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Comparison of optoacoustic and photothermal-lens determination of lipopolysaccharides

Mikhail A. Proskurnin[a,*], Elizaveta V. Ageeva[a], Vera V. Senyuta[a,b], Nataliya V. Orlova[a], Andrei V. Fokin[b], Oleg B. Ovchinnikov[b], Sergei V. Egerev[b]

[a] Chemistry Department, M.V. Lomonosov Moscow State University, Vorob'evy Hills d. 1, str. 3, 119991 GSP-1 Moscow, Russia
[b] N.N. Andreev Acoustics Institute, ul. Shvernika, d. 4, 117036 Moscow, Russia

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ABSTRACT

As a primary aim, several approaches to photothermal (thermal-lens) and optoacoustic determination of exogenous pyrogens (lipopolysaccharides) based on photometric procedures for their determination at the microgram level and below are compared. The limits of detection of lipopolysaccharides by thermal-lens spectrometry and optoacoustic spectroscopy are at a level of 2–100 ng/mL, and the conditions of optoacoustic and photothermal procedures are the same. Optoacoustic spectroscopy is advantageous in determining suspensions, while thermal lensing is superior in determining lipopolysaccharides from homogenous aqueous solutions. As a secondary aim, photometric procedures for lipopolysaccharides by the formation of their ion pairs with several dyes and by the reaction of 2-keto-3-deoxyoctulosonic acid as a part of a lipopolysaccharide molecule with thiobarbituric acid are optimized. In the case of the 2-keto-3-deoxyoctulosonic acid reaction, the sampling stage time is decreased twofold, and the possibility of substitution of the toxic metaarsenite for sulfite with better sensitivity is shown.

1. Introduction

Optoacoustic (OA) and photothermal (PT) spectroscopies are actively used in chemistry as most sensitive techniques of molecular absorption spectroscopy [1,2]. Recently, the progress in laser technologies has provided a sound basis for compact optical schemes and commercial applications of these methods [3–5]. However, despite a vigorous discussion of their advantages in chemical analysis, their joint use is still rather limited, although the methods, having many similarities, are complementary rather than competing. This situation is characteristic for analytical chemistry because OA/PT spectroscopies show spectral nonselectivity.

Apart from technical reasons, there seems to be a problem of methodology: contrary to PT spectroscopy, OA measurements are not very often used in chemical analysis of condensed samples, and the methodology of OA analytical measurements are not so developed as in photothermal spectroscopy [2,6]. Photometric determination can be substantially improved using PT spectroscopy [1]. As the most widespread PT method, thermal-lens spectrometry (TLS) provides the determination of various substances at the submicrogram level [1,2]. To the contrary, signal processing and data handing are more advanced in optoacoustics, and the joint studies involving both methods can provide a new quality of analysis and a mutual enhancement.

Previously, we showed that the use of TLS enhances the sensitivity of the determination of lipopolysaccharides (LPS) as the most infamous class of pyrogenic substances because it combines high instrumental sensitivity and the selectivity of chemical methods for LPS for their determination at the submicrogram level [7,8] and also calculated figures of merit for OA determination of LPS showing possible high sensitivity of such an application [9,10]. Hence, LPS seem to be a very good candidate for a comparison study of OA/PT spectroscopies. Thus, the aim of this work was to optimize the conditions for OA/PT determination of LPS by applying several photometric-reagent systems for sensitivity enhancement to the submicrogram level and to compare both methods from the viewpoint of sensitivity and methodology.

We selected three possible approaches to the determination of LPS, which can be distinguished by the size of the test molecules. The first approach is based on the decomposition of an LPS molecule to the three key parts: a polysaccharide, a lipid, and 2-keto-3-deoxyoctulosonic acid (KDO) following by the reaction with one of these parts [11–13]. In this study, we selected KDO as a characteristic part of LPS core only, which provides sensitive determination. The second approach is based on the fact that OA/PT signals depend on the existence of heterogeneity in the samples [14–17]. Large LPS molecules make it possible to see their contribution to OA/PT signals directly, without adding any chemical reagents to the test

* Corresponding author. Tel.: +7 4959393514; fax: +7 4959394675.
E-mail address: Michael@analyt.chem.msu.ru (M.A. Proskurnin).

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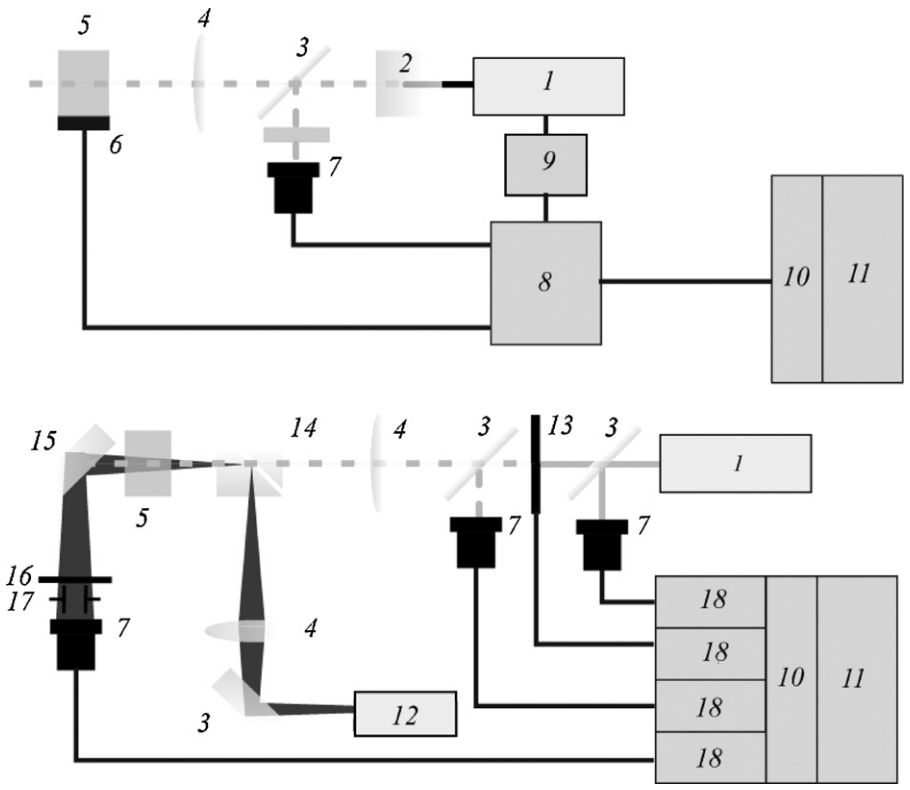

