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DEVELOPMENT OF A PREMIX BASED ON MICELLAR CASEIN FOR FORTIFICATION OF MEAT SYSTEMS WITH VITAMIN A

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Abstract
A novel approach to the protection of unstable fat-soluble vitamins, using retinol as an example, is presented in this work. This method is based on introducing vitamin A molecules into casein micelles. Protective properties of micellar casein towards different forms of retinol (native vitamin and palmitic acid ester) in vitro and in emulsion-type meat products are investigated. A technology of the introduction using micellar casein concentrate (MCC) has been developed. Conditions similar to those in which vitamin molecules can be in meat emulsions during heat treatment are simulated in vitro. The optimal time of "encapsulation" (2 hours) and the need for additional surfactant (tween-80) are identified. The use of the casein micelles protection made it possible to increase the number of retinol molecules that did not undergo decomposition under model conditions (in vitro) from ~30% to ~80%. Using the vitamin premix the degree of degradation of vitamin molecules does not exceed 4% after heat treatment. Data received allowed us to determine the efficiency of the protection properties of casein micelles for unstable vitamin A molecules.

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Introduction

The problem of vitamin deficiency among the inhabitants of Russia is quite common and relevant to this day. A more acute deficiency is observed in people living in the north of the country. For example, in one of the settlements of the northern region in the spring, approximately 20% of the surveyed had a lack of retinol. An effective method of solving this issue is the enrichment of food with vitamins [1]. However, some of them, due to their instability, need additional protection. Recent studies suggest that milk proteins can provide such preservation. Nowadays milk proteins found a wide application in the production of various products due to their availability, low cost, physicochemical properties, and high amino acid content [2–5]. Being a milk protein casein exists as micelles and consists of α_{S1}- and α_{S2}, β-, κ-caseins and calcium phosphate [6]. The properties of micellar casein began to be widely studied after the development of microfiltration methods for its isolation from milk. Simultaneously, an active search for ways to use casein in various areas of food production started. A range of articles can be found about the application of micellar casein concentrates in the production of sports nutrition, cheeses, yogurts, and high protein/low carbohydrate drinks [3,7]. Another direction of study is the use of casein and its components for the delivery of unstable products and medicines in human organisms [8–12]. This topic has been actively studied by many scientists in recent years and is of great interest.

The introduction of various organic compounds in the casein's structure allows us to control their concentration and ensure their transport in an unchanged form. Due to its micellar structure, casein exhibits a high ability to bind ions and small molecules, stabilizing them [13]. This property of micelles is especially seen in binding with water-insoluble compounds. It happens due to hydrophobic interactions, van der Waals forces, and hydrogen bonds [14]. Among the unstable hydrophobic molecules, fat-soluble vitamins A and D have a special place. These compounds are sensitive to light, UV radiation, and elevated temperatures. Casein can absorb radiation at 200–300 nm and protect these compounds [15]. Using vitamin D as an example, the ability of casein to protect molecules from exposure to high temperatures and destructive changes that occur during storage at low temperatures was shown. It favorably distinguishes the "casein" method of storing vitamins from, for example, storing a vitamin dissolved in vegetable oil or an aqueous emulsion stabilized by surfactants (e. g., tween-80) [14]. Generally, the recombination of casein is used to introduce vitamins in casein micelle. Initially, casein is taken not in its native micellar form, but as its processing product — sodium caseinate. After the introduction of vitamin to caseinate, solutions of K_2HPO_4, calcium citrate, and $CaCl_2$ are added to the reaction mixture. Under the action of these compounds, the micellar structure of casein is restored with vitamins presumably encapsulated in micelles. However, these restored micelles still differ from native ones [16,17].

In papers [15–19] researchers concluded that recombined and native micelles can protect some molecules (including retinol) by encapsulation. Heating β-carotene (vitamin A precursor) at 80 °C for 8 hours the concentration of carotene decreased by 83.5%, while using MCC the level of degradation was only 31%. Encapsulated vitamins also showed higher stability under high-pressure treatment. It is worth noting that the maximum decrease in the amount of β-carotene was observed in the first hours of heating; subsequently, its concentration ceased to change [18]. In the study of Mohan et al. [20] it was reliably shown that in milk enriched with vitamin A, most retinol molecules are contained in the casein fraction. To prove this, researchers separated milk proteins into different protein fractions using size-exclusion chromatography, and extracted vitamin A for quantitative determination by high-performance liquid chromatography (HPLC). After extraction, the composition of each protein fraction was determined by SDS-PAGE.

For a long time, micellar casein itself has been used during the production of various types of food products, including its application as vitamin transport [13,16,21–23]. However, in meat production, other additives based on milk proteins are mainly used, such as whey proteins, caseinates, and milk powder (the main protein of which is casein) [24–29]. Using the example of sausage production technology [30], it was shown that MCC has a positive effect on the functional and technological properties of minced meat systems and cooked products. Thus, analytical studies give reason to believe that the application of micellar casein concentrate in meat product formulations is an insufficiently studied topic, especially when used as a vehicle that transports and preserves vitamins in sausage products.

Based on these data, we hypothesized that the encapsulating ability of native casein can be effectively used to fortify boiled sausages with vitamin A. During production, these sausages are subjected to prolonged exposure to relatively high temperatures. Such heating, in the absence of protection, can lead to the degradation of most retinol. Thus, this study aimed to investigate the MCC protective properties towards unstable molecules of retinol and its palmitate *in vitro* and in model minced meat systems such as boiled sausages.

