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ARTICLES

Determination of Hyaluronic Acid in Aqueous Solutions Using Air as an Oxidant

B. K. Zuev[a], *, V. G. Filonenko[a], D. S. Nesterovich[b], and P. D. Polikarpova[b]

[a] *Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, Moscow, 119991 Russia*
[b] *Kosmetsevticheskii Inkubator JSC, Moscow, 127015 Russia*
**e-mail: zubor127@yandex.ru*

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Abstract—The development of new methods for the control of preparations widely used in cosmetics and medicine is a task of high current importance. Hyaluronic acid is one of the most often used components of anti-age cosmetics; its action and impact on the human skin depends on the concentration in the solution. The known methods of the determination of the concentration of hyaluronic acid are expensive, require complex sample preparation, and cannot be used in routine analyses. In this work, for the rapid determination of the concentration of hyaluronic acid in solutions used in the production of skin cosmetics, we propose the method of oxythermography, which does not require chemical reagents. The oxidant is air oxygen. The absolute limit of detection for hyaluronic acid is about 4 μg for a sample volume of 5 μL.

Keywords: oxythermography, hyaluronic acid, oxygen sensors in the gas phase, thermooxidation spectrum, high-molecular compounds, low-molecular compounds

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The development of the cosmetic industry and the appearance of new active components called for the development of new methods for the study of their properties and the determination of effective concentrations.

Hyaluronic acid is finding more and more wide application in cosmetics and is considered one of the most delicious cosmetic ingredients [1]. It is a white powder, completely dissolved in water and forming a gel in the concentration higher than 1 wt %. In contrast to the majority of the used cosmetic substances, hyaluronic acid demonstrates all its valuable properties in very low concentrations (0.01–0.1 wt % for the high-molecular form used in lipsticks, gels, and after-sun lotions); therefore, it is important to know the concentration of hyaluronic acid solution in cosmetic means, and also its effective concentration (limiting concentration of penetration into skin).

Today high-molecular compounds, such as peptides, synthetic polymers, and oligosaccharides are determined widely using the method of matrix-assisted laser desorption/ionization (MALDI). Its application to the determination of low-molecular compounds, for example low-molecular hyaluronic acid, is complicated because of the interfering effect of matrix ions in the low-molecular region [2]. The matrix usually consists of low-molecular compounds, complicating the determination of substances with close molecular weights; therefore, the concentration of low-molecular substances is also controlled by the method surface-assisted laser desorption/ionization (SALDI), in which matrixes are carbon materials, powdered metals and their oxides, etc. [3]. The wide use of these methods is complicated by the complexity of matrix selection, and also by the high laboriousness and long duration of sample preparation to the analysis.

Methods of a high-performance capillary electrophoresis and HPLC were developed for the determination of the concentration of gialuronic acid and its molecular weight [4, 5]. In contrast to MALDI and SALDI, these methods do not require the additional selection of a matrix, but take long time, are expensive, and also require laborious sample preparation. Therefore, the specified methods can hardly be widely used in the perfumery industry and beauty salons. It is clear that the development of reagentless, environmentally green, rapid, and rather cheap methods for the analysis of cosmetics is quite necessary.

The method of oxythermography is based on the programmed high-temperature oxidation of organic substances in a flow of a mixture of an inert gas with oxygen and the quantitative determination of molecular oxygen in the gas mixture obtained as a result of high-temperature oxidation. The method was patented in the Russian Federation [6]. In this work, we demonstrate a possibility of the development of the method of oxythermography and corresponding