Fig. 1. The schematics of (a) optoacoustic spectrometer and (b) coaxial dual-beam thermal-lens spectrometer: *1*, excitation laser; *2*, frequency doubler; *3*, mirror; *4*, focusing quartz lens; *5*, sample quvette; *6*, piezotransducer; *7*, an L-3DP3C Panasonic photodiode; *8*, digital oscilloscope; *9*, pulse generator; *10*, synchronization interface; *11*, a PC; *12*, probe laser; *13*, a mechanical chopper; *14*, a Glan prism; *15*, a ZR-88 type dichroic mirror; *16*, a passband filter; *17*, a pinhole; *18*, an analog signal amplifier and current-to-voltage converter.

samples. Finally, the third approach combines the chemical modification of the first approach while keeping the heterogeneity of the test sample, advantageous for OA/PT measurements, of the second approach. It is ion-pair formation of LPS molecules with dyes [18]. In this study, we used the reactions with the polysaccharide part of LPS molecule with cationic dyes of quinaldine and triphenylmethane series due to their sensitivity [19–21].

2. Experimental

2.1. Apparatus

2.1.1. Optoacoustic setup

Setup schematics is shown in Fig. 1a. A Nd^{3+}-YAG laser of the LTI type (Polyus Productions, Moscow, Russia) was used for the excitation of the sample in an optoacoustic Brodnikovsky-type cuvette cell (radius, 1 cm; Fig. 2) [17]. The second harmonics, λ_e = 532 nm, was selected to bring together the conditions of OA and PT experiments. The laser pulse energy was within the range 10–15 mJ and the pulse repetition rate was 1 Hz. The laser beam passed through the center of the cuvette, thus forming a pencil-shaped thermoacoustic array. The distance from the array to a PZT-19 piezoelectric transducer (Russia) was 15 mm (sensitivity, 20 μV/Pa; frequency band, 1 MHz). The energy was measured with a photodiode pre-calibrated in the necessary energy range with an IMO-2M laser power/energy meter (Russia). The signal was registered with a C9-8 digital oscilloscope (Russia), the energy measurements were synchronized with the signal measurements with the same oscilloscope using a G5-60 pulse generator (Russia). Generated pulse parameters are 5 V, 20 μs, 1 Hz.

2.1.2. Thermal-lens setup

A dual-laser coaxial-beam thermal-lens spectrometer with a single-channel detection system is used Fig. 1b [22]. It is set up with a coaxial dual-beam optical configuration. An excitation Ar^+ laser (Innova 90-6, Coherent, Palo Alto, CA 94303, USA) is used. A change in intensity in the center of the He–Ne probe laser beam (SP 106-1, Eugene, OR 97402, USA) is measured with a single L-3DP3C photodiode detector. The selection of the parameters of the instrument (linear dynamic range, optical-scheme design, instrumental sensitivity, etc.) is discussed elsewhere [22,23]. The data are gathered and handled online by the software written in-house [22]. The

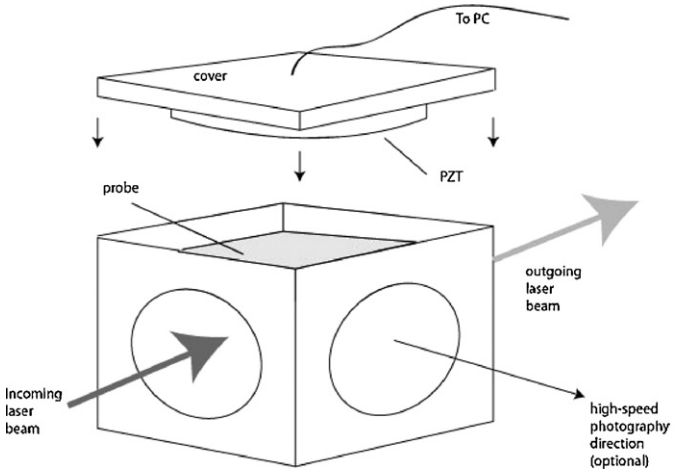

Fig. 2. The optoacoustic cuvette cell. See text for details.

Table 1
The main performance parameters for the thermal-lens and optoacoustic procedures of determination of LPS, photothermal experiments: excitation laser 514.5 nm, laser power varies, see below; optoacoustic measurements: excitation laser 532 nm ($n = 20$; $P = 0.95$).

Reagent	Spectrophotometry	Thermal-lens measurements						Optoacoustic measurements		
	LOD, ng/mL	P_e, mW	Φ_e, mJ	E_{theor}, Eq. (5)	E_{exp}	LOD, ng/mL	LOQ, ng/mL	Φ_e, mJ	LOD, ng/mL	LOQ, ng/mL
Thiobarbituric acid	600	45	18	7.0	6.5 ± 0.3	70	200	15	300	1000
Reagentless measurements	n/a	250	100	n/a	n/a	300	n/a	15	2[a]	n/a
Methylene Blue	200	95	20	4.8	4.77 ± 0.08	60	100	n/a	n/a	n/a
Methylthymol Blue	200	95	20	5.0	5.0 ± 0.1	40	100	15	4	10
Pinocyanol chloride[b]	200	100	20	13.5	13.3 ± 0.3	30	100	15	4	20

[a] For integrated OA measurements.
[b] 488.0 nm.

power of the excitation laser was selected so the absorbed energy Φ_e was the same as in OA measurements. Except for Pinocyanol chloride, the working wavelength was 514.5 nm as the nearest to the maxima of the absorption bands of the reaction products.

