# Estimation of dehydration of skin by refractometric method using optical clearing agents

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**Abstract.** Optical methods with highly sensitive to water content are important for the development of new diagnostic and therapeutic methods in medicine. The paper presents a quantitative assessment of the dehydration of skin when using optical clearing agents (glycerol and glucose) by the method of multi-wavelength refractometry for the visible and NIR spectral regions. The resulting decrease in the refractive index of the optical clearing solution made it possible to estimate the volume of the fluid extracted from the tissue. The possibility of using the method when conducting *in vivo* measurements is shown. An assessment was made of the degree of dehydration of rat skin in areas with a subcutaneous tumor neoplasm, which was three times less than for control (healthy) skin areas. © 2019 Journal of Biomedical Photonics & Engineering.

**Keywords:** multi-wavelength refractometry; skin; refractive index; dehydration; optical clearing; glycerol; glucose.

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## 1 Introduction

Currently, optical methods, such as photoacoustic and terahertz spectroscopy and visualization, and a number of other methods are widely used for the diagnosis and treatment of various diseases [1–9]. The use of optical methods is strongly limited to the small depth of probing tissues [9]. However, when exposed to hyperosmotic optical clearing agents (OCA) on tissue, it is possible to achieve a noticeable decrease in scattering and absorption in water bands in the entire range of working wavelengths due to dehydration of the tissue, respectively, an increase in optical transmission [9–14].

Enhancing image contrast due to dehydration and tissue optical clearing in the area of pathology is extremely important in the diagnosis of precancerous conditions, early stages of cancer and other diseases [1–6, 15–16]. In a number of works, the difference in the content of free and bound water in the tissues was shown and an increased content of free water in the tumor tissues was found [17–20]. It is assumed that protein molecules affect the free water content, which change as a result of the development of pathology. For example, it was shown in Ref. [19] that bound water interacts with the protein and is removed along with it. It should be noted that water is the main absorber in the terahertz frequency range and its reversible removal from tissue contributes to an increase in the depth of sensing of tissues by terahertz radiation [18–21]. The combination of studies in the optical and terahertz ranges opens up new possibilities for a more reliable diagnosis of a number of diseases [22, 23].

Traditional methods for quantifying the dehydration of tissue when exposed to OCA are measurements of changes in weight and geometric parameters [24–26]. However, these methods in practice are applicable only for *in vitro* or *ex vivo* measurements. The refractometric method allows the evaluation of the degree of dehydration for both *in vitro* (*ex vivo*) and *in vivo* measurements. And the measurement of refraction at the same time at several wavelengths makes it possible to obtain more reliable information about the degree of tissue dehydration due to the collection of a larger number of data and rely on determining the composition of the extracted fluid.

In this work, the further development of the refractometric method for estimating the degree of skin dehydration, previously described in Refs. [25, 26], and its application for *in vitro* and *in vivo* studies, which were conducted on laboratory animals with PC1 alveolar liver cancer tumors. *In vitro* measurements using 70% glycerol solution and 40% glucose solution as optical clearing agents were performed on a multi-wavelength Abbe refractometer DR-M2/1550 (Atago, Japan) for wavelengths of 486, 589, 680, 930, and 1300 nm. According to the obtained results, the refractive index of the antireflection solution decreased, which indicated the dehydration of the tissue and allowed a quantitative assessment of the volume of the extracted fluid. As an example, when conducting *in vivo* experiments, skin dehydration was evaluated in areas with a subcutaneous tumor neoplasm and in control (healthy) areas of the skin using the refractometric method for the wavelengths of 480, 486, 546, 589, 644, 656, 680, 800, 930, 1100, 1300, and 1550 nm.

## 2 Methods and materials

For *in vitro* measurements during the optical clearing experiment, skin samples of healthy laboratory rats were used as samples of the study. Using a micrometer, measurements were made of the thickness, width, and length of the samples before and after the experiment. On an analytical balance (Scientech, SA210, USA) with an accuracy of ±1 mg, the weight of the samples was measured before and after the experiment. Parameters were measured after the experiment and after complete removal of OCA on both sides of the sample.

The samples were placed in a closed sealed container, to which was added 2 ml of OCA. 70% glycerol solution and 40% glucose solution were used with the addition of saline as an OCA. Measurements of the refractive index of OCA were carried out before the start of the experiment and every 5 minutes after placing the skin sample in the solution with OCA for 20 minutes, then every 10 minutes for another 40 minutes. For the measurements, 10 μl of the solution was taken and three samples were taken for each solution.

