===== **ARTICLES** =====

Determination of Lipids and Their Oxidation Products by IR Spectrometry

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Abstract—We proposed a procedure for the IR spectrometric determination of lipid hydroperoxides in biological systems. The main bands in the IR absorption spectra of linoleic acid and its hydroperoxide were identified, and analytical bands suitable for the determination of both compounds in their mixtures were selected. It was demonstrated that *tert*-butyl hydroperoxide can be used as an external standard for determining fatty acid hydroperoxides. Using the external standard method (calibration curve) for *tert*-butyl hydroperoxides, we calculated the concentration of linoleic acid hydroperoxide in its mixture with linoleic acid; it agreed with the specified values. Using the developed procedure, we estimated the concentration of hydroperoxide groups in natural cardiolipin. The results were compared to those obtained by an independent method (activated chemiluminescence).

Oxidative stress (OS)—an imbalance of pro-oxidants and antioxidants in favor of the former ones—is the cause of a number of diseases, for example, neurodegenerative diseases, metabolic syndrome, diabetes, etc. The degree of oxidative stress can be assessed by the concentration of participants or products, that is, OS markers [1]. Lipid hydroperoxides are of the most interest among the oxidation products. On the one hand, they are products of lipid oxidation and can serve markers of lipid oxidative stress and, on the other hand, actively participate in peroxidation in the chain branching reaction by reacting with ferrous ions [1] or the heme center of the cytochrome *c*–cardiolipin complex [2]. Thus, the analytical determination of hydroperoxides in biological systems is a relevant and practically important problem of medicine and biology.

Since the fatty-acid residue of linoleic acid is a main component of polyunsaturated lipids, such as cardiolipin, which is of great importance in the regulation and initiation of apoptosis [3, 4], linoleic acid was selected as a model compound for the development of a procedure.

Several methods are known for determining lipid oxidation products. Iodometric titration is applicable both to the evaluation of the peroxide index in edible oils [5] and to the determination of lipid hydroperoxides, in particular, linoleic acid hydroperoxide, in cell membranes. For real objects, titration is hindered because a large amount of sample is required for analysis. The voltammetric determination of peroxides was also described [6–8], but these procedures are not applicable in the online mode.

In recent years, the chromatographic separation of lipids and their oxidation products to peroxides has been used with the subsequent identification of these substances by mass spectrometry [4, 9]. However, this method has not yet received widespread use in lipidomics because of its complexity, difficulties with data processing, and high cost.

A method for determining linoleic acid hydroperoxide (LOOH) in blood serum (plasma) was described based on recording of chemiluminescence (CL) in the reaction of hydroperoxides with a system of microperoxidase–isoluminol (or luminol) [10]. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol from soybean and phosphatidylserine from bovine brain were used as lipids [10]. *tert*-Butyl peroxide was added to phosphatidylcholine as a standard peroxide compound. The level of lipid hydroperoxides in human plasma was measured by HPLC–CL [11]. Hydroperoxides from different classes were separated by reversed-phase HPLC, and chemiluminescence was detected by a postcolumn reaction with isoluminol and microperoxidase. Using this approach, the stability of eicosatetraenoic acid hydroperoxide (15(S)-HPETE, (5E, 8Z, 11Z, 13Z, 15S)-15-hydroperoxyicosa-5,8,11,13-tetraenoic acid) and conjugated dienes on storage was studied. The determination of

Table 1. Concentrations of reference solutions of *tert*-butyl hydroperoxide (*t*-BuOOH)

Volume of *t*-BuOOH solution, µL	Final concentration of *t*-BuOOH, mM
10	1.0
30	3.0
50	5.0
70	7.0
80	8.0
100	10.0

lipid hydroperoxides and low-density lipoproteins (LDL) in human plasma by HPLC with chemiluminescence detection was described in [12]. In all these cases, the chemiluminescence detection of hydroperoxide was used after the separation of lipids in the serum by HPLC; this required special equipment and ultimately made the analysis time-consuming, difficult, and expensive.

