH is corrected by 1 M HCl (Tables 1 and 2).

Table 1 – Concentrations of electrolytes in Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) [29]

Constituent	SSF	SGF	SIF
	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}
K^+	18,8	7,8	7,6
Na^+	13,6	72,2	123,4
Cl^-	19,5	70,2	55,5
$H_2PO_4^-$	3,7	0,9	0,8
HCO_3^-, CO_3^{2-}	13,7	25,5	85
Mg^{2+}	0,15	0,1	0,33
NH_4^+	0,12	1,0	—
Ca^{2+}	1,5	0,15	0,6

After that, the specific amount of porcine pepsin solution made up of SGF is added, then the solution is incubated for 2 h at 37 °C under a stirring machine [26]. With this, the second step is done. The third stage starts with immediate simulated intestinal digestion by adding a specific amount of simulated intestinal fluid (SIF), 0.3 M $CaCl_2$, distilled water and enough bile extract solution to obtain correct bile salts in the final digestion volume [26]. At this stage, the pH is adjusted to 7 by adding 1 M NaOH. The solution is then incubated for an additional 2 h at 37 °C under a stirring machine [29].

Table 2 – Preparation of stock solutions of simulated digestion fluids

Constituent	Stock Conc.		SSF		SGF		SIF	
			pH 7		pH 3		pH 7	
			Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	g L^{-1}	mol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}
KCl	37,3	0,5	15,1	15,1	6,9	6,9	6,8	6,8
KH_2PO_4	68	0,5	3,7	3,7	0,9	0,9	0,8	0,8

End of table 2 – Preparation of stock solutions of simulated digestion fluids

Constituent	Stock Conc.		SSF		SGF		SIF	
			pH 7		pH 3		pH 7	
			Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	Conc. in SIF
	g L^{-1}	mol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}
$NaHCO_3$	84	1	6,8	13,6	12,5	25	42,5	85
NaCl	117	2	—	—	11,8	47,2	9,6	38,4
$MgCl_2(H_2O)_6$	30,5	0,15	0,5	0,15	0,4	0,1	1,1	0,33
$(NH_4)_2CO_3$	48	0,5	0,06	0,06	0,5	0,5	—	—
$CaCl_2(H_2O)_2$	44,1	0,3		1,5		0,15		0,6
For pH adjustment								
	mol L^{-1}		mL	mmol L^{-1}	mL	mmol L^{-1}	mL	mmol L^{-1}
NaOH	1		—	—	—	—	—	—
HCl	6		0,09	1,1	1,3	15,6	0,7	8,4

Note: source [30], [31]

Based on the above *in vitro* digestion antecedents and literature reviewed, the current research studied the bioavailability of microencapsulated vitamin A using cattle, and pigs simulated gastric and intestinal fluids models; by dissolving the vitamin A extracts in an *in vitro* digestive media, to analyze the concentration changes and determine the bioavailability of the models.

2. Methods

The study aimed to evaluate the bioavailability of a protected feed vitamin A in biorelevant environments of the gastrointestinal tract of farm animals. To achieve this goal, tasks were set to model the composition of biorelevant environments of the gastric intestinal tract of farm animals; to investigate the kinetics of microencapsulated vitamin A released in biorelevant media of the gastric intestinal tract of farm animals - pigs and cattle. Encapsulated samples of vitamin A (retinol acetate) produced by Research and Development (R&D) Agrobioeconomics of Arnika LLC (Vladivostok, Russia) with different shell composition and activity (measured in an international Unit, IU) were used as experimental samples of protected vitamin A: Sample No. 1 (500 IU); No. 2 (500 IU); No. 3 (1,000 IU); No. 4 (1,000 IU); No. 5 (500 IU), and No. 6 (500 IU).

The reagents and research materials were:

Components of biorelevant media: dry bovine bile (Cattle bile) obtained from the State Research Center for Applied Microbiology and Biotechnology (SSC PMB) (Obolensk, Russia); Soy lecithin obtained from Evalar Closed Joint Stock Company (CJSC) (Biysk Russia); Sodium hydroxide (chemically pure), Potassium phosphate monosubstituted (chemically pure) and Hydrochloric acid (chemically pure) from LenReaktiv (Saint Petersburg, Russia); Rennet “Beef Pepsin” from Modern technologies LLC (Moscow, Russia); Lipase and Bacterial high-temperature alpha-amylase from Biopreparat LLC (Moscow, Russia); Cellulase (Cellulase) liquid, and Mushroom alkaline protease from Concern Microbioprom LLC (Moscow, Russia); Ultrapure deionized water was obtained on the installation “SGWASSER Ultra Clear TWF/EL-ION UV plus Trademark (TM) (Siemens, Germany).