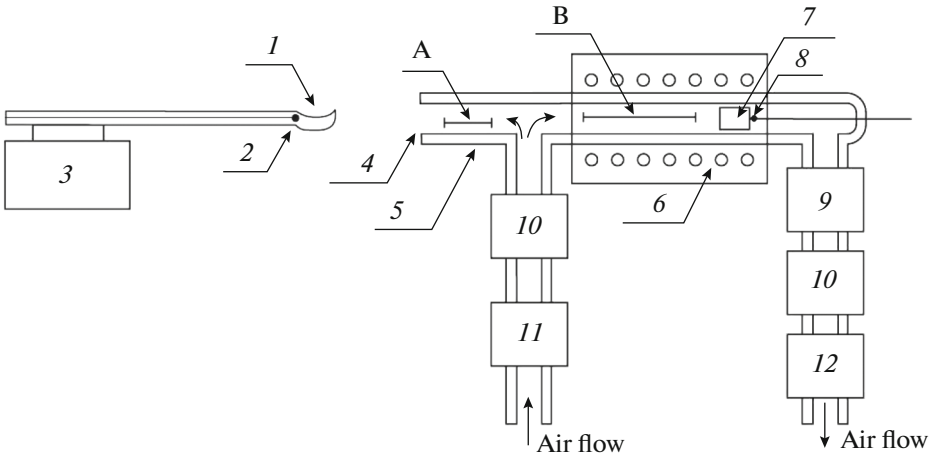


Fig. 1. Schematic diagram of an oxythermography installation: *1* is quartz boat, *2* is thermocouple for the control of boat temperature, *3* is mechanism for moving the boat according to the motion profile, *4* is entrance opening of the high-temperature reactor, *5* is reactor, a quartz tube with an appendix, *6* is high-temperature furnace for heating the reactor, *7* is catalyst, *8* is thermocouple for the control of reactor temperature, *9* is oxygen sensor, *10* is rotameter, *11* is gas compressor, *12* is gas flow activator; A is zone of water distillation and B is high-temperature section of the reactor, oxidation zone.

equipment for the analysis of cosmetics on an example of hyaluronic acid, widely used in cosmetology (sodium hyaluronate in aqueous solutions).

The aim of this work was to study of the analytical potentials and to optimize the method of oxythermography (thermooxidation spectroscopy) for the determination of the concentration of hyaluronic acid in aqueous solutions. In contrast to the previous study [7], in which organic substances were oxidized in a flow of a binary oxygen–argon mixture, in this work oxidation was performed in a flow of air. This made the proposed method, reagentless environmentally green, inexpensive, and free from the subsequent utilization of chemical reagents.

EXPERIMENTAL

We used sodium hyaluronate 3000 Da of the brand "Shancong Focuschem Biotech Co." as source of hyaluronic acid. The work was performed on an experimental installation, the schematic diagram of which is shown in Fig. 1. An analyzed liquid volume was dozed to sampling boat *1*. The material of the boat (quartz) did not interact with oxygen on heating in air. Thermocouple *2* controlling boat temperature in the course of heating was inserted into the boat. Using a specially developed graphics program (profile of boat movement, see Fig. 2), the researcher specified the algorithm by which the boat with the analyzed sample moved to the high-temperature quartz reactor through its entrance opening *4*. The profile was the dependence of boat position (mm) on time (sec) on its movement in the reactor. The initial position of the boat 0 is shown in the graph in Fig. 2. The reactor was preheated to the temperature 700°C, which was maintained by a special unit, manufactured by the OVEN company. Air in two flows was fed to the reactor through a tube located near the entrance opening of the reactor from a generator of pure air, manufactured by NPP Khimelektronika. One air flow left the reactor through the entrance opening. This flow can be used for distilling easily volatile liquids on heating the boat in zone A (see Fig. 1, distillation zone A). The air flow coming to the high-temperature section of the reactor was used for the oxidation of organic substances present in the sample on the introduction of the boat into high-temperature section of the reactor (see Fig. 1, oxidation zone B). To complete oxidation, at the exit from the reactor we arranged a platinum-based catalyst *7*. Oxygen sensor *9,* which continuously controlled the concentration of oxygen in the flow of air leaving the reactor, was located after the reactor. The oxygen sensor was a solid-electrolyte sensor for the control of the concentration of oxygen in exhaust gases [8, 9]. The information from the oxygen sensor with a specially created electronic unit and software was displayed on a computer monitor as a dependence of the concentration of oxygen (rel. unit in mV) on time.

When the quartz boat with the analyzed sample was in zone A, water present in the sample was distilled. In the movement of the analyzed sample in the high-temperature section of the reactor, the boat was heated and the organic substances remained on the boat surface were oxidized. The oxygen sensor detected the reduction of the concentration of oxygen leaving the reactor. The rate of heating was determined by the speed of boat motion; the profile of boat motion was set in the program file. The occurrence of the boat between zones A and B could lead to the disturbance

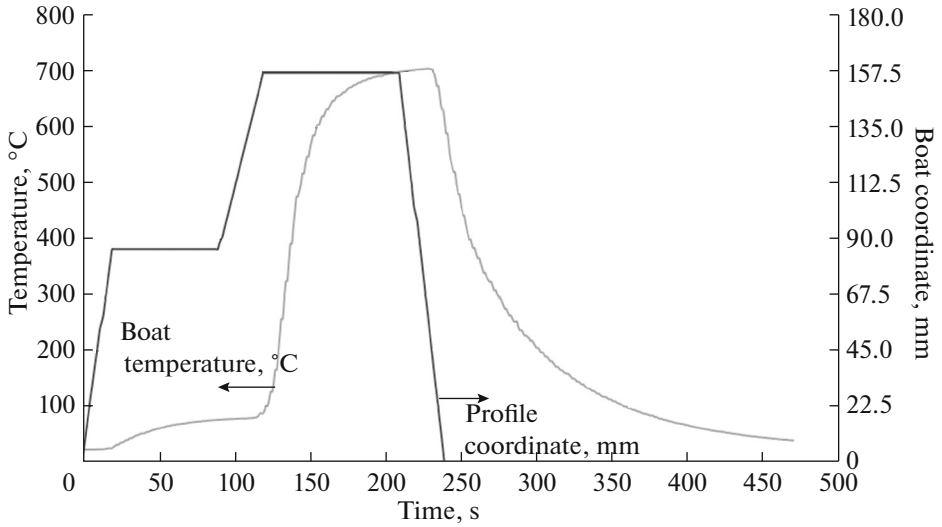


Fig. 2. Optimum profile of boat motion and a graph of the dependence of the change in boat temperature on the time of its motion.