A method for the determination of polyunsaturated fatty acids and lipid hydroperoxides in human body from the lip surface by attenuated total reflection (ATR)−FTIR spectrometry was proposed in [13]. Interestingly, the determination of lipid hydroperoxide was carried out on-site noninvasively (atraumatically). IR spectrometry was described as a rapid method for determining hydroperoxides of oils [14, 15]. Calibration samples were prepared by adding *tert*-butyl hydroperoxide and certain amounts of oleic acid and water to a series of oils. Recently, various instrumental methods have been developed to replace iodometric titration. The authors of [14, 15] used Fourier-transform IR spectrometry for determining the peroxide index of various food oils. IR spectrometry was used as a rapid method for determining hydroperoxides in oils [14−17] in the food industry; however, this method was not used for the determination of lipid hydroperoxides in biological systems.

Thus, the most popular methods for determining peroxide compounds used nowadays are typically time-consuming and expensive; they either yield poorly reproducible results or require large amounts of samples. The procedures were developed, as a rule, for determining the sum of lipid oxidation products in edible oils and are not suited for biological objects. Thus, the development of new procedures combining rapidity, sensitivity, and accuracy of data is an urgent task.

EXPERIMENTAL

Equipment and reagents. The work was performed using a Cary 630 FTIR spectrometer (Agilent, United States) with the MicroLab PC software. To assess the data, the OPUS 6.5 software was used.

The following reagents were used: linoleic acid (LA) (Sigma, United States, $M = 280$ g/mol, $d = 0.9$ g/cm^3); linoleic acid hydroperoxide (LOOH) ($M = 298$ g/mol); cytochrome *c* from horse heart (99%, Sigma, United States, $M = 12\,400$ g/mol) for biochemical research; 1,1',2,2'-tetraoleoyl cardiolipin (TOCL) (sodium salt, Avanti, United States); cardiolipin from bovine heart (BCL) (sodium salt, Sigma, United States); *tert*-butyl hydroperoxide (*t*-BuOOH) (MP, France); iron sulfate (FeSO$_4$, cp grade); coumarin C-525 (Al'fa Akonis); methanol (high-purity grade); Tris-HCl; and chloroform (cp grade).

Preparation of solutions. Aliquot portions were sampled using a set of Eppendorf micropipettes with the volumes 0.5−10, 2−20, 10−100, 20−200, and 100−1000 µL with an accuracy of ±0.05 µL. Tubes with solutions were stirred, if necessary, in a Vortex shaker at a rate of 1000 rpm. A Kern analytical balance (Switzerland) with an accuracy of 0.1 mg was used for weighing.

A stock 200 mM solution of linoleic acid was prepared by adding 62.9 µL of an individual acid to 1 mL of chloroform. Working solutions were prepared by serial dilutions of the stock solution with chloroform. Working solutions of linoleic acid hydroperoxide were prepared by two- to tenfold dilutions of a stock 50 mM solution with chloroform.

Recording of spectra. In recording IR spectra, the sample volume was approximately 50 µL. In using a Diamond ATR accessory, the sample was a linoleic acid hydroperoxide film. It was prepared as follows: a drop of a test solution was applied onto a substrate surface of the device with a zinc selenide crystal in the center. After the evaporation of the solvent for approximately 1 min, a dry film of the sample remained on the crystal. If necessary, an additional amount of sample was applied. The sample was pressed from the top by the second crystal, and a spectrum was recorded. In recording spectra of liquid solutions using a DialPath accessory, a drop of sample was spotted onto the substrate crystal, and spectra were recorded without drying the sample.

