Available online on 15.03.2024 at http://jddtonline.info

Journal of Drug Delivery and Therapeutics

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Research Article

# Development of a technique for determining the viability of dormant organisms in powdered substances

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Article Info:

Article History:

Received 13 Jan 2024
Reviewed 11 Feb 2024
Accepted 29 Feb 2024
Published 15 March 2024

Cite this article as:

Gaidashev IA, Nikolaeva YG, Syroeshkin AV, Development of a technique for determining the viability of dormant organisms in powdered substances, Journal of Drug Delivery and Therapeutics. 2024; 14(3):60-65

*DOI: http://dx.doi.org/10.22270/jddt.v14i3.6467*

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## Abstract

The purposes of pharmaceutical biotechnology and quality control of probiotics containing lyophilized bacterial preparations, as well as the purpose of ensuring biological safety, require methods for rapid determination of the viability of dormant forms. This paper describes an approach to determining the viability of dry powdered substances. Lyophilized powder of *Kalanchoe daigermontiana*, dry dormant eggs of *Artemia salina*, and dry powder of *Lycopodium sp.* spores were proposed as model objects. Suspensions of virus-like particles (VLPs) to SARS-CoV-2 were proposed as a cell-free reference material. The following methods of elemental analysis were used in the study: X-ray fluorescence spectroscopy (XRF) on an energy-dispersive device, atomic absorption spectroscopy with electrothermal atomization, and Zeeman background correction (GZ-AAS). The free water content was determined by NMR spin echo. A new method was also used for recording the kinetics of changes in broadband radio emission in the centimeter and millimeter wavelength ranges, with a measurement duration of up to 20 minutes. Live and inactivated powdered preparations of *K. daigermontiana* and *A. salina* were prepared and characterized by elemental composition, water content, and spin-spin relaxation time ($T_2$). For these live and inactivated preparations, it was determined that the flux density of intrinsic thermal radio emission decreases by an order of magnitude with the loss of viability. In the future, the results obtained will allow for a rapid test of the viability of lyophilized therapeutics (from medicinal plants to bacterial mixtures that stabilize the intestinal microflora).

**Keywords:** millimeter emission, X-ray fluorescence analysis, NMR spin echo, nanoparticles, powdered substances, medicinal plant raw materials

## INTRODUCTION

Monitoring the viability of pharmaceutical substances manufactured on a bacterial, plant or animal basis has the following main purposes:

- Preserving the viability of bacterial cultures in the lyophilized state, for example, to maintain the stability of the digestive system [1];

- Absence of live cells in dried medicinal plants for their stability at storage [2];

- Absence of active or potentially active life forms to prevent infection (prions [3], viral [4] or bacterial [5] forms, or invasions [6]).

Rapid determination of the presence of alive things is a non-trivial scientific task. The presence of alive things is always associated with slow conformational rearrangements of oligomeric intracellular complexes [7]. Slow conformational transitions are one of the main characteristics of oligomeric proteins and their complexes with membranes and other biopolymers as a result of the multiplicity of their conformational states that depend on the ligand environment, electric field strength on the biomembrane, temperature, and many other factors. Besides the multipoint Coulomb binding, which is well known for low-molecular substances, biopolymers [8-10] interact with a supramolecular target due to dispersion (Deryagin) "surface-to-surface" interactions [11]. A feature of these interactions is the Coulomb tuning of the flickering dipoles due to the mutual electromagnetic radiation of the surfaces. This interaction is dynamic, a mutual influence of quantum fluctuations on the electron density distribution occurs in complex matrices [12]. The contribution of dispersion interactions to the free energy of the adhesion reaction of surfaces is an order of magnitude larger than the interaction of single charges [13]. Therefore, the dynamic redistribution of dipoles on the surfaces, when a supramolecular therapeutic substance is binding to a supramolecular receptor, is a critical condition in the mechanism of the biological activity of nanoparticles. It would be expected that the electromagnetic emission of flickering dipoles can mimic the effect of a ligand on an enzyme, and then ligand-specific conformational transitions of supramolecular complexes can be induced by mimicking the effect of low- and high-molecular substances under changing thermal fields and emissions. Thus, thermal radio emission of macromolecules [14] can be used as a catalyst in biological reactions, as well as as an active substance that causes a change in the release of secondary messengers in the chain of ligand-receptor interaction.