The refractive index was measured on a multi-wavelength Abbe refractometer DR-M2/1550 (Atago, Japan). The radiation source in this installation is a high power incandescent lamp. For the selection of wavelengths, narrow-band interference filters for 486, 589, 680, 930, 1300 nm were used. The measurement error introduced by the device is ±0.0002. At the beginning of the measurements, the instrument was calibrated against the tabular value of the refractive index of distilled water. The temperature of the measurements during the experiment was 22 °C and was maintained with circulation thermostat.

Evaluation of the degree of dehydration of skin sample was performed using expression of Gladstone-Dale for a multicomponent solution [27, 28]:

$$n(\lambda,t)=\sum_i n_i(\lambda) f_i(t), \tag{1}$$

where $n$ is the refractive index of the solution, $i$ is the number of components of the solution, $n_i$ is the refractive index of the $i$-th component, $f_i$ is the volume fraction of the $i$-th component.

Expression (1) in our case will take the form:

$$\begin{aligned} n_{\exp}(\lambda,t) &= n_{OCA}(\lambda) f_{OCA}(t) + n_{NaCl}(\lambda) f_{NaCl}(t) + \\ &+ n_{ext\ fl}(\lambda) f_{ext\ fl}(t), \end{aligned} \tag{2}$$

where $n_{\exp}$ is the refractive index of the experimental solution, $n_{OCA}$ is the OCA refractive index, $f_{OCA}$ is the volume fraction of OCA (glycerol or glucose), $n_{NaCl}$ is the refractive index of saline, $f_{NaCl}$ is the volume fraction of saline solution, $n_{ext\ fl}$ is the refractive index of the extracted fluid, $f_{ext\ fl}$ is volume fraction of the extracted fluid.

From Eq. (2), the refractive index of the OCA is calculated from the measured and known in the literature data for the initial solutions. Initial calculations are performed under the assumption that the refractive index of the extracted fluid is equal to water.

Writing Eq. (2) for the solutions obtained after the enlightenment experiment, the volume fraction of the extracted liquid is calculated from the known data. The expressions for finding the volume fraction of the extracted liquid in glycerol and glucose solutions will be:

$$\begin{aligned} f_{ext\ fl}(t) &= \\ &= \frac{n_{\exp}(\lambda,t) - n_{OCA}(\lambda) + \left(n_{OCA}(\lambda) - n_{NaCl}(\lambda)\right) f_{NaCl}(t)}{\left(n_{ext\ fl}(\lambda) - n_{OCA}(\lambda)\right)}. \end{aligned} \tag{3}$$

The change in the volume fraction of the extracted liquid in the solution of OCA was estimated by the formula:

$$\Delta f = f_{60} - f_0, \tag{4}$$

where $f_{60}$ is the volume fraction of the extracted fluid in the OCA solution after 60 min exposure to the sample,

$f_0$ is the volume fraction of the extracted fluid in the OCA solution prior to the rat skin clearing experiment.

The found value of the volume fraction of the extracted liquid in the OCA solution was averaged over 5 wavelengths. Since this value is constant for all wavelengths, using also expression (2) one can determine the refractive index of the extracted liquid. Subtract the contribution of water, glycerol and glucose and compare the obtained dispersion dependence with the dispersion of water.

$$n_{ext\ fl}(\lambda) = \frac{n_{\exp}(\lambda,t) - n_{OCA}(\lambda) + n_{OCA}(\lambda) f_{ext\ fl}(t)}{f_{ext\ fl}(\lambda)} + \frac{\left(n_{OCA}(\lambda) - n_{NaCl}(\lambda)\right) f_{NaCl}(t)}{f_{ext\ fl}(\lambda)}, \quad (5)$$

*In vivo* studies were conducted on two laboratory rats of the Vistar line (sexually mature females weighing 300–400 g). The animal was vaccinated with a tumor, by subcutaneous injection into the area of the scapula by 0.5 ml of 25% tumor suspension of PC1 alveolar liver cancer strain in Hanks solution. Studies were conducted 16 days after injection. For the enlightenment experiment, a solution of glycerol 99.3% was poured into a special cuvette in the amount of 1 ml, which opened part of the skin on the skin to ensure full contact of the OCA with the skin surface for 30 min. After the impact of the OCA, he climbed out of the cell to measure the refractive index. The refractive index of glycerol solutions, as in the experiment described earlier *in vitro*, was measured on an multi-wave Abbe refractometer (Atago, Japan). Interference filters for 480, 486, 546, 589, 644, 656, 680, 800, 930, 1100, 1300, and 1550 nm were used to select wavelengths. Measurements were performed three times for each test solution. The measurement error introduced by the device was ±0.0002. The temperature of the solution during the measurements of the refractive index was 27 °C and was kept constant by means of a circulation thermostat. The volume of the extracted fluid was calculated by the Eq. (3).