To implement this idea, it was necessary to develop a method for encapsulating vitamin A molecules in casein micelles which would include such requirements as easy scalability and implementation into production. Because native micellar casein was the object of our studies, relatively simple approaches using caseinate recombination in micelles were not of interest. Also approaches based on encapsulations in native micelles accompanied by rather specific conditions, such as excessively high pressure did not suit us. The second goal was to estimate the vitamin's degree of degradation under model conditions in an unprotected form, as well as with protection in the form of casein micelles. The last task was to study the preservation rate of vitamin A in the meat system. This work is devoted to the solution of these issues.

Objects and methods

The objects of this study were retinyl palmitate (pharmaceutical drug, 50 mL, JSC "Retinoids"), retinol (derived from retinyl palmitate), concentrate of micellar casein Lactoprima Pro MicCC85 (BaltMilk, Lithuania), retinol mixed with MCC, retinol mixed with MCC and tween-80 (IGL, India), retinyl palmitate, retinyl palmitate mixed with MCC and tween-80, vitamin premix based on micellar casein concentrate, minced meat system.

The studied minced meat system was manufactured according to the formulation presented in [30]. This system was an emulsion for the production of meat products such as boiled sausages. The heat treatment included three stages (drying, frying, and cooking) until the center of a sample reached an internal temperature of 72 °C, while the temperature in the thermal chamber reached 80 °C [30].

The solvents were purified and dried by standard methods.

^{1}H NMR spectra were recorded on a DPX-250 (Bruker, Germany) (250 MHz, Scientific and Educational Laboratory of Resonance Spectroscopy, Department of Natural and High Molecular Compounds Chemistry of Southern Federal University) spectrometer using $CDCl_3$ as a solvent with the solvent residual peaks as the internal standard.

High-performance liquid chromatography was carried out using the Gilson analytical HPLC system (USA).

Products were purified by chromatography (chromatography columns were used) on Al_2O_3 (Brockmann activity III). The progress of reactions and the purity of products were monitored by thin-layer chromatography (TLC) on Al_2O_3 plates and developed with iodine vapor or UV light (UV-viewing cabinet (Spectroline, USA)).

Stirring was carried out using an overhead stirrer SH-II-6C (Huanghua Faithful Instrument Co., China).

Distillation was conducted on a Hei-VAP Core rotary evaporator (Heidolph, Germany).

For ultrasonic exposure, an ultrasonic bath Sonorex Super RK 31 (Bandelin, Germany) was used.

In the experimental part, optimal procedures are indicated (taking into account the yield of retinol).

To select model conditions various technological processes and heat treatment parameters used in the production of boiled sausages were investigated and analyzed. Studies showed that the temperature of the chamber does not usually exceed 80 °C and the time of heat treatment is less than 3 hours. Thus, we chose the harshest conditions as a model one in which molecules of the vitamin can be in meat emulsions. These conditions included maintaining a constant temperature in the range of 78–80 °C and varying the time the sample was exposed to temperature, ranging from 0 to 3 hours. It is worth noting that the processes took place in the absence of light sources.

Isolation of native retinol from a drug

A mixture of the drug (50 mL), EtOH (200 mL), and 50% aqueous KOH (80 mL) was saponified for 30 min at 80 °C with vigorous stirring. Then H_2O (300 mL) was added, and the reaction mixture was allowed to cool to rt. The crude product was extracted with *n*-hexane (4×50 mL). Most of the solvent was distilled off, and the product was purified by column chromatography (Al_2O_3, *n*-hexane). A yellow-colored fraction luminous in UV was isolated. After evaporation of the solvent yellow-orange oil (solutions have a weak green fluorescence in UV) with a total weight of 259 mg was afforded.

The resulting mixture was dissolved in 8 mL of EtOH, 0.5 mL was taken and evaporated. 3 mg of anthracene was added to the sample containing 0.5 mL of ethanol solution and the mass of retinol in the sample was calculated using the ^{1}H NMR method. There were no antioxidants in the spectrum, the mass of vitamin A was 9.1 mg, therefore, the content of retinol in the resulting mixture is 56% (145 mg).

The degree of homogenization of an MCC and retinol mixture

The study was carried out at stirring for 1, 2, and 3 h. Ultrasound (ultrasonic exposure was carried out in an ultrasonic bath) and tween-80 were used as additional factors leading to homogenization. A control sample was stirred without the addition of tween-80 or ultrasonic exposure: 1 g of casein was hydrated in a ratio of 1:20. Then 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1, 2, and 3 h in darkness. A sample with tween-80 was prepared by adding surfactant (0.5 mL) and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) to hydrated casein. With the additional action of ultrasound, the sample was placed in an ultrasonic bath for 15 minutes before stirring. Then it was subjected to ultrasound every 30 min for 5 min.

The study of the preservation of the vitamin depending on the stirring time

1 g of casein was hydrated in a ratio of 1:20. Then tween-80 (0.5 mL) was poured in, the mixture was stirred and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1, 2, and 3 h in darkness and heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating dibutylhydroxytoluene (BHT) (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

4 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield after 1 h was 5.2 mg, 2 and 3 h — 6.9 mg.

Retinol stability study

A mixture of H_2O (10 mL) and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow-orange oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield was 2.6 mg, therefore, the content of retinol decreased by 71%.

Retinol stability study using a surfactant

A mixture of H_2O (10 mL), tween-80 (0.5 mL), and 0.5 mL retinol solution in EtOH (9.1 mg of retinol) was stirred at rt for 30 min in darkness. Then the reaction mixture was heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield was 3.3 mg, therefore, the content of retinol decreased by 64%.