An advantageous model for studying conformational transitions in macromolecules are the dormant eggs of the brachiopod crustacean *A. salina* because of their ability to maintain viability under extremely unfavorable environmental

conditions. Dormant eggs have the ability to fast hydrating-dehydrating, which is an extremely important factor in primary (molecular) stability [15]. Fast dehydrating increases the viscosity in the cell and preserves non-freezing water, which has a beneficial effect on the low temperature tolerance, since there is no crystallization or mechanical damage to the cell [16].

Under unfavorable conditions, with small amounts of water in the cell, a significant decrease in the conformational mobility of high-molecular compounds occurs, which leads to a decrease in the degradation of the molecular structural-functional components of the system [17-18]. Conformational mobility is regulated by the balance of the interaction of water with polar groups of proteins and the hydrophobic interactions of the lipophilic parts of protein molecules. Drying proteins under vacuum results in a significant loss of water, however, it often does not lead to irreversible changes in the protein structure. Using the lyophilization method shows a more complete removal of water, which can lead to irreversible changes in their structure. Thus, the practice of preparing reference samples for trace element analysis using unstable and complex organic matrices widely uses sample stabilization by removing liquid through lyophilization. It is also noted that this procedure affects the organic matrix, which is due to irreversible changes in the conformation of proteins. It was shown that dormant eggs of *A. salinae* can remain viable even after drying by lyophilization [19].

This study proposes a rapid method for the viability control of powdered dry preparations. Lyophilized powder of *Kalanchoe daigermontiana*, dry dormant eggs of *Artemia salina*, and dry powder of *Lycopodium sp.* spores were proposed as model objects. Alive and inactivated preparations were characterized by elemental composition and intracellular water content. Suspensions of virus-like particles (VLPs) to SARS-CoV-2 were proposed as a cell-free reference material. It was shown that the listed model inactivated samples lost up to 90% of the density of their intrinsic flux of thermal radio emission in the millimeter band.

## MATERIALS AND METHODS

### Objects of study

The following dormant forms were chosen as objects for studying thermally induced conformational transitions in the organic matrix: dormant eggs of the crustacean *A. salinae,* spores of *Lycopodium sp.*, VLP SARS-CoV-2 vaccine, fresh shoots, shoot homogenate, and a reference sample made from *K. dagermontiana.* Samples of dormant eggs of *A. salina,* spores of *Lycopodium sp., K. dagermontiana* were tested for monodispersity [20-22]. Maximum size distribution: *A. salina* - 250 μm, *Lycopodium sp.* - 32 μm, reference sample of *K. dagermontiana* - 63 μm. Alive forms of *A. salina and Lycopodium sp*. germinated by 85% (dead forms - 0%).

### Element analysis

Elemental content was monitored using two methods: XRF with the sample preparation technique used for making a reference sample based on *K. daigremontiana* and GZ-AAS as the reference method. Sample preparation of dormant eggs of *A. salina* using the GZ-AAS method was carried out in the same way as for a sample of the medicinal plant *K. daigremontiana* [22] using microwave digestion in Teflon bombs under pressure.

### Determination of the flux density of thermal radio emission

The flux density of thermal radio emission in the microwave wavelength range was determined using a TES - 92 instrument (TES Electrical Electronic Corp., Taipei, Taiwan) with a sensor configured for anisotropic measurement along the Z axis. The measurement results are the maximum average flux density value with incremental averaging every 300 ms. The measurements were carried out both without opening the primary package and with opening the package. The preparations were heated to 37 $^{o}$C using a solid-state thermostat with Peltier elements. The temperature of the samples was controlled by a remote laser infrared thermometer. The installation geometry did not change and was strictly observed for each sample. The spatiotemporal distribution of microwave background emission was controlled before each measurement with height, width, and length increments of 50 cm. The background radiation did not exceed 1 mW/m$^2$. Aqueous solutions were applied in drops of 100 ml to the center of sterile 10-cm Petri dishes. Drops were applied in the axial direction along the Z axis at a distance of 10 mm from the measuring sensor of the device. To assess the validation characteristics of in-lab precision, all measurements were performed at least seven times [23].