Determination of lipid hydroperoxides by chemiluminescence. Reagents are listed in the order of their addition to a cuvette of the chemiluminometer. Ten microliters of a 10 mM FeSO$_4$ solution was placed in the cuvette (final concentration, 100 µM), and chemiluminescence was recorded for 1 min. Next, a mixture of *tert*-butyl hydroperoxide of different concentration and of a required amount of a 20 mM Tris−HCl buffer solution (pH 7.4) was added (volumes and final concentrations of the solution are given in Table 1). The volume of the system was always 1000 µL. The resulting solution was stirred, sampled into a 2-mL syringe, and injected into a chemiluminescence cell. Chemiluminescence was recorded for 5 min. A quick flash in the chemiluminescence curve was obtained; its amplitude was used as an analytical signal. A calibration
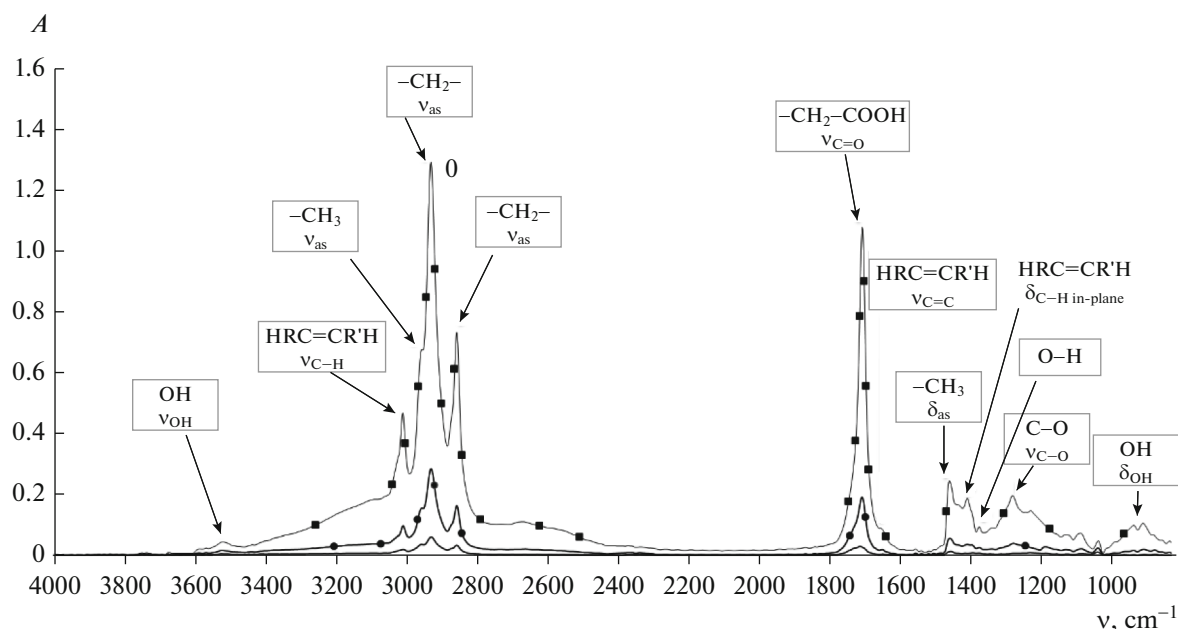
Fig. 1. An IR absorption spectrum of pure linoleic acid; optical path length, 100 μm; number of scans, 8; background, air; c_{LA}, mM: (■) 200, (·) 50, and (—) 10.

dependence of the quick flash amplitude on the concentration of *tert*-butyl hydroperoxide was plotted.

RESULTS AND DISCUSSION

Determination of linoleic acid by IR spectrometry. Linoleic acid is a monobasic carboxylic acid with two isolated double bonds. The residue of linoleic acid is a part of cardiolipin mitochondrial phospholipid.

Spectra of pure linoleic acid and its solutions in chloroform are presented in Fig. 1. The functional groups and types of vibrations are indicated for the most intense bands.