Retinol stability study using MCC

1 g of casein was hydrated in a ratio of 1:20. Then tween-80 (0.5 mL) was poured in, the mixture was stirred and 0.5 mL of retinol's solution in EtOH (9.1 mg of retinol) was added. The resulting mixture was stirred at rt for 1 or 3 h in darkness and heated at 80 °C in silicone oil (Merck, Germany) for oil baths for 3 h with constant stirring. After heating BHT (30 mg), 50% aqueous KOH (15 mL), and EtOH (30 mL) were added. Saponification was carried out at 80 °C for 30 min. Then H_2O (10 mL) was poured, and the reaction mixture was allowed to cool to rt. The product was extracted with *n*-hexane (4×10 mL). After the evaporation of the solvent yellow oil was afforded.

3 mg of anthracene was added to the sample. The amount of retinol was calculated using ^{1}H NMR spectroscopy. The yield after 1 h was 8.3 mg (the content of retinol decreased by 9%), 3 h — 7.0 mg, hence the content of retinol decreased by 23%.

Preparation of a premix based on MCC for its further incorporation into sausages

Tween-80 (0.15%, this volume is within the range of recommended safe doses for its use, and also provides a good degree of homogenization [31,32]) was added to hydrated

(1:4) MCC (8 kg of MCC, 2 kg of water). Then retinyl palmitate (1.8 mL) was gradually poured into the mixture. The resulting mixture was stirred for 2 h. After stirring a premix was cooled to 4 °C and added to the cutter according to formulation [30].

Determination of vitamin A by HPLC

Sample preparation for HPLC was carried out according to GOST 32307–2013[1]. After the isolation of vitamin A, it was dissolved in acetonitrile (0.05 mL). Then 0.02 mL of solution was taken and studied by HPLC. The amount of a vitamin was calculated using the formula presented in GOST 32307–2013. For example, the mass concentration of the vitamin in the calibration solution is C_{st} mg/cm^3, peak area of an individual vitamin in a sample S_x, peak area of an individual vitamin in a calibration solution — S_{st}, the volume of solvent taken to dissolve the dry residue is V_s mL, mass of the test sample — m g.

$$X = \frac{C_{st} \times S_{st} \times 0.5 \times 1000}{S_x \times m} \text{ (mg/kg)} \quad (1)$$

Each experiment was repeated 15 times. The main variable is measured in metric scale, therefore, to select statistical processing, a comprehensive check of the normality of data distribution was carried out (graphical analysis of histograms and calculation of the Shapiro-Wilk test). Since the variables almost always showed a distribution other than normal, non-parametric Wilcoxon T test (for dependent samples, comparisons between phases of the same experiment), Kruskal-Wallis H test and Mann-Whitney U test (for independent samples, comparison of the final mass of retinol in stability studies under different conditions) were applied. In order to study the preservation of the vitamin depending on the stirring time, we also used the calculation of the Spearman's rank correlation coefficient.

Results and discussion

The most common method for the quantitative determination of fat-soluble vitamins is HPLC. However, this method, like any other, has its drawbacks. In this work, a fast and effective method for the quantitative determination of fat-soluble vitamins using nuclear magnetic resonance spectroscopy was successfully used. It is consistent with the results obtained previously [33].

Firstly, the optimal conditions were selected to introduce vitamin into casein. Vegetable oil and ethanol were chosen as solvents. The "encapsulation" process was accompanied by intensive stirring and took place at room temperature. Stirring time varied from 1 to 3 hours. Ultrasound (US), surfactants (tween-80), and their combined effect were applied as factors contributing to the transition of vitamin molecules into micelles (Table 1). The addition of surfactants is common in the enrichment of milk and milk products with fat-soluble vitamins [34]. The idea of ultrasound application is based on the obvious need to disperse fat globules of vitamin mixtures. For a preliminary visual determination of the interaction effectiveness, the degree of reaction mixture homogenization was assessed. This method of vitamin protection is easier than the ones described earlier based on the recombination of protein [11,15,17,18]. Besides, this approach allows one to preserve protein micelles in their native state.

Table 1. Homogenization of a mixture of micellar casein concentrate and retinol with stirring

	$T_{stir.} = 1$ h				$T_{stir.} = 2$ h				$T_{stir.} = 3$ h			
	—	*US*	*Surf.*	*US + Surf.*	—	*US*	*Surf.*	*US + Surf.*	—	*US*	*Surf.*	*US + Surf.*
Retinol in EtOH	—	—	*	**	**	**	***	***	**	**	***	***
Retinol in oil	—	—	*	**	*	*	***	***	*	*	***	***

The degree of homogenization: —, no homogenization; *, poor; **, good; *, excellent. $T_{stir.}$ — time of stirring**

Since micellar casein is an emulsifier, although not as good as sodium caseinate, it has been suggested that a vitamin solution with hydrated MCC be kept under vigorous stirring. However, visible results were achieved only after 2–3 hours of stirring. As can be seen from the table, homogenization and, as a result, encapsulation proceed slowly without the use of auxiliary factors. Due to the hydrophobic nature of the vitamin, oil makes it more difficult to introduce it into casein. Ethanol mixes with water and creates conditions for effective penetration of retinol into the interior and pores of casein micelle. Short-term exposure to ultrasound does not significantly affect emulsification, while long-term exposure is undesirable due to the instability of the vitamin. At the same time, the use of tween-80 significantly accelerates the mixing and encapsulation processes. After an hour of stirring polysorbate introduction leads to partial homogenization with a gradual separation of the solution without stirring. After two hours of stirring the solution looks mostly homogenized. Additional stirring for one more hour does not lead to any visible changes. There were not any noticeable differences when the combination of ultrasound and surfactant was used. Thus, based on preliminary tests the MCC/tween-80 system was chosen to produce vitamin premix (the addition of surfactant leads to more effective homogenization).