### NMR spin echo technique

Instruments used in the study of proton spin-spin relaxation: XL-100 (Varian, Paolo Alto, California, USA), Avance 600 (Bruker, Billerica, Massachusetts, USA). The NMR instrument has several components [24]: 1) Computer for visualizing and averaging signal amplitudes and differentiating decay curves; 2) Unit that determines the sequence of 90$^{o}$ and 180$^{o}$ pulses to determine $T_1$, $T_2$; 3) Generator of radio frequency pulses that create a high-frequency magnetic field to rotate magnetic moments; 4) Oscillator circuit inductor, 0.4 T; 5) Oscilloscope – measures the spin echo signal and the transmitter signal. Temperature changes were provided by a unit with liquid nitrogen. The summation and accumulation of the results of individual measurements, the division of a multicomponent signal decay curve into individual components and the determination of $T_1$, $T_2$ for each component were carried out by a software specifically created for this installation. Nuclear magnetic resonance provides a wide range of parameters (shape, width, area, and position of the resonance line; time of spin-lattice $T_1$ and spin-spin relaxation $T_2$), that reflect the dynamics of molecules. The operating frequency of the installation is about 20 MHz. The measurement accuracy does not exceed 7%. The linearity of the installation's operation was continuously monitored using the aqueous reference solution of $CuSO_4$. NMR allowed obtaining information about changes in the conformation of organic macromolecules and mobile protons. The amplitude characteristics make it possible to determine the water content in the sample. Samples of *A. salina* dormant eggs were dried in a freeze-dryer. At the last stages of drying, the sample was heated to room temperature. For measurements, a loose sample of 0.5 g of dormant eggs was placed in a glass ampoule with a plug, and then the ampoule was placed between the poles of a magnet. After exposure of the sample to high-frequency pulses, the system of nuclear spins returns to equilibrium, that is, the nucleus transfers its energy to similar nuclei as a result of spin-exchange; this process of energy exchange between nuclei occurs according to the mechanism of spin-spin relaxation of nuclei ($T_2$). $T_2$ can be measured in the range from $10^{-5}$ to $10^2$ s. In our experiments, the components of immobile and low-mobility molecules were not recorded. A significant advantage of the NMR method is the possibility to study objects (including living ones) without violating their integrity or state.

## RESULTS

### Characteristics of live and inactivated samples of dormant eggs of *A. salina*

As is known, the state of water in the bulk phase, in the electrical double layer, and in the tightly bound state in crystalline hydrates and colloidal particles can be characterized by the spin-spin relaxation time ($T_2$) using the NMR-spin echo

method [24, 25]: $T_2$ for column water molecules is 2 s, for loosely bound water –100-200 ms, for tightly bound water (not removed by drying – 5-15 ms, for ice – 50 μs.

To determine the kinetics of changes in content during storage in dormant eggs of *A. salina*, the NMR spin echo method was used. The data obtained illustrate a significant reduction, from 2.5 to 1 mg of $H_2O$ per 1 g of biomass (Figure 1) when stored in a climatic chamber with a constant temperature of t=23 °C and humidity of 75%.

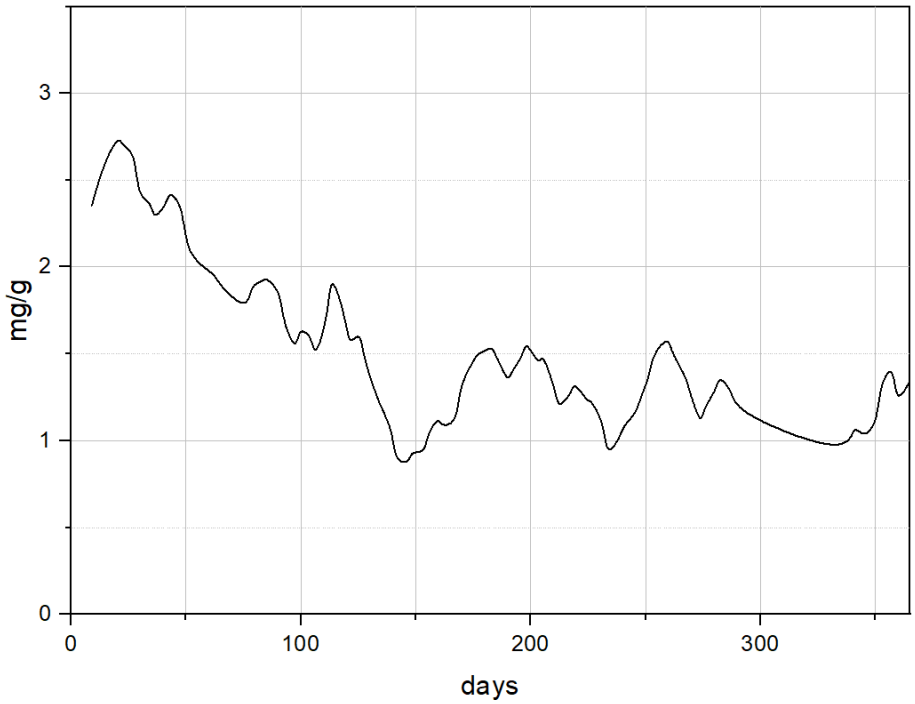


**Figure 1: Change in the content of loosely bound water ($T_2$=220 ms) in dormant eggs of *A. salina* according to NMR spin echo data**

Such slow drying does not affect the viability of dormant eggs, as it reflects the background metabolism of alive eggs frozen in the early gastrulation stage.