## 3 Results and discussion

The resulting *in vitro* measurements of the dependence of the refractive index of the OCA solution on time are shown in Fig. 1. The approximation of the dependences obtained was performed using the exponential function:

$$y(x) = y_0 + Ae^{Bx}, \quad (6)$$

where $y_0$, $A$ and $B$ are constant values.

The approximation is shown on the graphs in solid lines.

In Fig. 1, it is noticeable that the refractive index of the 70% glycerol solution decreased more strongly, compared to the refractive index of the 40% glucose solution. It is well known that glycerol is highly viscous and hygroscopic. However, the process of interaction of glycerol with the skin is rather complicated and is not fully understood today, despite the extensive literature on this subject [29]. Due to its high hygroscopicity and high viscosity at relatively short time intervals (1–2 hours), it mainly acts as a hyperosmotic agent, extracting interstitial free and weakly bound water from the tissue, penetrating to the tissue to a lesser extent due to diffusion [14, 30]. Glucose is also one of the common OCA. In Ref. [31], it was shown that the optical clearing when using glucose occurs three times faster than when using glycerol.

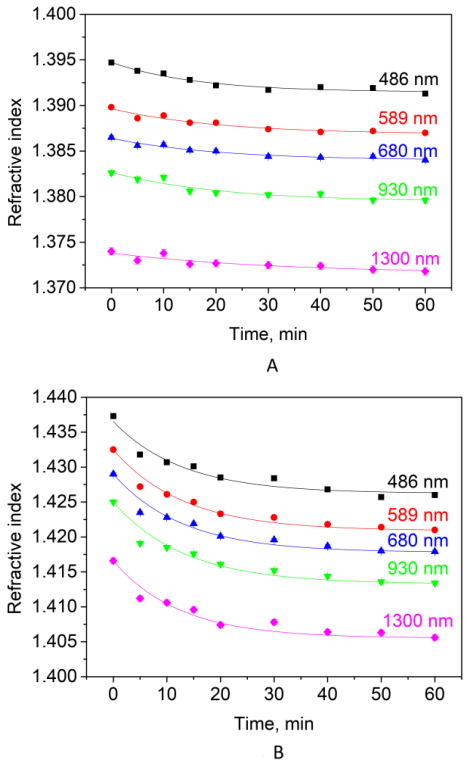


Fig. 1 Dependence of the refractive index of the antireflection solution on time: a) 40% glucose solution; b) 70% glycerol solution.

The measured values of the refractive index according to Eq. (3) were used to calculate the volume fractions of water in the studied solutions for five wavelengths of the visible and near IR regions. The data averaged over five measurements for skin samples and solutions of glucose and glycerol are shown in Table 1 and Table 2.

Fig. 2 shows the increase in the volume fraction of the extracted liquid in a 70% solution of glycerol and a 40% solution of glucose after 60 min of exposure to OCA on a sample of rat skin, calculated by Eq. (4).

In Fig. 2, it can be noted that 70% glycerol solution leads to greater dehydration of the skin compared to 40% glucose solution. The increase in the volume fraction of the extracted liquid in the 70% solution of glycerol was 8.11%, and in the 40% solution of the glucose solution – 2.02%. Due to its high hygroscopicity, glycerol is one of the most effective hyperosmotic OCAs, which confirms our result.

Table 1 Data for skin samples and OCA when exposed to 40% glucose solution.

| | Weight sample (mg) | Sample thickness (mm) | Sample length (mm) | Sample width (mm) | The volume of the OCA (ml) | Refractive index | λ (nm) | The volume fraction of water and extracted fluid in the OCA, % | The average volume fraction of water and extracted fluid in the OCA, % |
|---|---|---|---|---|---|---|---|---|---|
| Before exposure to OCA | 756±35 | 1.55±0.20 | 12.6±0.15 | 10.05±0.20 | 2 | 1.3947±0.0002<br>1.3898±0.0002<br>1.3865±0.0002<br>1.3826±0.0003<br>1.3740±0.0004 | 486<br>589<br>680<br>930<br>1300 | 60<br>60<br>60<br>60<br>60 | 60 |
| After 60 min exposure to OCA | 640±25 | 1.55±0.20 | 10.50±0.17 | 7.00±0.18 | 1.5* | 1.3913±0.0002<br>1.3870±0.0002<br>1.3840±0.0003<br>1.3796±0.0003<br>1.3718±0.0004 | 486<br>589<br>680<br>930<br>1300 | 62.39<br>62.01<br>61.80<br>62.15<br>61.74 | 62.02±0.27 |

* the volume of solution remaining on the sample after it was taken out of solution was not taken into account.