The in vitro setup used the constituent electrolyte stock solutions shown in Tables 1 and 2 above; enzymes, CaCl2, and water were added to the simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). The pH concentration of the simulated gastrointestinal fluid was attained by adding 4 parts of the electrolyte stock solution plus 1 part of the water in the 1.25-concentrated electrolyte stock solutions as described by [19].

Vitamin A released from capsules was evaluated by the spectrophotometric method by measuring the optical density of the biorelevant medium at a wavelength of 850 nm in an RTS-1 bioreactor (Biosan, Latvia).

The typical temperature of a mature cow is about 38.5°C and 38.7 – 40 °C for pigs [33]. The time taken into consideration is to give an overview of how and when the microencapsulated vitamin A is starting to be available to the simulated gastrointestinal fluid (GIF); and when it attained its optimum and declines. The speed is to simulate the normal movement of the organisms and this too might be an influencing factor on the bioavailability. Microencapsulated vitamin EO-3 (manufactured in China) was used as a control sample.

3. Results

After the experiments were done, the bioavailability as well the above parameters, were observed and found to correlate with the bioavailability of vitamin A. Under the condition of microencapsulation, the vitamins have extra security and stability

without being affected by heat and other digestive enzymes. Thus, it facilitates the bio-accessibility and finally bioavailability of the vitamins.

Modeling of the process of dissolution kinetics of microencapsulated samples of vitamin A was carried out the conditions of the gastrointestinal tract of farm animals from the literature.

Figures 1, 2 show the total and relative bioavailability of experimental samples and industrial reference sample EO-3 (PRC).

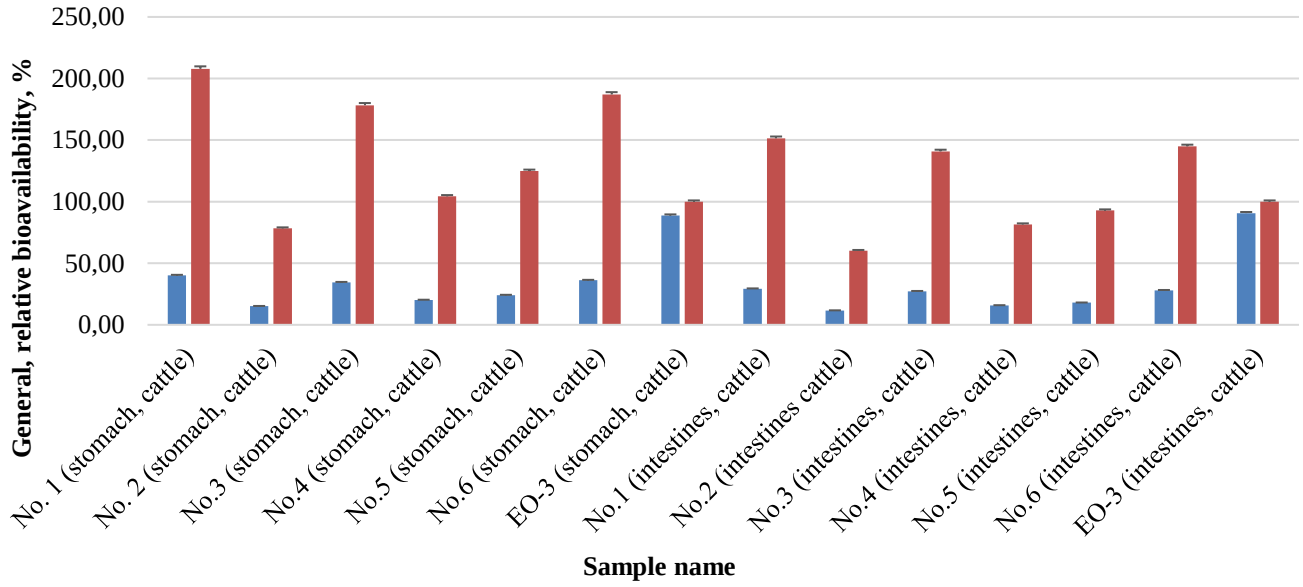

Fig. 1 – Total and relative bioavailability of vitamin A in biorelevant environments of cattle

It follows from Figure 1 that microcapsules, which include gelatin (about 38%) and starch (about 18%), dissolve better than other experimental samples in the biorelevant environment of the stomach and intestines of cattle - sample No. 1. In the conditions of the biorelevant environment of cattle, samples No. 3 and No. 5 dissolved better than samples No. 2 and No. 4. Under the conditions of the biorelevant environment of the stomach of cattle, the relative bioavailability is higher in the experimental sample No. 1, in the conditions of the intestine of the cattle - in the samples No. 1, No. 3, No. 6 compared with the industrial sample EO-3 (PRC)