Under accelerated drying (9 hours at 150 °C), dormant eggs lose their viability (that is, they die), and the water content drops to 0.5 mg/g.

The spin-spin relaxation time $T_2$ of protons in live dormant eggs under heating and that of the control sample of killed dormant eggs at 20°C was 220 and 95 ms. Thus, the spin-spin relaxation time in killed dormant eggs significantly decreases, which indicates a change in the fraction of mobile protons and is confirmed by a decrease in water content (Table 1).

**Table 1: $T_2$ proton relaxation time and total water content in samples of *A. salina* dormant eggs, alive and killed at 150°C for 9 hours**

| Sample *A. salina* | $T_2$ relaxation time; ms | Water content; mg/g |
|---|---|---|
| Live | 220±30 | 2.5 |
| Inactivated | 95±7 | 0.5 |

It should be noted that the histological study detected no degenerative changes in the tissue.

Earlier, we proposed a method for distinguishing between live and killed dormant eggs based on the characteristics of elemental profiles obtained by the GZ AAS method under microwave digestion and XRF [26]. In this study, we used XRF to characterize the alive and killed homogenate and lyophilisate of Kalanchoe shoots.

**Elemental analysis of *K. daigremontiana* shoots**

We developed a reference sample from *K. daigremontiana* shoots for elemental analysis. The stability of indicators in the case of analysis by X-ray fluorescence spectroscopy of such a sample is ensured only in the absence of alive cells in the lyophilized powdered material. As part of monitoring the conformational transitions of the organic matrix using the XRF method, a prototype of a reference sample was developed using the following stages: 1) homogenization of the original substance; 2) lyophilization of the homogenate; 3) grinding the lyophilisate by direct knife blow; 4) sifting through a nylon sieve with a mesh diameter <63 μm. It is known that the complex organic matrix significantly influences the X-ray fluorescence yield. The sample preparation conducted allows for stabilizing the matrix and ensuring the preservation of repeatability and reproducibility over a long period of time. The key step in sample preparation is drying the sample. This study used freeze drying until the condensate flow completely stopped, which led to the removal of water retained by the electrical double layer and, as a consequence, a significant decrease in conformational mobility (increased stability over time) [22]. Figure 2 shows the difference in the elemental profile of a live shoot homogenate and a shoot lyophilisate that does not contain live cells.

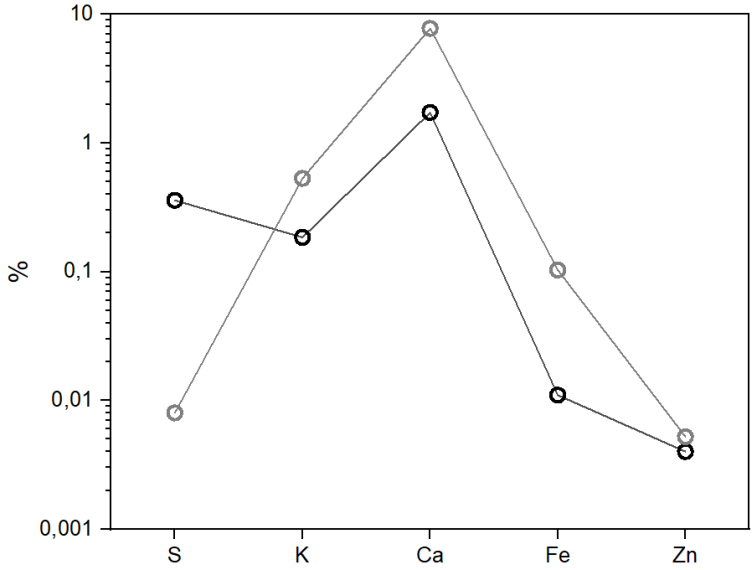


**Figure 2: Elemental profile in fresh homogenate and dried sample of *K. daigremontiana.* Determined by XRF method, the element content was recalculated according to the protocol of the equipment manufacturer, Shimadzu. Black circles indicate the fresh sample, gray circles indicate the dried sample.**