of the gas flow coming to the high-temperature section of the reactor in the course of water distillation.

The result of analysis is an oxythermogram (thermooxidation spectrum), the dependence of the change in the concentration of oxygen in the gas flow leaving the reactor on time or on the sample temperature in its heating in the high-temperature section of the reactor. The oxythermograms are specific for organic and other oxidized substances.

RESULTS AND DISCUSSION

The first stage of the work included the optimization of the volume of solution dosed to the quartz boat and the choice of a sample heating profile. We used a two-step heating of a sample of a solution on continuously registering the concentration of oxygen in the flow of air leaving the high-temperature reactor. At the first step, water was distilled at the temperature lower than 100°C (see Fig. 2, temperature profile) and, at the second step, in the fast motion of the boat to the high-temperature section of the reactor, hyaluronic acid remaining on the boat surface was oxidized at the temperature about 700°C. A specific feature of the chosen mode of boat motion (motion profile) was that the position of the boat with an aqueous solution in the water distillation mode was selected specially, so that only a small portion of water vapors entered the high-temperature section of the reactor in water evaporation. This resulted in the disturbance of the gas flow in the high-temperature section and in the appearance of the first negative peak in the recorded spectrum. This approach allowed us to control the completeness of water distillation from the analyzed sample.

We varied the volume of hyaluronic acid solution with the concentration 1.0 wt % dozed to the boat in the range 5–20 μL and the time the sample was kept in the water distillation zone from 30 to 120 s. It was found that sample volume of 5 μL and time of water distillation 70 s were sufficient for the determination of hyaluronic acid in an aqueous solution. An increase in sample volume over 5 μL led to an increase in the time of distillation and, correspondingly, to an increase in the duration of analysis. The reduction of sample volume led to the reduction of the analytical signal against the baseline. The optimum profile of boat motion and, correspondingly, sample heating as a function of time are presented in Fig. 2.

Using the optimum profile of boat motion, we studied the possibilities of the method of oxythermography for the determination of the concentration of hyaluronic acid with the molecular weight 3000 Da. We prepared aqueous solutions of hyaluronic acid of different concentrations in the range from 0.1 to 2.0 wt %. Typical oxythermograms, i.e., thermooxidation spectra of the analyzed solutions, are shown in Fig. 3. It can be seen that, in using the optimum profile of boat movement, water was the distilled completely. The first negative peak characterizing the process of water distillation at 100 s reached almost background values. The second negative peak was due to the presence of hyaluronic acid in the sample. The analytical signal is the area of the negative peak, which depends on the concentration of hyaluronic acid in the sample.

The areas of the recorded peaks were related to the corresponding amount of hyaluronic acid in a sample volume of 5 μL. We obtained a linear dependence of the peak area of hyaluronic acid on its amount for a sample volume of 5 μL. The equation of the calibra-

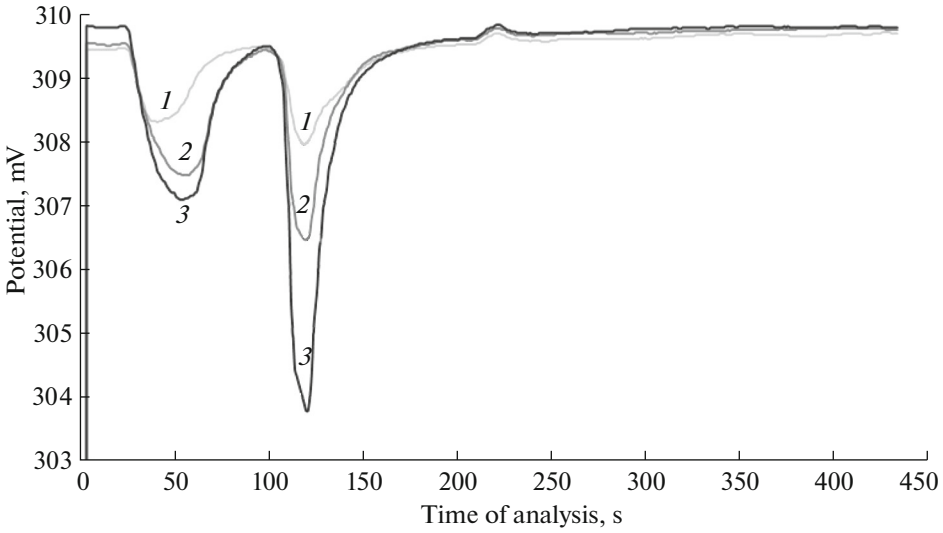


Fig. 3. Oxythermograms, thermooxidation spectra of hyaluronic acid solutions with molecular weight 3000 Da. The volume of the analyzed sample is 5 μL. The concentration of the solution of sodium hyaluronate, wt %, are as follows: *1*, 0.5; *2*, 1.0; *3*, 2.0.