2.1.3. Spectrophotometric and other auxiliary measurements

Spectrophotometric measurements (200–900 nm) were made with a UVmini 1240CE spectrophotometer (Shimadzu, Japan). Quartz photometric cells with the optical pathlength of 10 mm (sample volume 2.5 mL) were used throughout. The temperature of the sample cell was kept at 20.0 °C with the precision of ±0.1 °C using a U15 MWL thermostat (Poland). A Wirowka MPW-2 centrifuge (Poland, 8000 rpm) was used. Dissolution and disaggregation of lipopolysaccharides and degasification of prepared solutions were made in a Branson ultrasonic bath (1 W, 30–60 min).

2.2. Data treatment

2.2.1. Optoacoustic measurements

Amplitude optoacoustic signal A_p (pressure, in Pa) is connected with the molar concentration c_M with the equation [17]:

$$A_p = 2.303 r^{-1/2} \left(\frac{\Phi_e}{\omega_{0e}^{3/2}} \right) \frac{\beta v_s^2}{4\pi C_P} \varepsilon c_M. \quad (1)$$

Here, r is the distance from the beam axis to the detection point, Φ_e is the excitation pulse energy, ω_{0e} is the radius of the excitation beam waist, β is thermal expansion coefficient; v_s is the speed of sound, C_P is isobaric heat capacity, ε is molar absorptivity, and c_M is molar concentration. The signal was analyzed within the band of up to 250 kHz (a lowpass 5th order Butterworth digital filter with a corresponding cut-off frequency). OA signal measurements were accumulated in series of 50–500 readings.

To increase the sensitivity for low OA signals, we also used cumulative OA signals [9], which were calculated as the total signal A_p accumulated at the detector at the moment t:

$$\bar{A}_p(t) = \int_0^t A_p(\tau)\, d\tau \quad (2)$$

where τ is the integration variable.

2.2.2. Thermal-lens measurements

Thermal-lens signal ϑ was determined as a relative change in the intensity of the probe beam at a far-field detector plane [2] and was calculated according to [1] as

$$\vartheta = 2.303 B l \left(\frac{\Phi_e}{\omega_{0e}^2} \right) E_0 D_T \varepsilon c_M \quad (3)$$

where B is the geometry factor, l is optical pathlength, E_0 is thermal-lens enhancement factor, and D_T is thermal diffusivity [2]. The thermal-lens enhancement factor for the excitation-laser power of 1 mW was calculated from

$$E_0 = \frac{-dn/dT}{\lambda_p k}. \quad (4)$$

Here, dn/dT is temperature coefficient of the refractive index, λ_p is the probe laser wavelength, and k is thermal conductivity of the medium [2]. The theoretical increase in the sensitivity for thermal lensing (Table 1) was calculated as a ratio of expected sensitivities for thermal lensing and spectrophotometry:

$$E_{theor} = 2.303 P_e E_0 \varepsilon_{phot} \varepsilon_{tls}^{-1}, \quad (5)$$

where P_e is excitation power, and ε_{phot} and ε_{tls} are molar absorptivities at wavelengths used for spectrophotometric measurements and thermal lensing, respectively. The experimental value of the enhancement factor, E_{exp} (Table 1) was calculated as a ratio of corresponding calibration slopes for thermal-lens and spectrophotometric procedures.

The limits of detection (LOD) were calculated as 3σ—according to the IUPAC 1998 recommendations for the presentation of the results of chemical analysis. Absolute limits of detection of LPS by thermal lensing were calculated using the signal-generating volume of thermal-lens as a cylinder with the cross-section of the diameter of the thermal-lens (equal to 1.05 mm under these conditions) and the height l, which is equal to 40 μL [24].

2.3. Reagents and solvents

All the used reagents and solvents were at least of analytical grade; unless otherwise stated, they were from Reakhim (Moscow, Russia). Doubly distilled deionized water was used (specific resistance not less than 18.2 MΩ cm, thermal-lens signal $(2 \pm 3) \times 10^{-4}$). The following solutions were used throughout: 0.1 and 0.06 M sulfuric acid, 0.5 M hydrochloric acid, a 2.6% (w/v) solution of sodium metaarsenite in 0.5 M hydrochloric acid, 0.04 M sodium sulfite in water, 0.04 M periodic acid in 0.06 M sulfuric acid, 0.6% (w/v) solution of thiobarbituric acid, sodium hydroxide, 0.2 M sodium carbonate, cp Methylthymol Blue dye (Chemapol, Czech Republic), cp Methylene Blue, cp Pinocyanol chloride (ICN, USA), and cp the 2-keto-3-deoxyoctulosonic acid from Sigma–Aldrich (Germany). Bacterial cultures of lipopolysaccharides from *Salmonella typhi 110* and *E. coli* were produced by the N.F. Gamaleya Plant of Biological Preparations (Moscow, Russia). All the working solutions were prepared in doubly distilled water daily before the experiments. All the glassware, OA and thermal-lens cells were kept in the conc. nitric acid and washed out with doubly distilled pyrogen-free water.