Thus, determining the elemental composition by XRF requires compliance with complex criteria to achieve the greatest reliability of the results. The elemental profile of the dried sample shows significant quantitative differences compared to the fresh *K. daigremontiana* shoot homogenate. The decrease in S content and a slight increase in Zn are due to the presence of sulfur-containing volatile compounds, which are removed by drying the sample, as well as a change in the localization of elements in the organic microenvironment in the case of zinc. The previous study showed that organic matrix stabilization by freeze-drying has the greatest effect on the increase in repeatability. Also, a stability test was performed on the reference sample for 210 days, the results of which showed a slight deviation in the content of elements from the original values.

To distinguish between live and killed medicinal plants, it is necessary to carry out long-term elemental analysis with complex sample preparation. For the samples of animal and plant origin presented in the first and second sections, we applied a new method of monitoring their own thermal radio emission, which allows rapid determination of their viability.

**Rapid viability test for powdered substances**

In a recent study, we showed that biologically active nanoparticles of irregular shape are capable of radio emission in the millimeter band [14]. The flux density of this emission depends on the temperature, structural features of nanoparticles, their nativeness, and their concentration, which makes it possible to use this method for chemical-analytical control of infusions of medicinal plants and vaccines [27].

The conformational mobility of live and killed dormant eggs was proven by monitoring the thermal radio emission of dormant forms of powdered dormant eggs of *A. salina*, spores of *Lycopodium sp*, VLP-SARS-CoV-2 vaccine suspension, and fresh homogenized shoots of *K. daigremontiana.* When measured over 20 minutes, these samples showed a significant increase in thermal radio emission from 7 μW/m² to 35 μW/m², with background values <1 μW/m² (Figure 2).

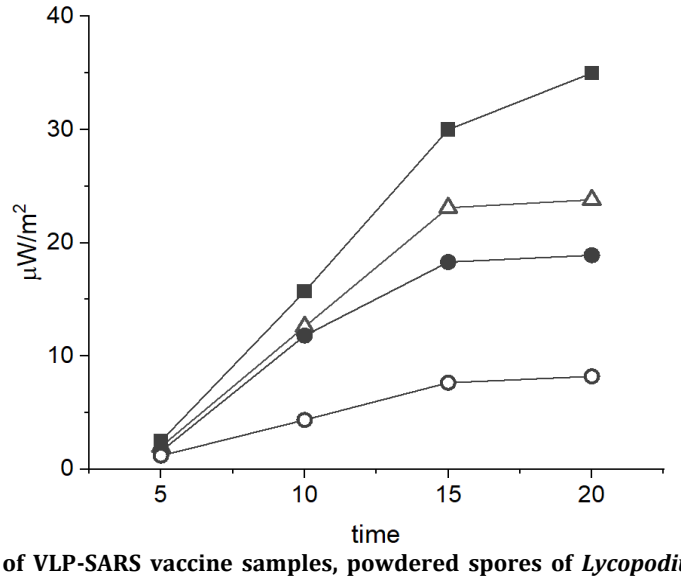


**Figure 3: Thermal radio emission of VLP-SARS vaccine samples, powdered spores of *Lycopodium sp.,* are indicated by a triangle, powdered dormant eggs of *A. salina* are indicated by a filled circle, and homogenized leaves of *K. daigremontiana* are indicated by a hollow circle.**

Biologically active nanoparticles of plant or animal origin are isolated from living tissues and retain the properties of living things in an artificial environment that maintains them in their native state (buffer, given ionic strength, osmolality, limited temperature range). As is known, changing the storage conditions of isolated nanoparticles leads to their denaturation with minute kinetics. We assumed that nanoparticles of a live cell (oligomeric membrane and surface proteins, nucleoproteins, and peptides) would emit only as part of a live cell. Indeed, the flux density of thermal radio emission of powdered forms and suspensions drops by 3-10 times in killed samples (Table 3).