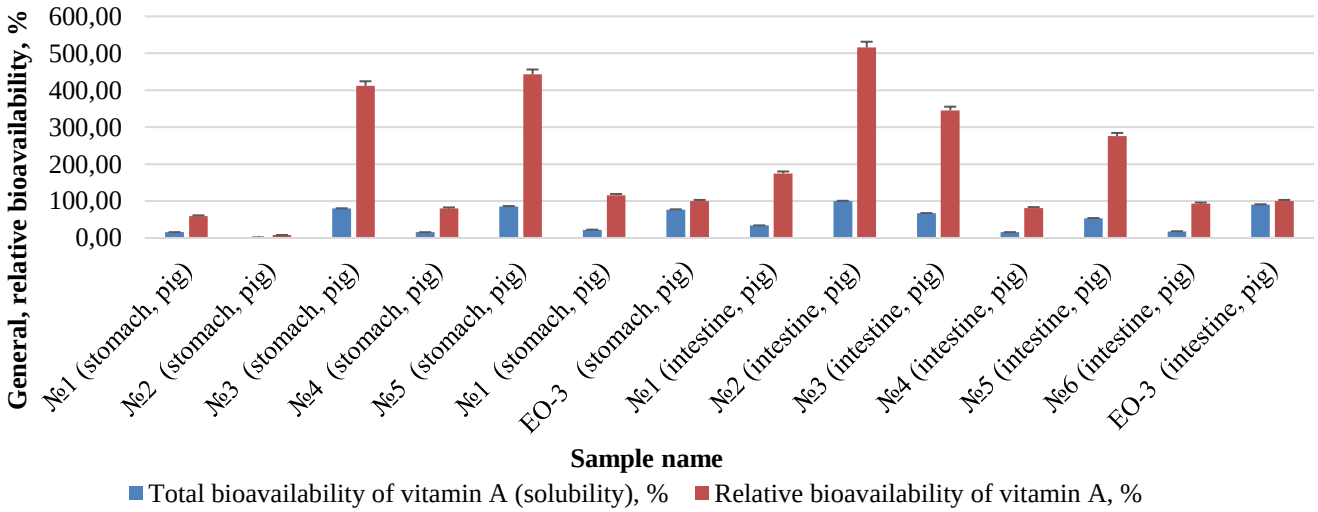

Fig. 2 – Total and relative bioavailability of vitamin A in biorelevant environments of the pig

It follows from Figure 2 that microcapsules containing gelatin (about 38%) and starch (about 18%) dissolve better than experimental samples No. 3 and No. 5, No. 2 (intestine) in biorelevant environments of the stomach and intestines of pigs. In the conditions of biorelevant environments of pigs, samples #3 and #5 dissolved better than samples #2 and #4. The relative bioavailability of vitamin A in the conditions of the stomach of pigs is higher in experimental samples No. 3, No. 5, No. 6, in the conditions of the intestines of pigs - in samples No. 1, No. 2, No. 3, No. 5 compared with industrial sample EO-3 (China).

In the conditions of biorelevant environments of the stomach of pigs and farm birds, samples of feed microencapsulated vitamin A dissolve better than in the conditions of biorelevant environments of the intestines of pigs and farm birds, except for sample No.

Samples No. 3 and No. 5 had the best solubility in biorelevant environments of the stomach and intestines of pigs and poultry. Samples No. 2 and No. 4 had the lowest solubility in the conditions of biorelevant media of the stomach of pigs and farm birds; sucrose was not included in the composition of the shell.

This study made it possible to establish the rate and degree of release of vitamin A into the dissolution medium from the encapsulated form under normalized conditions, which to some extent imitates the behavior of samples in the conditions of the gastrointestinal tract. It also allowed us to indirectly assess the bioavailability of vitamin samples and compare them with each other. Initially, model compositions of biorelevant media in the gastrointestinal tract of farm animals - pigs and cattle were developed. The use of these biorelevant media allowed for the screening stage of studying the in vitro bioavailability of vitamins and feed additives. This will reduce costs, time and speed up the stages of development and industrial production of the most effective samples of feed fortified with vitamins for the agro-industrial sector. The release kinetics of samples of microencapsulated vitamin A make it possible to conclude that it is expedient to include microencapsulated vitamin A samples 1, 3 and 4 for pigs; and samples 1 and 2 for cattle in the composition of feed and premixes for farm animals.

From the result of this study, it can be concluded that microencapsulation technology can increase the bio-accessibility and bioavailability of vitamin A. The findings of this work unequivocally demonstrated that the level of vitamin A intake given by the standard would be insufficient if administered conventionally. Thus, it is likely not to provide cattle and pigs with the nutrients needed for rapid growth. The growth of these animals may be enhanced by incorporating modern technologies like microencapsulation for better delivery of vitamins and other macromolecules. This indicates the need for further research on how different technologies could be integrated to deliver vitamins is required.

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Финансирование

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Conflict of Interest

None declared.

Конфликт интересов

Не указан.

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