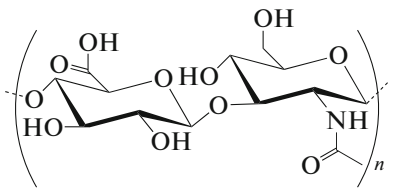
tion dependence was $y = (770 \pm 0.5)\,x + (240 \pm 20)$, where x was the concentration of hyaluronic acid in the sample, wt %; y was peak area, mV s. The relative standard deviation was 5%.

The absolute limit of detection was calculated by statistical methods using the Chebyshev inequality in accordance with [10]:

$$c_{\min} = \frac{3s_{\text{blank}}}{S},$$

where c_{blank} was the standard deviation of the analytical signal of the background, and S was sensitivity limit. The minimum amount of hyaluronic acid that can be found by this method is 4 μg in sample of 5 μL. This value ensures the use of the oxythermography method for the diagnostics of human skin in the contact sampling of organic substance to the surface of a special sampler [11].

We investigated the effect of the molecular weight of hyaluronic acid (HA) on the process of its oxidation by oxygen. The theoretical calculation of the amounts of oxygen consumed for the oxidation of hyaluronic acid of molecular weights 3000 and 750000 Da showed that the HA with the average molecular weight 3000 Da, on an average, contained 8 monomeric units (Scheme 1) and, therefore, corresponded to the formula $C_{14}H_{21}NO_{11}$ and the molecular weight 3034 g/mol.



Scheme 1. Structural formula of a monomeric unit of hyaluronic acid.

Taking into account the reaction equation

$$(C_{14}H_{21}NO_{11})_8\,H_2 + 110.5O_2 = 112CO_2 + 85H_2O + 4N_2,$$

the amount of oxygen consumed for the oxidation was 110.5 times greater than the amount of HA. In the oxidation of 5 μL samples of hyaluronic acid with the concentration 1.0 wt %, the amount of oxygen participating in the reaction was 1.82 μmol. Similar calculations for the oxidation of hyaluronic acid with the molecular weight 750000 Da showed that the amount of consumed oxygen was equal to 1.81 μmol. Therefore, it is clear that the molecular weight of the acid has virtually no effect on the amount of consumed oxygen.

The effect of the molecular weight of the HA on the amount of oxygen consumed for its oxidation was also estimated experimentally. We chose solutions of low-molecular (3000 Da) and high-molecular hyaluronic acid (>500 kDa) with the concentration 1.0 wt %. It was shown that, with an increase in the molecular weight of sodium hyaluronate, the amount of oxygen consumed for the oxidation slightly increased for equal weights. The results obtained (S is the peak area of oxygen equal to 972 ± 49 mV sec for low-molecular acid and to 1175 ± 22 mV sec for high-molecular acid) indicate that the peak areas of low-molecular and high-molecular acids differ by no more than 17%.

To check the accuracy of the data obtained, we analyzed hyaluronic acid of the DNCSwiss brand bought in a drugstore with the declared concentration 1.0%. As the molecular weight of the acid was not specified on the packing and on the web site of this substance, for the analysis we used a calibration curve for low-molecular hyaluronic acid (3000 Da), because low-molecular substances are most often used in such means. We obtained the area of the thermooxidation

peak of the acid equal to 959 ± 42 mV sec, which allowed us to determine the concentration of the acid 0.98 ± 0.04 wt % by the calibration dependence; it coincided with the certified value of 1.0% of hyaluronic acid in the Switzerland preparation.

Therefore, a possibility of the rapid determination of hyaluronic acid by oxythermography in aqueous solutions without using chemical reagents was proved; the absolute limit of determination was found at a level of 4 μg, which ensures the use of the method for the diagnostics of human skin in the study of the transdermal properties of the preparation.

As hyaluronic acid is an active component of antiage means applied on skin, the research performed is necessary for the study of the kinetics of the penetration (creation of permeability profiles) of this substance into the skin of humans. In further, we are going to develop a procedure of the study of the penetration of other active cosmetic components into skin by oxythermography in combination with other methods of substance analysis.

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