Table 2 Data for skin samples and OCA when exposed to 70% glycerol solution.

| | Weight sample (mg) | Sample thickness (mm) | Sample length (mm) | Sample width (mm) | The volume of the OCA (ml) | Refractive index | λ (nm) | The volume fraction of water and extracted fluid in the OCA, % | The average volume fraction of water and extracted fluid in the OCA, % |
|---|---|---|---|---|---|---|---|---|---|
| Before exposure to OCA | 805±41 | 1.55±0.20 | 12.20±0.20 | 10.50±0.23 | 2 | 1.4373±0.0002<br>1.4325±0.0002<br>1.4290±0.0002<br>1.4250±0.0003<br>1.4166±0.0003 | 486<br>589<br>680<br>930<br>1300 | 30<br>30<br>30<br>30<br>30 | 30 |
| After 60 min exposure to OCA | 530±34 | 1.55±0.20 | 10.00±0.24 | 8.10±0.21 | 1.7* | 1.4260±0.0002<br>1.4210±0.0003<br>1.4179±0.0002<br>1.4134±0.0003<br>1.4056±0.0004 | 486<br>589<br>680<br>930<br>1300 | 37.96<br>38.16<br>37.91<br>38.27<br>38.26 | 38.11±0.17 |

*the volume of solution remaining on the sample after it was taken out of solution was not taken into account.

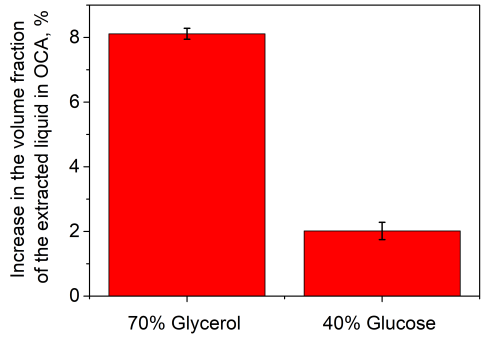


Fig. 2 The change in the volume fraction of the extracted liquid in a 70% solution of glycerol and a 40% solution of glucose after the optical clearing of the sample of rat skin.

Measurement of the refractive index at several wavelengths allowed us the calculation of the dispersion dependence of the refractive index of the extracted substance using Eq. (5) and a comparison with the dispersion of water. Dispersion dependencies for water, 70% glycerol, 40% glucose, and the extracted fluid after skin interaction with glucose and glycerol solutions are shown in Fig. 3.

Presented in Fig. 3, the dispersion curve for the fluid displaced from the skin after exposure to 40% glucose solution is close to the dispersion dependence for water, but larger values of the refractive index for all wavelengths except 486 nm indicate the presence of proteins and salts in the extract. After exposure to 70% glycerol solution, the dispersion is overestimated for all wavelengths in the visible region and coincides with the dispersion of water in the NIR region, which can also be explained by the displacement of intercellular fluid from the tissue, which includes not only water, but also proteins and salts. It can be assumed that the contribution of the protein component is somewhat higher when exposed to glycerol, since protein molecules have a high absorption in the UV region, which leads to a noticeable contribution of anomalous dispersion in UV and corresponding changes in the short-wave visible region due to the wings of the absorption bands.

Table 3 Water content in the glycerol solution after an *in vivo* experiment on clearing of rat skin.

| Sample | Volume fraction of glycerol, % | Volume fraction of water, % |
|---|---|---|
| Area over healthy tissue | 97.0±0.3 | 3.0±0.3 |
| Tumor area | 98.9±0.2 | 1.1±0.2 |
| Glycerol solution | 99.3 | 0.7 |

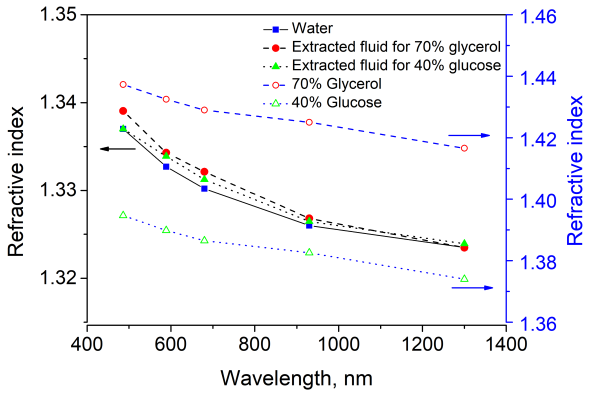


Fig. 3 Dispersion dependencies for water, 70% glycerol, 40% glucose and the isolated fluid after the skin interacts with glucose and glycerol solutions.