As noted earlier, quantification was carried out using NMR spectroscopy. A given amount of anthracene was introduced into the samples as a standard. In this case, an important factor in the selection of a standard substance is the absence of signals in the spectrum that would intersect with the characteristic peaks of the substance studied. Anthracene signals are at 7.46 (dd, J = 6.5, 3.3 Hz, 4H), 8.00 (dd, J = 6.5, 3.3 Hz, 4H), 8.43 (s, 2H) ppm. Retinol peaks are at 1.03 (s, 6H), 1.46 (m, 2H), 1.61 (m, 2H),

[1] GOST 32307–2013 "Meat and meat products. Determination of fat-soluble vitamins by high performance liquid chromatography". Retrieved from https://docs.cntd.ru/document/1200107182. Accessed August 05, 2023

1.71 (s, 3H), 1.89 (s, 3H), 2.00 (s, 3H), 2.02 (t, J = 6.1 Hz, 2H), 4.32 (d, J = 7.0 Hz, 2H), 5.69 (t, J = 7.0 Hz, 1H), 6.09 (d, J = 11.2 Hz, 1H), 6.10 (d, J = 14.1 Hz, 1H), 6.14 (d, J = 13.9 Hz, 1H), 6.28 (d, J = 15.1 Hz, 1H), 6.64 (dd, J = 15.1, 11.3 Hz, 1H) ppm [33]. In the ^{1}H NMR spectrum of vitamin A, upfield signals cannot be used for quantitative assessment, since other aliphatic signals are present in the region of 1–3 ppm as well as water. In the downfield region, there are signals from the methylene unit associated with the OH group and C_{sp^2}–H protons, which do not intersect with other signals and can be used to estimate the content of retinol in the sample (Figure 1).

To estimate the influence of stirring on the "encapsulation" degree of retinol molecules into protein micelles, we carried out experiments with time of preliminary stirring varied from 1 to 3 hours. After stirring for a given time samples were subjected to three hours of heating under the same conditions. Figure 2 shows selected signals in ^{1}H NMR spectra obtained in our experiments. We calculated the amount of retinol using an anthracene singlet at 8.43 ppm (marked in orange) and a triplet from one of the CH retinol units (5.69 ppm), marked in green on the structure.

Initially, we supposed that there is a dependence on retinol concentration from stirring time. To test this

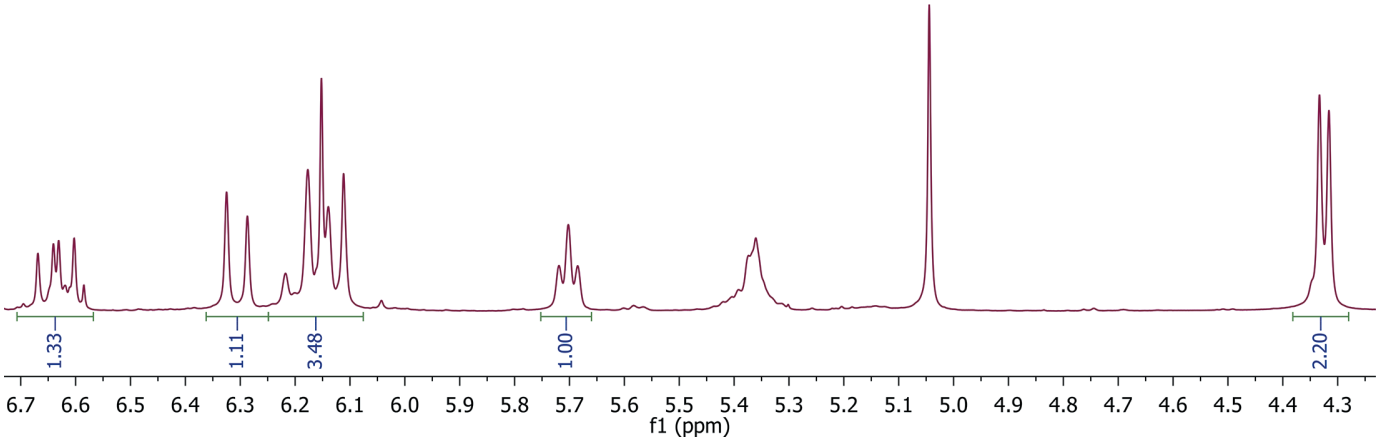

Figure 1. Fragment of the ^{1}H NMR spectrum of retinol isolated chromatographically from a drug