2.4. Procedures

2.4.1. Procedure 1. LPS determination by the reaction of KDO with thiobarbituric acid

A 1-mL portion of 0.1 M sulfuric acid is added into a test-tube with 0.025–0.125 mg/mL of LPS (or 0.6–6 μM of free KDO). The mix-

ture is heated at 90 °C for 30 min for the KDO release from LPS, cooled to room temperature, and centrifuged at 8000 rpm for 5 min. Next, 0.5 mL of the centrifugate is transferred to another test-tube and 0.2 mL of 0.04 M periodic acid in 0.06 M sulfuric acid is added, and the mixture is left for 20 min. Next, 1 mL of 0.04 M sodium sulfite (or sodium metaarsenite in 0.5 M hydrochloric acid) is added to eliminate unreacted periodate, and the mixture is kept until the total disappearance of the brown color of aqueous iodine. Next, 0.5 mL of 0.6% (w/v) thiobarbituric acid is added, and the mixture is boiled for 5–7 min until a pink color appears. Next, 1 mL of DMSO is added to the hot solution to stabilize the color, the solution is stirred and cooled to room temperature. The absorbance (at 550 nm) or thermal-lens signal (514.5 nm, 95 mW) are measured against a blank solution (prepared as described above by adding HIO_4 to 1 mL of distilled water). Optoacoustic signal is measured at 532 nm (pulse energy 15 mJ), with the number of readings of 100.

2.4.2. Procedure 2. Reagentless OA/PT determination of LPS

A 3-mL aliquot of the test sample is placed into a photometric or optoacoustic cell. The thermal-lens signal (514.5 nm, P_e = 250 mW) is measured in series of 1000–2000 readings at a rate of the ADC board (27.5 kHz). Optoacoustic signal was measured at 532 nm (pulse energy 15 mJ), with the number of readings of 300. In the presence of an inert colorant (ferroin, 1.3 μM, 0.5 mL aliquot) thermal-lens measurement conditions do not change, while the number of readings in OA measurements is decreased to 100, other conditions being the same as in TLS.

2.4.3. Procedure 3. LPS determination by ion-pair formation

LPS solutions are prepared in doubly distilled water or sodium carbonate (pH 11, for Methylthymol Blue) and disaggregated in an ultrasonic bath (1 W) for 10 min. A working solution, 20–100 nM (aliquot of 1.0 mL), is placed into a test-tube, and 0.2 mL of a Methylene Blue, Methylthymol Blue or Pinocyanol chloride solution with the concentration of 40 μM (spectrophotometry) or 30 μM (thermal lensing) is added. The solution volume is adjusted to 2 mL with doubly distilled water or 0.2 M sodium carbonate (pH 11, for Methylthymol Blue). Absorbance is measured at 560, 585, or 490 nm, respectively (the absorption-band maxima of the formed ion pairs) against a reagent blank. Thermal-lens signals are measured at 514.5 nm for Methylene Blue and Methylthymol Blue (excitation power of 95 mW) or at 488.0 nm (excitation power of 100 mW) for Pinocyanol chloride. The blank solution is made in the same way, except a lipopolysaccharide solution is not added. Optoacoustic signal is measured at 532 nm (pulse energy 15 mJ), with the number of readings of 100, other conditions being the same as in TLS.

3. Results and discussion

3.1. LPS determination by the KDO reaction with thiobarbituric acid

2-Keto-3-deoxyoctulosonic acid is a key part of an LPS as it belongs to the inner part (core) of its structure and mediates between the lipid and polysaccharide fragments [11,13]. The determination of LPS by KDO is based on a soft acidic hydrolysis that leads to the decomposition of LPS into two parts: a water-insoluble lipid and a water-soluble polysaccharide with two KDO molecules [13]. The lipid part can be precipitated by centrifugation. The interaction scheme is the following [13]. Adding periodic acid to the mixture leads to the oxidation of KDO to formylpyruvic acid. The excess periodate is reduced (usually with arsenite [13,25]). Finally, thiobarbituric acid is added to the reaction mixture, and a chromophore substance of an unknown structure with the primary absorption maximum at 550 nm is produced [13]. Previously, we succeeded in the optimization of the original spectrophotometric conditions for this reaction and showed the possibility of TLS determination of LPS [7]. However, for a number of reasons, the conditions for LPS determination cannot be considered optimum yet. Hence, in this paper, apart from comparing OA/PT measurements, we also paid our attention to more in-depth optimization of this experimental routine.

3.1.1. General optimization of the procedure

The procedure [11,13] is characterized by a time- and labor-consuming sample preparation (3 h). It includes boiling of the endotoxin sample with sulfuric acid (the soft acidic hydrolysis to free the KDO molecule from the LPS), centrifuging the mixture, keeping it for 20 min after adding periodic acid and the reductant, and boiling again with thiobarbituric acid to form the colored product. We shortened the stage of sample preparation considerably.

Firstly, no precipitation of the lipid fraction from LPS for working concentrations was observed. Inasmuch as low LPS concentrations are considered (which is the case of OA/PT measurements), we supposed that lipid residue does not interfere with the reaction of KDO. Thus, we excluded the centrifugation stage, which not only shortened the analysis time but diminished the loss of KDO that may co-precipitate or be absorbed with the separated lipid fraction and, thus, increased the overall sensitivity. Secondly, due to the trace concentrations of LPS and, thus, KDO, we reduced the time of the reaction of KDO with periodic acid. It was found that the reaction time in the range 1–20 min affects the absorbance of reaction products only slightly. Thus, the stage time of 1 min was selected. Thirdly, at the final stage, we reduced the time of boiling the reaction mixture with thiobarbituric acid, taking into account the kinetics of the reaction (a stable intense pink color of the products). As a whole, the time for sample preparation was decreased from 3 to 1.5 h.

3.1.2. Reductant selection and periodate–sulfite ratio

The reductant in this reaction scheme is needed for removing the surplus periodic acid only and does not interact with KDO. Usually, it is highly toxic and expensive sodium metaarsenite [13]. Moreover, high reagent concentrations lead to a high blank signal that degrades the sensitivity of the procedure. Thus, we tested several well-known reducing agents used in organic synthesis for removing surplus oxidants like formaldehyde and sodium sulfite [26]. Using formaldehyde was proved inexpedient as it affects the reaction products. To the contrary, sulfite reduces periodic acid to iodide and does not produce any other colored substances in the visible region. The absorption spectra (Fig. 3) for the KDO–thiobarbituric acid reaction by *Procedure 1* and for metaarsenite [13,25] show that reaction products for both reductants are the same, and sulfite increases the absorbance under the same conditions compared to metaarsenite. Thus, using sulfite in LPS determination by the KDO reaction makes this method cheaper and less hazardous.