**Table 3: Thermal radio emission flux density of live and killed dormant eggs of *A. salina*, native and denatured VLP-SARS-CoV-2 vaccine, freshly prepared homogenate, and lyophilized homogenate of *K. dagermontiana shoots***

| Sample | Original sample, % | Processed sample, % |
|---|---|---|
| *A. salina*[1] | 100 | 14 |
| VLP-SARS-CoV-2[2] | 100 | 10 |
| Shoots of *K. dagermontiana*[3] | 100 | 30 |

*1 - 100% corresponds to the emission flux density of 19 μW/m² from 1.0 g of A. salina dormant egg preparation at 37°C.*

*2 - 100% emission corresponds to the emission flux density of 35 μW/m² from 0.1 ml of the VLP-SARS-CoV-2 preparation with a protein concentration of 160 μg/ml at 23°C.*

*3 - 100% emission corresponds to the emission flux density of 7 μW/m² from 1.0 g of freshly prepared homogenate.*

A lower flux density for the reference sample and killed dormant eggs indicates the cessation of the conformational activity of the supramolecular formations in the organic matrix and is another evidence of the significant influence of the sample preparation on the stability of the sample. Supramolecular emitting formations are apparently distributed in cellular structures that have absorptive capacity, which is leveled out by homogenization and release of these structures from the cellular matrix. This phenomenon may be species-specific due to structurally different supramolecular emitters. A significant change in the flux density of thermal radio emission in live dormant eggs indicates a change in the dielectric constant of the medium, which affects the yield of X-ray fluorescence caused by conformational transitions. No such increase was observed in killed dormant eggs.

## DISCUSSION

It should be emphasized that XRF determination of elements in organic therapeutics is impossible without a reference sample: this is how international QA/QC conditions are met. Also, as part of the homogeneity test, a dispersion analysis was carried out using the LALLS method, showing the presence of a major fraction (by mass fraction) of about 65 μm and a minor fraction of about 39 μm. Stability tests of the prototype reference sample were carried out using the XRF method for 7 months, demonstrating less than 5% deviation from the original values.

A change in the organic matrix and, as a consequence, in the X-ray diffraction signal when heating the preparation from 25 °C to 37 °C, manifesting an effect similar to the heterogeneous catalyst, will make it possible to develop an enzyme-catalyzed synthesis controlled by spatiotemporal characteristics. Thus, we demonstrated the same before for the anomalous kinetic isotope effect, neutron control, and mechanochemical conformational transitions [26, 28].

Changes in the rate of hatching of dormant eggs when irradiated under thermal neutron irradiation may indicate a modulation of redox reactions due to the generation of free radicals. This phenomenon is observed as a result of the decay of unstable isotopes of elements with the release of high-energy photons, followed by ionization of the substance. One of the significant factors influencing the mutual tuning of flickering dipoles, and, accordingly, the intensity of thermal radio emission of particles, is the microelement environment.

When the elemental composition changes in a particular sample, the flux density of thermal radio emission will undergo changes. This phenomenon is explained by the redistribution of dipoles in supramolecular formations and changes in the dielectric constant of the medium. Since the results obtained by X-ray fluorescence spectroscopy directly depend on the organic microenvironment, monitoring the microelement composition with the proper level of reliability in order to analyze conformational transitions in the complex organic matrix using the XRF method is impossible without making a reference sample due to specific matrix effects affecting the X-ray fluorescence yield [29].

Sample preparation by the technique of preparing a reference sample is accompanied by a change in the organic matrix, which is also observed when *A. salina* is heated for 9 hours at t = 150 °C and, as a consequence, a change in the XRF signal.

It has been shown that various biological objects, such as *A. salina*, *Lycopodium sp.*, intact Kalanchoe leaves, also emit in the thermal radio band when heated from 25 °C to 37 °C. The Kalanchoe leaf showed the lowest flux density of thermal radio emission due to absorption. When the test sample was heated above 37 °C and further the organic matrix was destroyed, that is, in the temperature denaturation or accelerated aging method, a decrease in the thermal radio signal was observed, as well as a change in the intensities of the elements contained in the samples. This phenomenon allows for the development of technique for quality control of catalysts with an organic matrix for the induction of an active conformation.

This study proposes a rapid method for viability control of powdered dry preparations. Lyophilized powder of *Kalanchoe daigermontiana*, dry dormant eggs of *Artemia salina*, and dry powder of *Lycopodium sp.* spores were proposed as model objects. Live and inactivated preparations were characterized by elemental composition and intracellular water content. Suspensions of virus-like particles (VLPs) to SARS-CoV-2 were proposed as a cell-free reference material.

It was shown that the listed model inactivated samples lost up to 90% of the density of their intrinsic flux of thermal radio emission in the millimeter band. The vitality of lyophilized therapeutics (from medicinal plants to bacterial mixtures that stabilize the intestinal microflora) can be characterized in a rapid manner.

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