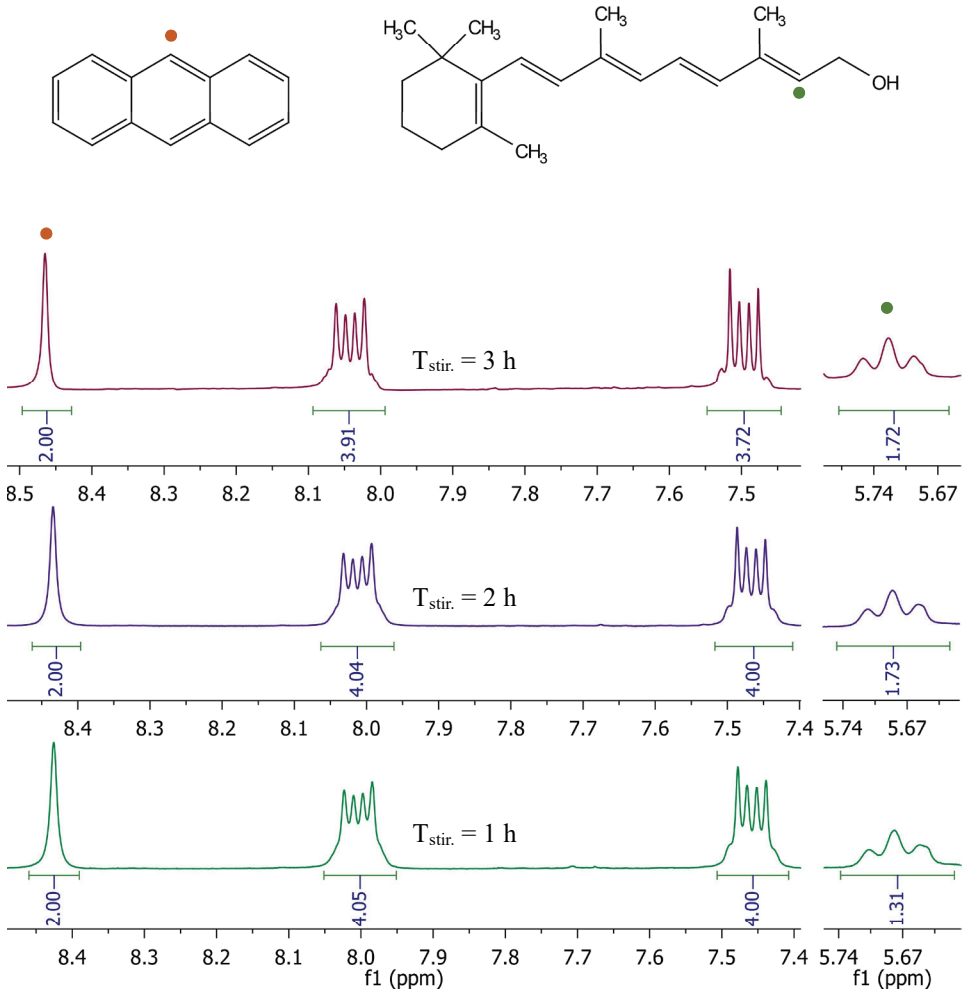

Figure 2. Fragments of ^{1}H NMR spectra of anthracene and retinol mixture with stirring for 1, 2, and 3 h

hypothesis, we calculated Spearman's correlations between two variables. Indeed, the resulting correlation coefficient confirms that the stirring time and the mass of the vitamin in the mixture are directly proportional to each other (positive correlation $r = 0.799421$, $p \leq 0.005$). To determine when the most significant increase in retinol concentration occurs, a comparative analysis was carried out.

Significant differences between the yield of retinol depending on stirring time were tested using the Wilcoxon test. Table 2 shows the results of calculating the criterion for pairwise comparisons of experimental phases, indicating the empirical value of the T-criterion and the level achieved.

Table 2. Results of comparison of the retinol mass in the samples depending on the stirring time

		Comparison between different points		
	M ± sd, mg	Before stirring	1 h	2 h
Before stirring	9.1			
1 h	5.18 ± 0.161	T = 0, p = 0.00065		
2 h	6.86 ± 0.073	T = 0, p = 0.00065	T = 0, p = 0.00065	
3 h	6.90 ± 0.065	T = 0, p = 0.00065	T = 0, p = 0.00065	T = 0, p = 0.06789

M — mean; sd — standard deviation; T — empirical value of the Wilcoxon test; p — significance level

In this work, significant differences are those in which the significance level is $p \leq 0.05$. Figure 3 shows a graph of the average retinol mass values depending on the stirring time. To visualize the spread of values curves with minimums and maximums are presented. The level of significance of differences ($p \leq 0.005$) between phases was also noted.

The result of this comparison allows us to conclude that there is a significant ($p = 0.0007$) difference in the mass of retinol before mixing and after 1 hour (the concentration decreases by 42.86%). Comparing the retinol concentration in the mixture after 1 and 2 hours the level of vitamin A increased almost by 20% which is also a statistically significant difference ($p = 0.0007$). There is no statistical difference between retinol mass after 2 and 3 h of stirring ($p = 0.067$). Thus, the optimal stirring time is 2 hours as the additional hour gives no significant difference for the increase in the vitamin concentration.

To estimate the efficiency of vitamin protection by casein it was necessary to understand at what rate retinol itself would undergo thermal degradation. Experiments were carried out using retinol solutions in sunflower oil or ethanol. The saponification stage was carried out in both cases since this stage is mandatory for the quantitative determination of vitamins in sausages. Saponification results in the removal of casein since its peptide bonds are subjected to alkaline hydrolysis. As described earlier, the alcohol solution mixes more easily with casein, so it was chosen to be used in further experiments. In Figure 4A fragments of ^{1}H NMR spectra are shown. They allow one to calculate the amount of vitamin A in samples without casein's protection after heating at 80 °C for 3 hours.