Previously recommended ratio [13,25] for periodic acid (0.04 M):reducing agent (0.04 M) = 1:1 is not optimum as periodic acid is not completely reduced under these conditions, which manifests itself in a weak brownish color of iodine and the appearance of yellowish opalescent flakes after adding thiobarbituric acid (it is oxidized with the remaining periodate). The optimum ratio of periodic acid and sulfite was found by the measurement of light scattering of the final solution and the absorption spectra of the colored product. It was found that the ratio of 1:5 provides negligible interference from all of the byproducts.

The colored product has two absorption bands with maxima at 450 and 550 nm (Fig. 3). The peak at 550 nm is characterized by a lower reagent blank and higher calibration slope. An increase in the amount of periodic acid increases the peak height at 550 nm and decreases the peak at 450 nm (see the inset for Fig. 3). The

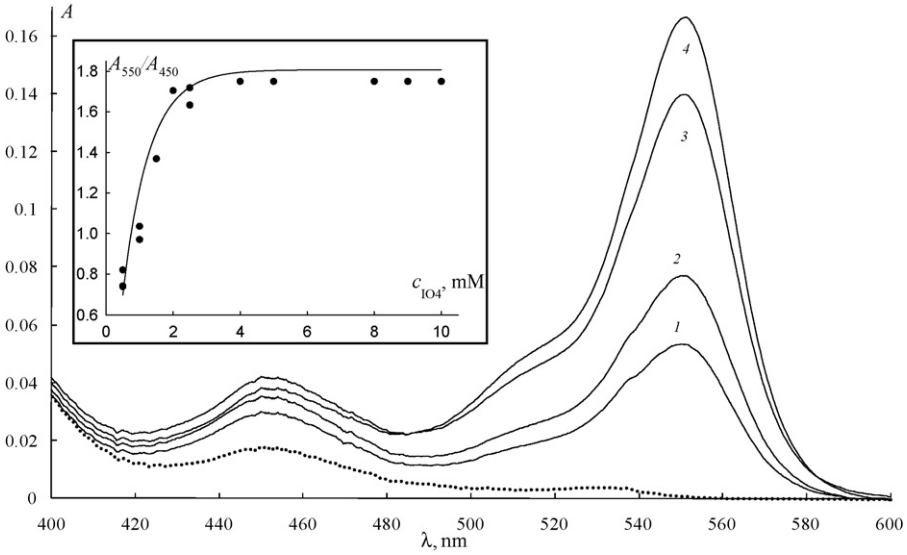

Fig. 3. Absorption spectra of the products of the reaction of KDO with thiobarbituric acid for the concentrations of KDO of: dashed line, reagent blank; *1*, 2.5 μM; *2*, 3.8 μM; *3*, 5.1 μM; *4*, 6.4 μM. Other concentrations are HIO_4, 2 mM, Na_2SO_3, 9 mM; thiobarbituric acid, 8.1×10^{-2}% (w/v). Insert: the dependence of the maxima at 550 and 450 nm on the concentration of periodic acid in the solution.

optimum ratio was taken for the final concentration of periodic acid in solution of 2 mM.

3.1.3. Spectrophotometric measurements

In this part, we primarily used molar concentrations to facilitate the comparison of the analytical parameters obtained for free KDO solutions and the KDO from LPS preparations. The equation of the calibration curve at 550 nm for pure KDO solutions was obtained (within the error of the experiment, the intercept is negligible; Table 2). The limit of detection is 10 nM. Next, model LPS solutions were analyzed (Table 2) by the optimized procedure (*Procedure 1*). The limit of detection is 30 nM (the absolute limit of detection is 0.5 nmol). Thus, the determination of KDO from real LPS shows no significant change in the calibration slope compared to free KDO solutions, and the LOD is degraded due to an increase in the reagent blank in the *Procedure 1* only. Taking into account the sophisticated sample-preparation stage of the procedure, this result is very satisfactory. The accuracy of LPS determination by the reaction with KDO was verified by spiked solutions:

$$A_{550} = (3.1 \pm 0.2) \times 10^4 c_M + (0.020 \pm 0.001),$$
$$r = 0.993; P = 0.95; n = 14. \quad (6)$$

Thus, the calibration slope also differs insignificantly from KDO equations in Table 2. From Eq. (6) we estimated KDO contents in LPS. It was found to be 2.5 ± 0.2 μM, the absolute contents in the LPS solution was 5 nmol. The calculation of KDO concentration from the pharmacopoeia concentration provided by the documentation of the N.F. Gamaleya Plant of Biological Preparations and the molecular mass of the test LPS is 2.5 μM, which differs insignificantly from the KDO concentration calculated from Eq. (6).

3.1.4. Thermal lensing and optoacoustic spectroscopy

The optimized spectrophotometric procedure (*Procedure 1*) was applied for OA/PT measurements without any changes in the sample-preparation process (Table 2). The limit of detection of a free KDO solution with thiobarbituric acid is 1 nM (20 ng/mL). For the determination of KDO the linear calibration range of LPS is 0.07–100 μg/mL. The limit of detection of KDO released from LPS is 3.5 nM (70 ng/mL, the absolute limit of detection is 80 pmol). As in the case of spectrophotometric measurements, the slopes of equations for free KDO and the KDO from LPS differ insignificantly. The OAS LOD is 12 nM (300 ng/mL, the absolute limit of detection is 300 pmol), which is higher than for thermal-lens determination for the same excitation energy (Table 1). The comparison of the data of KDO determination by the spectrophotometry and PT spectroscopy shows that LOD decreases by an order with no changes in the reaction conditions, such an increase is obtained despite a twofold decrease in molar absorptivity on the working wavelengths

Table 2
Calibration plots for the determination of LPS by spectrophotometry, optoacoustic spectroscopy (Φ_e, 15 mJ) and thermal-lens spectrometry (P_e, 45 mW) ($P = 0.95$; $n = 16$).