To construct calibration curves, we selected the most intense absorption bands: 2975–2950 cm^{-1} (asymmetric stretching vibrations of −CH$_3$), 2940–2915 cm^{-1} (asymmetric stretching vibrations of −CH$_2$−), 2870–2860 cm^{-1} (symmetric stretching vibrations of −CH$_2$−), and 1715–1680 cm^{-1} (stretching vibrations of C=O), which did not overlap with the most intense absorption bands of chloroform. The equations of calibration curves for the selected analytical bands and performance characteristics are listed in Table 2.
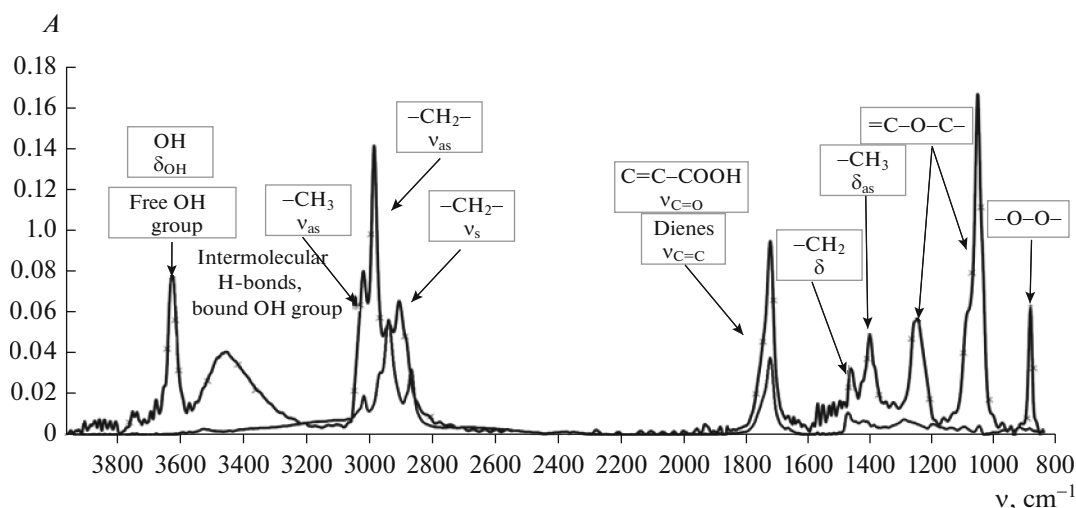
Determination of linoleic acid hydroperoxide by IR spectrometry. Linoleic acid hydroperoxide is soluble in many solvents, including dimethyl sulfoxide, methanol, and chloroform. An IR absorption spectrum of linoleic acid hydroperoxide is demonstrated in Fig. 2 in comparison with the spectrum of linoleic acid; the main absorption bands are indicated. The following absorption bands of linoleic acid hydroperoxide were selected as analytical: 3620 cm^{-1} (stretching vibrations of the free OH group), 3575–3200 cm^{-1} (vibrations of intermolecular hydrogen bond), and 2973 cm^{-1} (asymmetric stretching vibrations of −CH$_2$−); the band at 879 cm^{-1} (stretching vibrations of the −O−O− fragment) can supposedly serve as a marker of hydroperoxide. These bands also do not overlap with the main absorption bands of chloroform.

Spectra of linoleic acid hydroperoxide solutions of different concentrations in chloroform are presented in Fig. 3. The peak in the region of 3600–3230 cm^{-1} is asymmetric; therefore, a calibration dependence was constructed based on the area under the peak. Peaks were integrated using the OPUS software. Integration was performed from 3600 to 3230 cm^{-1}. The calibra-

Table 2. Performance characteristics of the determination of linoleic acid ($n = 5$, $P = 0.95$)

| Absorption band, cm^{-1}	Regression equation	r	LOD, mM	RSD, %
2930	$A = (0.0065 \pm 0.0003)c + (0.0161 \pm 0.0009)$	0.999	2.7	6
2854	$A = (0.0055 \pm 0.0004)c + (0.049 \pm 0.004)$	0.999	10.1	7
1711	$A = (0.0034 \pm 0.0003)c + (0.0070 \pm 0.0005)$	0.999	2.9	7

Fig. 2. IR spectra of 50 mM linoleic acid and 25 mM linolenic acid hydroperoxide; optical path length, 100 μm; number of scans, 8; background, chloroform; (−×−) 25 mM LOOH, (—) 50 mM LA.

tion curve equations and performance characteristics are listed in Table 3.