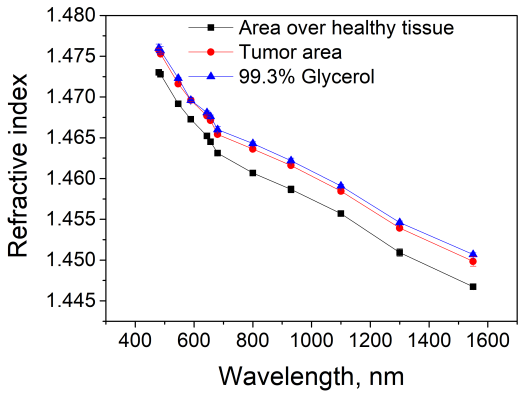


Fig. 4 Dispersion dependence of the refractive index of 99.3% glycerol solution and glycerol solutions after an in vivo experiment on skin on healthy tissue and on a tumor.

Based on the literature data and previously obtained results, the glycerol solution was chosen as an OCA for *in vivo* studies that performed a comparison of the degree of dehydration of tissue in a healthy skin area and in a skin area over a tumor. Based on the obtained values of the refractive index using the Eq. (3), the content of the volume fractions of glycerol and water in the solutions after the *in vivo* experiment was calculated. The obtained volume fractions were averaged for 12 wavelengths for each solution. The data obtained are shown in Figs. 4, 5 and Table 3.

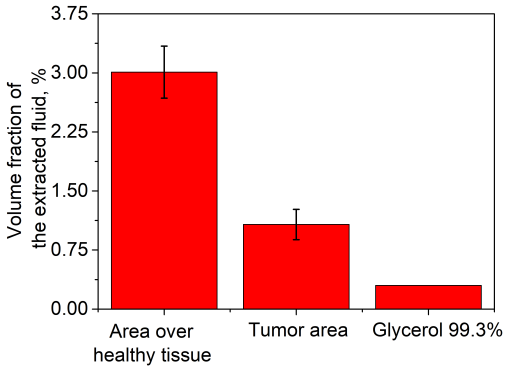


Fig. 5 The volume fraction of water in a glycerol solution after an in vivo experiment on skin areas over healthy tissue and on a tumor, calculated from the refractive index at 12 wavelengths, followed by averaging.

According to the results, the dehydration of a healthy rat skin area was 3.0 ± 0.3%, and for a rat skin area above a tumor it was 1.1 ± 0.2%. This result is generally consistent with the data from the Tromberg group [17], obtained on the basis of spectral measurements for areas of healthy tissue and carcinoma of the female breast, for which the volume of bulk water in healthy tissue was on average 15–16%, and in the tumor about 30%. This, in accordance with the results of [32, 33], means that with a taken concentration of glycerol solution with a water content of 30%, the flow of water extracted from the malignant tissue should be small, while for healthy tissue it is relatively high. Thus, the refractometric method allows one to quickly and simply perform an assessment of the dehydration of the skin in an *in vivo* experiment on optical clearing. The result obtained allows determination of a healthy skin area and a skin area above a tumor, which can be used to develop optical methods for determining the boundaries of a tumor. Also in Fig. 3, it can be noted that for the NIR, the region of the difference in the refractive indices of glycerol solutions after the *in vivo* experiment on areas without a tumor and with a tumor is most noticeable. The difference between the refractive indices of the initial glycerol solution and the refractive index of the glycerol solution after *in vivo* measurements on the skin over healthy tissue is 0.0029 for the visible region (480-680 nm) and 0.0036 for the NIR region (930–1550 nm), and over the tumor, these values are 0.0004 and 0.0007, respectively, can be attributed to the strong contribution of anomalous

dispersion due to water absorption in the NIR of the spectral region.

## 4 Conclusions

The method of refractometry allows one to quickly and easily to assess the degree of dehydration of tissue when exposed to OCA. Measurements at several wavelengths in the visible and NIR ranges allowed the possibilities of this method to be expanded, including for the analysis of the dispersion curves of the extracted interstitial fluid. The application of the method makes it possible to establish differences in the degree of dehydration for the skin of healthy tissue and a tumor. In the future, the multi-wavelength refractometric method for assessing the degree of dehydration of tissue can be applied to develop new rapid methods for diagnosing and monitoring oncological diseases and increasing the effectiveness of the methods currently used in practice.

## Disclosures

All authors declare that there is no conflict of interests in this paper.

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