Reaction	Spectrophotometry	Optoacoustics (532 nm)/thermal lensing (514.5 nm)
Free KDO	$A_{550} = (3.0 \pm 0.1) \times 10^4 c_M$ ($r = 0.998$)	$A_p = (4.8 \pm 0.3) \times 10^5 c_M + (3 \pm 1) \times 10^{-3}$, ($r = 0.979$) $\theta = (1.8 \pm 0.1) \times 10^5 c_M + (0.040 \pm 0.008)$ ($r = 0.994$)
KDO from LPS	$A_{550} = (2.9 \pm 0.1) \times 10^4 c_M + (0.004 \pm 0.003)$ ($r = 0.995$)	$A_p = (5.0 \pm 0.3) \times 10^5 c_M + (3 \pm 1) \times 10^{-3}$, ($r = 0.967$) $\theta = (1.9 \pm 0.3) \times 10^5 c_M + (0.11 \pm 0.04)$ ($r = 0.997$)
Methylene Blue	$A_{560} = (0.067 \pm 0.004)c + (0.008 \pm 0.001)$ ($r = 0.995$)	$A_p = (0.5 \pm 0.1)c + (3 \pm 1) \times 10^{-3}$, ($r = 0.991$) $\theta - \theta_{blank} = (0.32 \pm 0.02)c + (0.06 \pm 0.02)$, ($r = 0.997$)
Methylthymol Blue	$A_{585} = (0.034 \pm 0.002)c + (0.005 \pm 0.001)$ ($r = 0.991$)	$A_p = (0.3 \pm 0.1)c + (4 \pm 1) \times 10^{-3}$, ($r = 0.992$) $\theta - \theta_{blank} = (0.17 \pm 0.08)c + (0.03 \pm 0.02)$ ($r = 0.997$)
Pinocyanol chloride	$A_{490} = (0.039 \pm 0.001)c + (0.008 \pm 0.001)$ ($r = 0.990$)	$A_p = (0.8 \pm 0.2)c + (4 \pm 1) \times 10^{-3}$, ($r = 0.984$) $\theta - \theta_{blank} = (0.52 \pm 0.02)c + (0.05 \pm 0.02)$[a] ($r = 0.993$)

c_M is molar concentration, c is concentration in μg/mL.
[a] 488.0 nm.

of OA/PT setups in comparison with the maximum of the absorption band of reaction products (Fig. 3).

3.2. Reagentless determination of LPS

As LPS are biological macromolecules, their aqueous solutions exhibit properties of colloidal solutions [11]. OA spectroscopy as well as thermal lensing can be used for the determination of colloidal substances and their properties [14,15]. This is based on a change effect of colloidal particles on thermophysical parameters, first of all on thermal conductivity, Eq. (1) for OAS and thermal conductivity and thermal diffusivity Eq. (3) in TLS. It is well-known that these techniques can be used for determining surfactants in their aqueous solutions and other fine suspensions at the level below their cmc [15,16]. Within the scope of thermoacoustic conversion mode of OA/PT spectroscopies, the response of a disperse liquid sample irradiated with a short laser pulse is influenced by the process of temperature exhibiting an additional low-frequency constituents of the response due to energy transfer through phase interface, thus affecting the efficient thermophysical parameters and changing the amplitude of OA/PT signals [9,16,17]. Thus, such response changes may serve for enhancing sensitivity of OA/PT spectroscopy [10]. It is obvious that the most convenient variant of OA/PT determination of LPS based on this approach when no other reagents are used. Within the frames of this study we used it for the direct (reagentless) determination of LPS by its effects on thermal-lens and OA signals of water. Doubly distilled water showed both OA and TL signal at the background noise level.

For OA measurements, the amplitude signal A_p starts to significantly differ from the pyrogen-free water signal for LPS at 10 ng/mL (the ratio of OA maxima for LPS-containing and LPS-free samples of 1.24), which can be considered as the LOD for this technique [10]. The transfer parameter of the OA setup used gives the signal as a continuous oscillation process, which nevertheless obviously shows an insignificant difference in the oscillograms prior and after their time integration.

For thermal lensing, the calibration plot (*Procedure 2*) for 514.5 nm (c in μg/mL) is

$$\theta = (3.6 \pm 0.1) \times 10^{-4} c + (1.7 \pm 0.1) \times 10^{-2}, \quad (r = 0.955; P = 0.95; n = 10). \tag{7}$$

The PT limit of detection of LPS is 3 μg/mL. This value is higher than the limits of detections of LPS by spectrophotometry (Table 1) and is roughly the same as the cmc of LPS [11]. However, the values of the thermal-lens signal (0.001–0.002) are out of the range of the optimum precision of the spectrometer.

Thus, the direct determination of LPS is possible but not significantly sensitive. The simplest way to get more reproducible results is to use inert colorants that increase the absorbance and decrease the error of measurements. Such inert colorants are used to increase OA/PT signal only due to an increase in the linear absorption coefficient of the sample, Eqs. (1) and (3).

We selected iron(II) *tris*-(1,10-phenanthrolinate) (ferroin), which has the absorption-band maximum (510 nm) near the working wavelengths of the excitation lasers used, and does not affect the ionic strength of the solution and show no significant interaction with LPS [27]. The concentration of ferroin was varied within the range from 0.25 to 1.0 μM. The optimum concentration was 4 μM for TLS and 0.64 μM for OA spectroscopy. Adding an ferroin solution to water and an aqueous suspension of LPS showed no increase in the maximum optoacoustic signal compared to reagentless measurements (Fig. 4a). The LOD under these conditions is 10 ng/mL, which is 30-fold lower compared to TLS.