Study of *tert*-butyl hydroperoxide as a reference compound. We studied a possibility of using *tert*-butyl hydroperoxide as a standard compound under the selected conditions. Infrared spectra of a series of *tert*-butyl hydroperoxide solutions of different concentrations in chloroform are shown in Fig. 4. The calibration dependence of absorbance on the concentration of *tert*-butyl hydroperoxide was built using absorption

bands corresponding to vibrations of the peroxide group. As in the case of linoleic acid hydroperoxide, the peak in the range of 3480–3180 cm^{-1} was asymmetric, so it was advisable to plot the calibration dependence using the area under the peak. The equations of calibration curves and performance characteristics are presented below (Table 4). Thus, *tert*-butyl hydroperoxide can be used as a standard substance in the determination of lipid hydroperoxides under the selected conditions.
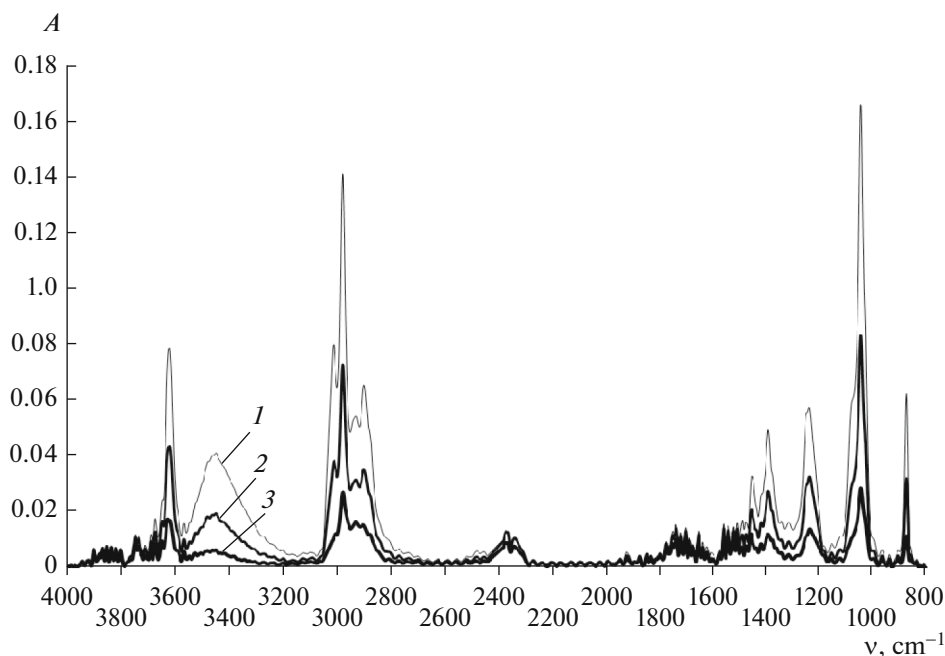
Fig. 3. An IR absorption spectrum of linoleic acid hydroperoxide of at different concentrations; c_{LOOH}, mM: (*1*) 25.0, (*2*) 12.5, and (*3*) 5.0.

Table 3. Performance characteristics of the determination of linoleic acid hydroperoxide at different concentrations ($n = 5$, $P = 0.95$)

Absorption band, cm^{-1}	Regression equation	r	LOD, mM	RSD, %
3627	$A = (0.0028 \pm 0.0003)c - (0.004 \pm 0.0005)$	0.999	1.6	7
3454	$S_A = (0.29 \pm 0.03)c - (0.49 \pm 0.04)$	0.999	2.2	6
880	$A = (0.0024 \pm 0.0002)c - (0.0011 \pm 0.0001)$	0.999	1.5	6

**Table 4.** Performance characteristics of the determination of *tert*-butyl hydroperoxide ($n = 7$, $P = 0.95$)

Absorption band, cm^{-1}	Regression equation	r	LOD, mM	RSD, %
3490–3070	$S_A = (160 \pm 20)c - (28 \pm 3)$	0.998	0.17	7
880	$A = (0.579 \pm 0.006)c - (0.0508 \pm 0.005)$	0.989	0.08	6

Analysis of natural and synthetic cardiolipin. Residues of linoleic acid are present in cardiolipin, a mitochondrial phospholipid. Their oxidation, according to an available hypothesis, proceeds in the initiation of apoptosis; therefore, the determination of hydroperoxide groups in cardiolipin is of interest for the study of the mechanism of this process. Natural cardiolipin (in this case, BCL), bearing residues of linoleic and linolenic acids, is easily oxidized, while synthetic TOCL containing oleic acid residues is not oxidized. It was interesting to test the developed procedure for both types of cardiolipin and to determine the numbers of hydroperoxide groups in natural cardiolipin and TOCL.