The first spectrum shows the initial concentration of the vitamin (Figure 4, A1). Since retinol was isolated from a drug and subjected to purification by column chromatography, it was decided to remove only the most mobile fraction which contained antioxidants to optimize the time. Thus, the isolated vitamin contained some impurities that did not affect the accuracy of the experiment, but the initial amount of the vitamin needed to be determined spectroscopically. NMR reliability in the determination of fat-soluble vitamins was verified by repeated quantitative evaluation of the same sample by HPLC. The discrepancy between the two methods is no more than 5%. The same results were obtained in work [33] in which pharmaceutical drugs were studied by the NMR method. Re-determination of the concentration is possible because ^{1}H NMR spectroscopy is a non-destructive method of analysis. Using the Wilcoxon test, we confirmed that the decrease in retinol concentration as a result of three-hour heating is significant, most of the retinol is decomposed, and its residual content does not exceed 30% (Figure 4, A2;

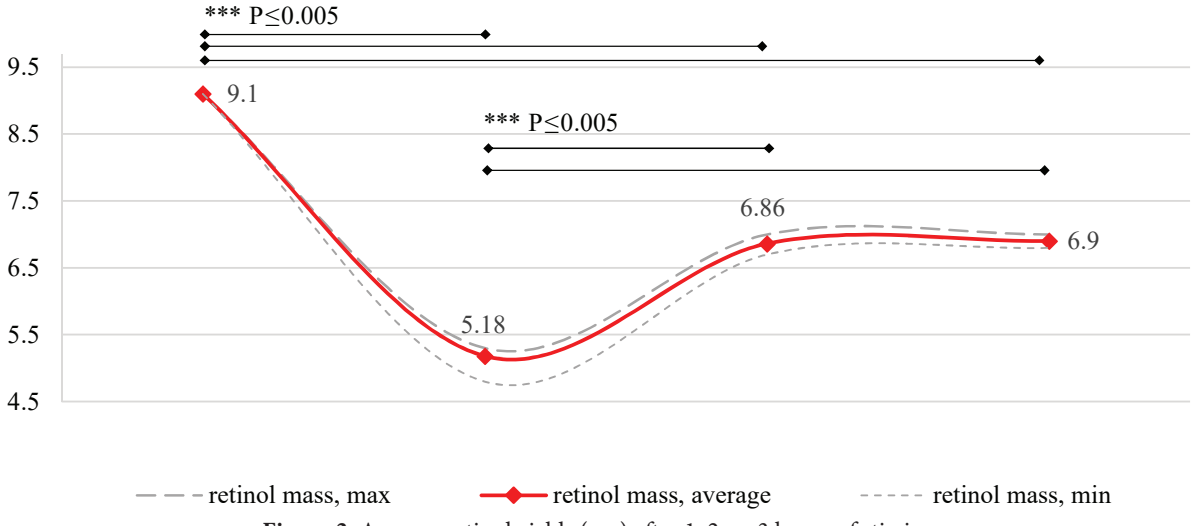

Figure 3. Average retinol yields (mg) after 1, 2, or 3 hours of stirring

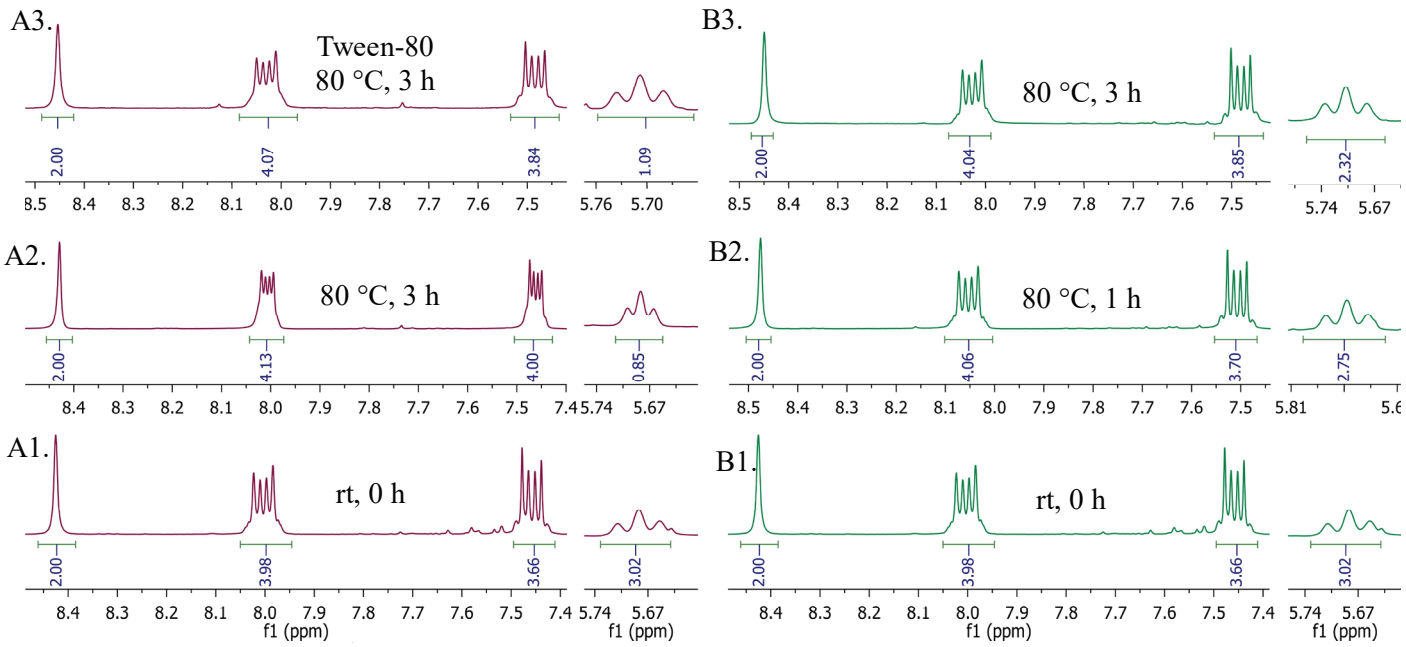

Figure 4. Fragments of ^{1}H NMR spectra of anthracene and retinol mixture without casein (A) and with the addition of MCC (B) without heating and with heat treatment for 1 and 3 h

Figure 5). The reduction of vitamin concentration in such conditions is in agreement with statistical calculations at the significance level $p \leq 0.005$.