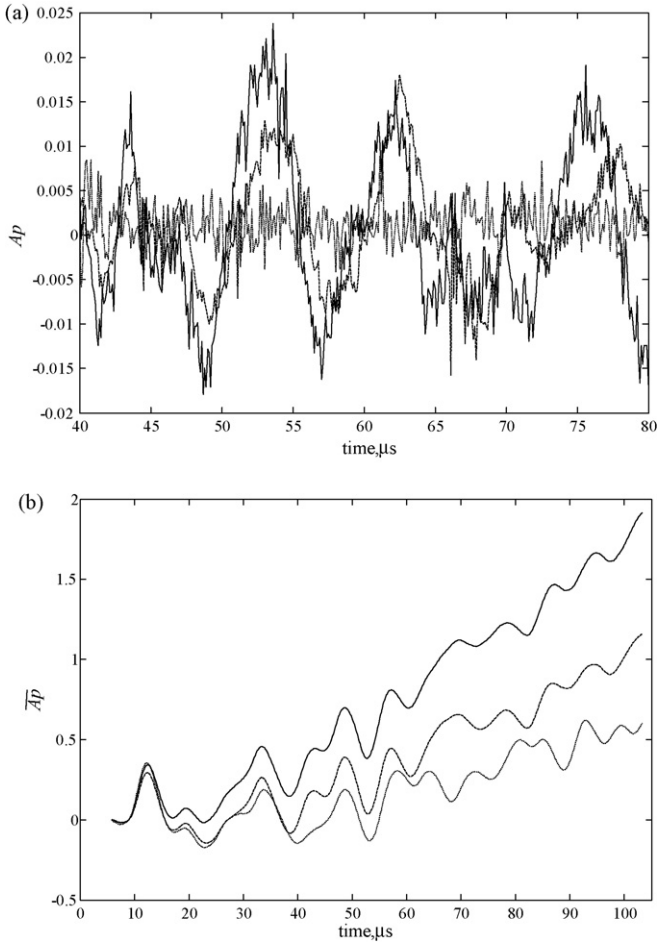

Fig. 4. Optoacoustic signals: (a) averaged signals (n = 100): dotted line, filtered distilled water with the excitation beam off; dashed line, filtered distilled water; solid line (b) Integrated (cumulative) optoacoustic signals: dotted line, filtered distilled water; dashed line, LPS 4.0 nM; and solid line, LPS 8.0 nM.

There are two ways to decrease the limit of detection of such samples with OAS. The first is the analysis of frequency and amplitude parameters as the shape of the signal for pure water and an LPS sample differ significantly. As an example, in the study, we used integrated (cumulative) signals, Eq. (2). Due to the nature of OA signal, the cumulative signal accumulates the changes due to difference in solution properties, which can be used for qualitative detection of LPS. Fig. 4b shows the growth of the cumulative optoacoustic signal with the LPS concentration. As a whole, the ratio of maxima for LPS-containing and LPS-free samples increased from 1.24 without ferroin to 1.38 with ferroin. The LOD under these conditions decreases to 2 ng/mL (2 nM) which is fivefold lower than for normal OA signal and more than 100-fold lower than the TLS LOD.

The second approach to decrease the OA LOD is the signal fluctuation analysis by the use of the histogram method. The existing of particles with constantly changing configuration provides a random constituent of the signal [28,29]. These features are observed as a functions of dynamic (response profile) and statistic (amplitude fluctuation) parameters of OAS and PLS signals and disperse-phase parameters. Such different mechanisms results in of optoacoustic response that is not well described with optoacoustic models developed for ideal solutions. The irradiation of disperse and turbid media by a train of laser shots yields a histogram of acoustic signal magnitudes which is very informative about the content of suspensions [16,17]. However, this was out of the scope of this paper.

3.3. Determination of LPS by ion-pair formation

As reagents, we selected Methylene Blue [20], Methylthymol Blue, and Pinocyanol chloride [21] as they provide a contrast effect upon the formation of ion pairs with LPS in the range 480–550 nm and, thus, are very advantageous for lasers used as excitation sources in OA/PT spectroscopies [7]. The study of the stability of these ion pairs to the laser irradiation (0.1–5 μM) showed a stable signal at the excitation-laser (480–530 nm) power 300 mW for more than 30 min.

3.3.1. Spectrophotometric measurements

The optimum concentrations of the reagents were selected from the balance of two opposite factors: the minimum value of the blank signal and the maximum value of the calibration slope. In the presence of LPS, Methylene Blue absorption maximum shifts from 470 to 560 nm. For Methylthymol Blue, all the measurements were made at pH 11 (0.2 M sodium carbonate) to keep it in a neutral form showing a maximum contrast upon the formation of ion pairs with LPS [19]. The absorption-band maximum of formed ion pairs lies at 585 nm, and an increase in the LPS concentration within the range 0.01–1 μM results in a linear increase in the absorbance within the range of 480–650 nm (*Procedure 3*). For Pinocyanol chloride, the formation of ion pairs with LPS produces the most contrast effect, and the absorption maximum of the product lies at 490 nm [21]. This reaction is the most promising for OA/PT setups used as the absorption maximum coincides with the excitation-laser wavelength (see Table 1). Thus, in this case, we selected the concentration of the dye so that it provided the minimum signal of the reagent blank, giving up the wideness of the calibration range. The concentration of the dye in the reaction mixture of 50 μM was selected, which provided an absorbance of the free dye 0.007 abs. units at the selected wavelength (*Procedure 3*).

The calibration equations are summarized in Table 2. The limits of detection for all the three dyes are the same and equal to 0.2 μg/mL (10 nM) (Table 1). The linear calibration ranges for Methylene Blue and Methylthymol Blue are also roughly the same, 1–80 μg/mL (0.05–4 μM). As expected, the procedure with Pinocyanol chloride shows the narrowest linear calibration range, 1–20 μg/mL. The repeatability RSD within the calibration ranges for all the dyes is no higher than 5%. The sensitivity of all the proposed procedures is high enough for transferring these conditions to OA/PT measurements.