Infrared spectra of TOCL are presented in Fig. 5; the main bands were assigned. The spectrum does not contain bands of peroxide groups, and, as was expected, almost all bands observed for linoleic acid appeared in the spectrum, i.e., 2977 cm^{-1} (asymmetric stretching vibrations of $-CH_3$), 2920 cm^{-1} (asymmetric stretching vibrations od $-CH_2-$), 2859 cm^{-1} (symmetric stretching vibrations of $-CH_2-$ and symmetric stretching vibrations of $-CH_3$), and 1730 cm^{-1} (stretching vibrations of C=O) (Fig. 5). Furthermore, vibrations of the phosphate moiety appeared in the regions of 1230 and 1050 cm^{-1}.

Figure 6 demonstrates a spectrum of BCL as compared to the spectrum of TOCL, in which vibrations of
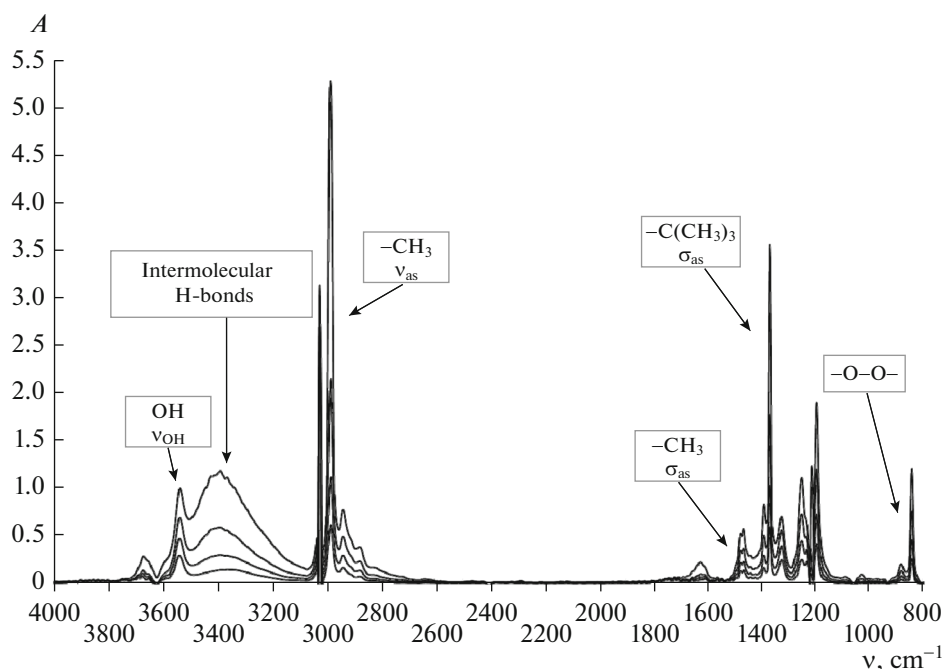
Fig. 4. IR absorption spectra of extracts of *tert*-butyl hydroperoxide at different concentrations; optical path length, 100 μm; number of scans, 8; background, chloroform.
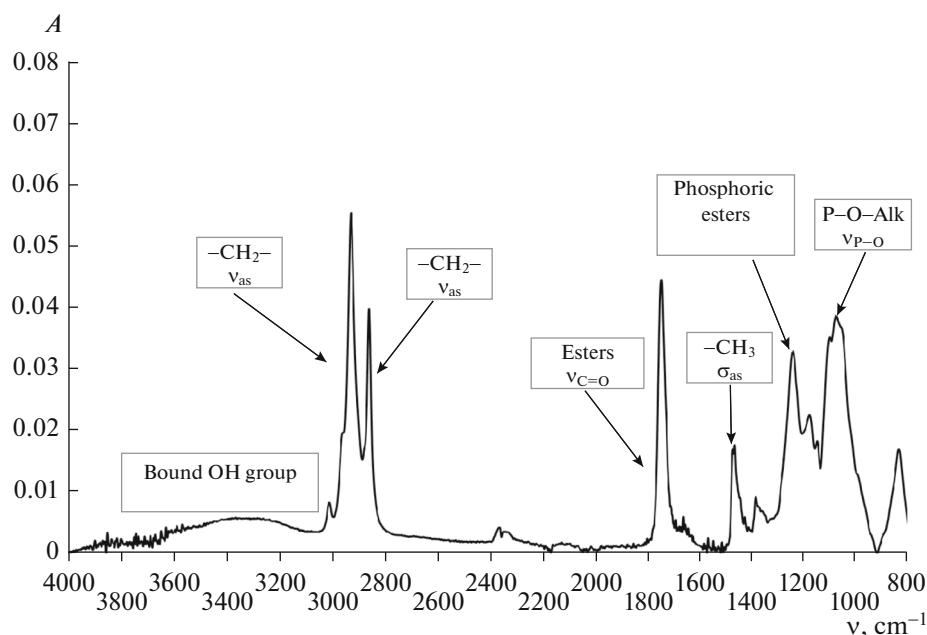
Fig. 5. IR spectra of 1,1',2,2'-tetraoleoyl cardiolipin; optical path length, 100 μm; number of scans, 8; background, chloroform.