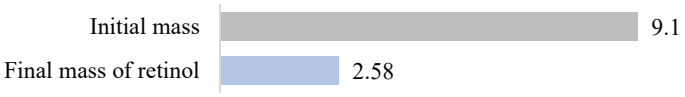

Figure 5. The average yield of retinol (mg) before and after the test was carried out (T = 0, p = 0.00065)

As expected, retinol in its pure form without any protection shows the worst results even in contrast to a vitamin sample emulsified with polysorbate (Figure 4, A3). Most likely, the micellar shell created by surfactant molecules can protect vitamin molecules to some extent. In general, it is seen that retinol at this temperature is largely subjected to destructive changes, the residual content of the vitamin after heating for 3 hours, even with the use of tween-80, is no more than 40% (Figure 4, A3). The Wilcoxon T-test allowed us to validate that the decrease in retinol amount in this experiment is significant. The difference between the mass of the vitamin in the mixture before and after heat treatment shows a statistically significant reduction ($p \leq 0.005$) (Figure 6).

These results are consistent with a study on the preservation of β-carotene by recombined casein, in which after 8 hours of heating at 80 °C the amount of vitamin A precursor was 16.5% [18].

The retinol stability using MCC was studied at 1 and 3 hours. The results of descriptive statistics are shown in Table 3.

Figure 4B shows the spectra of three retinol samples prepared using MCC and polysorbate. The first one is the sample that did not undergo any heating used for the determination of initial concentration (Figure 4, B1). The second sample was subjected to heating for 1 hour (Figure 4, B2) and the third one was heated for 3 hours (Figure 4, B3). According to the spectra, after an hour of heating, only 9% of the total number of vitamin molecules underwent destruction. After three hours, 77% of retinol was retained in the sample. All the described dependencies are presented for greater clarity in the form of a graph in Figure 7. Thus, the use of micellar protection provided more than a twofold increase in the residual content of retinol after three hours of heating at 80 °C. All experiments were repeated several times and showed high reproducibility. The results are consistent with similar data obtained by Sáiz-Abajo et al. [18].

Figure 6. The average yield of retinol (mg) before and after heat treatment (T = 0, p = 0.00065)

Table 3. Main descriptive statistics for experiments with a vitamin premix based on MCC

	Average amount, mg	Standard deviation
Initial mass	**9.1**	**0**
Mass after 1 h, t = 80 °C; Vitamin A + surf. + MCC	**8.3**	**0.151186**
Mass after 3 h, t = 80 °C; Vitamin A + surf. + MCC	**7.0**	**0.109978**

A comparison of the final retinol concentration in formulations obtained by different technologies was conducted in two stages. Firstly, in the Kruskal-Wallis test the variable H was calculated (a nonparametric analog of analysis of variance, since the data is not normally distributed) (Table 4).

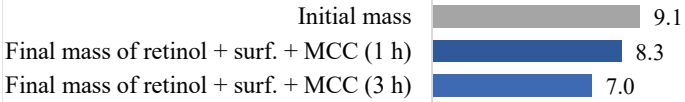

Figure 7. The average yield of retinol (mg) before and after 1 and 3 h of heating (T = 0, p = 0.00065)

Table 4. Primary results of comparison of the retinol mass in samples obtained by different techniques

	Kruskal-Wallis ANOVA by Ranks Kruskal-Wallis test: H = 55.75857 p ≤ 0.005		
	Number of measurements (N)	Sum of ranks	Average of ranks
Mass after 3 h, t = 80 °C; pure vitamin A	15	120	8
Mass after 3 h, t = 80 °C; Vitamin A + surf.	15	345	23
Mass after 1 h, t = 80 °C; Vitamin A + surf. + MCC	15	795	53
Mass after 3 h, t = 80 °C; Vitamin A + surf. + MCC	15	570	38

Pairwise comparisons were performed using the Mann-Whitney U test for independent samples. Since all the shifts were typical (i. e., between any two groups, changes in the concentration of the substance were observed only in one direction, and the sets did not intersect), the result of calculating the criterion for all pairs was similar and amounted U = 0, p = 0.000003393, which corresponds to the required significance level p ≤ 0.005. A visual comparison of the stability of vitamin A at heating at 80 °C for 3 hours in the presence of additional protection (tween-80 or its combination with MCC) is shown in Figure 8.

Even though we did not set the task of determining the localization of retinol molecules and whether they undergo encapsulation, we can indirectly assume that this probably occurs. This is supported by the rather effective protection of vitamin A provided by casein, as well as the fact that a shorter stirring (encapsulation) time leads to a decrease in the degree of protection.

The final stage of the research was to estimate the preservation of vitamin A in model minced meat systems such as boiled sausages. These systems were based on poultry meat partially replaced with micellar casein concentrate. The technology of manufacturing minced meat systems with minor modifications (the stage of obtaining hydrated casein) is presented in patent № 2801108, and the original formulation is given in Table 5.