3.3.2. Thermal lensing and optoacoustic spectroscopy

The selection of the working wavelength of the thermal-lens spectrometer (514.5 nm for Methylene Blue and Methylthymol Blue and 488.0 nm for Pinocyanol chloride, see Table 1) was dictated by its vicinity to the maximum of ion pairs of the absorption-band maxima of LPS with selected dyes. The optimum concentration for Methylene Blue for thermal lensing is 0.03 mg/mL, which is only twofold lower than the spectrophotometric concentration of the dye. Similarly, the optimum concentration of Methylthymol Blue for thermal lensing is 1.5 μM. Such a decrease in the reagent concentrations compared to spectrophotometry was dictated by the error curve of thermal lensing [30], which shows a decrease in the measurement precision for thermal-lens signals over $\theta = 3$. The selected reagent concentrations provided thermal-lens signals of the blank at a level of 0.10–0.15 (Table 2), which was a compromise between a change in the absorbance at the working wavelength upon the formation of ion pairs and high instrumental precision of thermal lensing (RSD for this range of signals is about 0.02). For Pinocyanol chloride, the reagent concentration was left unchanged as the contrast of the reaction is high and the thermal-lens reagent blank, $\theta = 0.05$, is low enough. In all the cases, as the concentration of the ion-pair reagent is much higher than the concentration of LPS, the loss of the reagent for the ion-pair reaction was considered insignificant. Except for selecting the optimum concentration of the dye, no procedure optimization compared to thermal lensing (*Procedure 3*) was made.

The TLS linear calibration ranges are 0.1–200 μg/mL (5 nM to 1 μM), the repeatability RSD within this range is no higher than 8%. The limits of detection of LPS (Table 1) are 30–60 ng/mL. The data in Table 1 show that the values of E_{theor} (Eq. (5)) and E_{exp} for TLS are in good agreement, and thermal lensing provides an increase in the overall sensitivity of LPS determination by a factor of 5–13.5, which confirms our previous data [7,8].

Based on thermal-lens LODs, Methylthymol Blue and Pinocyanol chloride were selected for OA measurements. The optimum concentrations were 0.4 μM for both dyes. The ratios for optoacoustic maxima for LPS-containing and LPS-free samples were 1.35 for Methylthymol Blue and 1.80 for Pinocyanol chloride, respectively; which are significantly higher than in the absence of dyes. The limits of detection for both dyes were 4 ng/mL (1 nM), which is 10-fold lower compared to thermal-lens determination, and is in good agreement with the previous theoretical evaluations of the figures of merit [10].

3.4. Comparison of the developed photothermal and optoacoustic procedures

The achieved limits of detection of LPS by spectrophotometry and OA/PT spectroscopies are summed up in Table 1. First of all, it is important that under the same energy, the LODs for LPS by OAS and TLS are at the same level. In other words, the methodology of analytical measurements developed in TLS [7,8] can be readily transferred to OAS resulting in a considerable increase in the sensitivity compared to spectrophotometry. The comparison of limits of detection shows that an increase in the instrumental sensitivity provides most OA/PT limits for detection below the level of 0.1 μg/mL, which is a rather good sensitivity for chemical methods for LPS and is comparable to that of the state-of-the-art biological test for LPS, limulus amoebocyte lysate (LAL) [27,31].

It is essential that a good selectivity potential of the OA method for disperse solutions was observed even without sensitivity-enhancing dyes (Table 1), as the OA time spectra from pure water and endotoxins solutions differ (Fig. 4). As previously predicted [9,17], the low-frequency constituents appear in the response from endotoxins as macromolecules leading to an increase in cumulative signals. Combining OA spectroscopy with the sensitivity-enhancing dyes (ion pairs) serves for further increasing its potential (Table 1). In this case, we can say that the sensitivity of OA spectroscopy in disperse samples is 100-fold higher than TLS under the same conditions. We believe that some extra experiments in this direction may result in the development of a simple OA/PT tests that can be used for screening for LPS before a more complicated biological or chemical method for LPS.

Among all the tested OA/PT procedures, the most sensitive are based on ion-pair formation. And in this case the sensitivity of OAS and TLS is the same (Table 1). However, the reasons are different: in OAS, the sensitivity enhancement compared to spectrophotometry is due to disperse LPS solutions as discussed above, and the increase in the signal is higher as the laser radiation is absorbed by colored particles with more heating compared to uncoloured solutions. However, an overall increase in the acoustic response for formed ion pairs compared to reagentless measurements is minor as it governed by the density of the sample rather than absorbance. In the case of thermal lensing, the sensitivity enhancement compared to spectrophotometry results from the reaction conditions resulting in ion pairs strongly absorbing at the excitation-laser wavelength leading to a more distinct temperature gradient affecting the PT signal, Eq. (3). Hence, the sensitivity of the thermal-lens proce-

dures is limited by a significant decrease in the molar absorptivity when shifting from the ion-pair absorption maximum (560 nm for Methylene Blue and 585 nm for Methylthymol Blue) to the working wavelength of thermal-lens (514.5 nm). Therefore, the most advantageous is Pinocyanol chloride as in the case the spectrophotometric and thermal-lens procedures use the same wavelength. Thus, this reagent shows the lowest thermal-lens LOD.

From this viewpoint of the blank signal, the KDO procedure, despite its lower sensitivity is much more advantageous in TLS as it is very selective [13]. In our opinion, the successful substitution of traditional metaarsenite for sulfite is a crucial success of this study, and may aid in more widespread uses of this approach in the pharmaceutical analysis. However, the use of such a 'traditional' photometric reaction in OAS did not provide a significant increase in the sensitivity compared to ion-pairing reagents. Probably, this requires a finer approach in the sample cell in OAS, e.g. layered-prism geometry [32].

As a whole, the application of OA/PT spectroscopies for the determination of LPS provided a significant decrease in the limits of detection and with the similar excitation energy and the methodology of measurements. This may be considered as a first step for a combination of two methods in a single setup for chemical analysis.

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