the peroxide group appeared in the range of 3600–3100 cm^{-1}. Using a calibration curve for the area under the peak versus the concentration of *tert*-butyl hydroperoxide, we estimated the concentration of hydroperoxide groups in bovine cardiolipin, which was equal to (8.8 ± 0.2) mM.

To verify the accuracy of the results, we determined lipid hydroperoxides in bovine cardiolipin by activated chemiluminescence. A calibration equation was obtained and performance characteristics were evaluated: $I_{CL} = (0.94 \pm 0.09)c$ (mM) ($P = 0.95$, $n = 5$); $r = 0.99$; the detection limit was 6.2 mM. The concentration of lipid peroxides found by the method of activated chemiluminescence was $c_{CL} = 8.87 \pm 0.03$ mM.
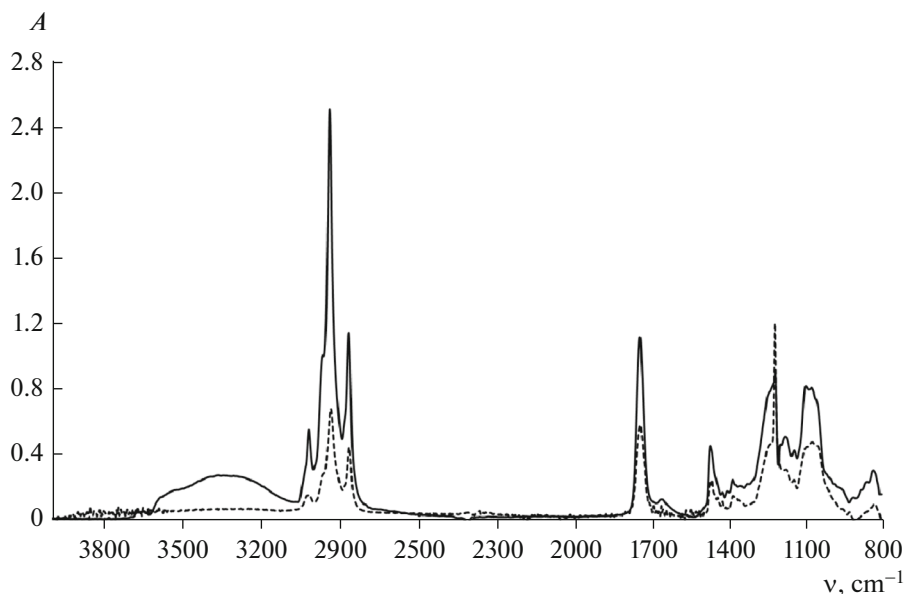

Fig. 6. Comparison of IR spectra of 1,1',2,2'-tetraoleoyl cardiolipin (TOCL) and bovine cardiolipin (BCL): (– – –) TOCL, (—) BKL.

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