Table 5. Formulation of model minced meat system

Component	Mass (kg/100 kg of minced meat)
Poultry meat (chicken fillet)	80
Pork rind	20
Spice mixture "Munich sausages"	0.7
Salt	2.5

A developed vitamin premix of retinyl palmitate based on MCC was added to meat systems. Casein concentrate was hydrated in a 1:4 ratio to achieve a protein percentage close to that in poultry meat. Then it was added to the recipe replacing 10% of raw meat. Firstly, we calculated the amount of vitamin added to the premix. To do this, data on the adults' physiological requirement for vitamin A (900 μg RAE/d) was taken as a basis[2]. According to the order of the Ministry of Health of the Russian Federation dated August 19, 2016, № 614[3], the standard consumption of poultry meat per year is 31 kg or about 85 g per day. The formulation of the studied systems with the partial meat replacement with MCC provides for the use of 72 kg of poultry meat per 100 kg of raw materials. It means 120 g of the finished product will contain the recommended daily intake. To eliminate the possibility of vitamin A overdose, the required dose of retinol was calculated as 10% (90 μg RAE) of the daily intake of vitamin per 100 g of the product. Thus, 90 mg of the vitamin would be used for 100 kg of the product. Taking into account the fact that the retinyl palmitate drug used contains about 55 mg of retinyl palmitate in 1 mL, 1.8 mL of the drug should be taken per 100 kg of raw materials. At the same time, the content

[2] MR2.3.1.2432–08 "Balanced diet. Norms of physiological needs for energy and nutrients for various groups of the population of the Russian Federation". Retrieved from https://fcgie.ru/download/elektronnaya_baza_metod_dokum/mr_2432–08.pdf. Accessed August 09, 2023

[3] Order of the Ministry of Health of the Russian Federation (August 19, 2016 No. 614) "On approval of recommendations on rational standards of food consumption that meet modern healthy nutrition requirements". Retrieved from https://nadn.ru/upload/iblock/58d/58df042069fa850e7d425d9f2b06244f.pdf. Accessed August 09, 2023

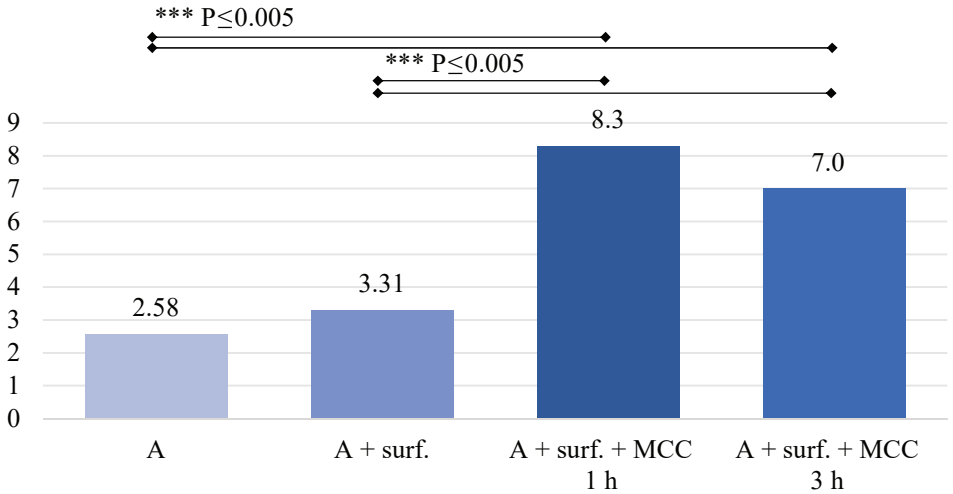

Figure 8. The average yield of retinol (mg) in samples obtained with and without using tween-80 or MCC and tween-80 (U = 0, p ≤ 0.005)

of vitamin A in the raw materials used for the production of sausages can be not taken into consideration due to its extremely low content in cooked products, which was established in different studies [35,36].

It is also worth noting that the quantitative ratio of casein/retinol in the product is much lower than in model systems. This allowed us to reduce the surfactant's amount required for more efficient homogenization to 0.15% vol.

Determination of retinol at sufficiently high concentrations was conducted by ^{1}H NMR in experimental studies. However, with the addition of vitamin A into the product according to the recommendations given, its amount would not exceed 1 mg in 1 kg of sausages. For such measurements, the nuclear magnetic resonance method is not applicable. Also due to the large mass of the sample tested, its preparation for analysis becomes difficult. Therefore, in this case, the concentration of retinol was determined by HPLC.

It was found that the model samples contained an average of 0.87 ± 0.05 mg of vitamin per 1 kg of finished product. This confirms the effectiveness of fortification of boiled sausages with a retinyl palmitate premix based on MCC.

Conclusion

It has been found that casein micelles are indeed able to protect unstable hydrophobic molecules (retinol in this case). Technology has been developed for the introduction of vitamin A into MCC, which involves the use of surfactants and intensive stirring at room temperature for two hours. With a decrease in time, the degree of protection reduces, and with an increase, it remains practically unchanged. When the vitamin is heated in the presence of casein for 3 hours at 80 °C (model conditions), the residual content of retinol reaches almost 80%, while without milk protein the maximum yield is 36%.

The study and verification of deep processes that occur during the so-called "encapsulation" is fascinating but at the same time difficult task for further research. At the same time, this goal is more fundamental than practical. In our opinion from a practical point of view, the results obtained, are enough to talk about the effectiveness and expediency of using MCC in the fortification of food products, in particular sausages, with such important substances as fat-soluble vitamins.

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