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Modified noninvasive method of study of the oxidation of lipids of airways

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ABSTRACT

To improve existing noninvasive method of study of the oxidative modification of lipids of airways we suggest to extract lipids from exhaled breath condensate by the Blich-Dyer method and register the UV-absorbance spectra of the obtained lipid extracts. To assess the degree of lipid oxidation in exhaled breath condensate samples 5 indices should be used: A_{206nm} - unoxidized lipids, A_{233nm} - conjugated dienes, A_{278nm} - conjugated trienes and ketodienes, A_{233}/A_{206} - a ratio of absorbance of dien conjugates to absorbance of unoxidized lipids, and A_{278}/A_{206} - a ratio of absorbance of conjugated trienes and ketodienes to absorbance of unoxidized lipids. The use of the described method shows the increased oxidation of lipids in airways of patients with asthma compared with healthy people. This increase has been expressed in larger extent in asthma patients with hyperresponsiveness to hyposmolar stimulus.

Key words: Exhaled breath condensate, UV-absorbance spectra, lipid peroxidation, asthma.

INTRODUCTION

One of the noninvasive methods of study of airways is based on the collection of exhaled breath condensate (EBC) [1]. In particular, EBC is used as a biomaterial for the measurement of the oxidative modification of airway lipids [2,3]. There are methods of estimation of the content of lipid peroxidation products in lipid extracts by measuring the values of absorbance in the UV region of the spectrum [4,5]. In the original method extraction of lipids from EBC samples is performed with heptane: isopropanol 1:1. The measurement of absorbance of heptane and isopropanol phases of extracts at 220 nm, 232 nm and 278 nm allows to express the content of unoxidized lipids (A_{220nm}), conjugated dienes (A_{232nm}), conjugated trienes and ketodienes (A_{278nm}) in relative values and calculate two indices of lipid oxidation: A_{233nm}/A_{220nm} and A_{278nm}/A_{220nm} . [6]. Some authors also propose to measure the absorbance at 400 nm and attribute it to the Schiff bases. The original method does not provide for the registration of the absorbance spectra of the EBC lipid extracts and is bounded by measuring the absorbance at the fixed abovementioned wavelengths. It is not clear how lipids of EBC are distributed between heptane and isopropanol phases and it is evident that isopropanol phase contains water of EBC, which may influence the absorbance in the UV region of the spectrum. There are two classical methods for the extraction of lipids from biomaterial, namely methods suggested by Folch [7] and Blich-Dyer [8]. In the former method lipids are extracted with the mixture of chloroform: methanol 2:1 and in the latter with the mixture of chloroform: methanol 1:2. Blich-Dyer method is preferable for the extraction of lipids from samples with high water content. Both methods assume the separation of chloroform and water-methanol phases. Total lipids are contained in the chloroform phase. We have modified the original method for the study of lipid peroxidation products in EBC, namely we used the Blich-Dyer method for the extraction of lipids from EBC and registered UV-absorbance spectra of the lipid extracts from EBC instead of measurement of absorbance in particular wavelengths (220, 232, 278 and 400 nm). We used the modified method of measurement of lipid peroxidation products in EBC for the study of osmotic airway responsiveness of asthma patients with the help of hyposmolar bronchial provocation test.

MATERIALS AND METHODS

Subjects

Sixty one patient with mild-to-moderate asthma aged 35 ± 1.4 years old (height - 166 ± 1.2 cm, weight - 73.4 ± 2.1 kg) as well as 15 healthy persons aged 32 ± 2.9 years old (height - 168 ± 1.5 cm, weight - 69.6 ± 2.7 kg) were recruited to the study. Detailed characteristics of asthma patients and healthy persons were described previously [9]. Patients with asthma were divided into two groups – with hyperresponsiveness to a hyposmolar stimulus (3-minute inhalation of ultrasonically nebulized distilled water) - group 1 and with normal responsiveness - group 2.

Airway response to the hyposmolar stimulus

To study the airway response to hyposmolar stimulus the bronchial provocation test with inhalation of ultrasonically nebulized distilled water was performed. Ultrasonic Nebulizer Thomex L-2 (Poland) was used for the generation of aerosol. The study included two consecutive inhalations of aerosols of isotonic NaCl solution and distilled water, respectively, for 3 min each. Control spirometric tests were performed by Flowscreen (Erich Jaeger, Germany) prior to the start of bronchial provocation test and after it at the 1st and 5th min of the recovery period. Airway hyperresponsiveness to a hyposmolar stimulus was diagnosed when a fall of forced expiratory volume for 1st sec after inhalation of distilled water exceeded 10% of the initial values [9].

EBC sample collection

EBC samples were collected in the morning after a double rinsing of the mouth with distilled water for 20 minutes during quiet breathing of a subject with a help of ECoScreen II (Erich Jaeger, Germany), equipped with a valve system, which allows to carry out breath of atmospheric air, and exhale vapors into the unit for their condensation. The volume of collected EBC samples varied from 0.5 to 3 ml. The tubes with the samples of EBC were kept in a freezer at -70°C . Before assay microtubes with EBC samples were vigorously shaken at the centrifuge-vortex ELMI CM -70V.07.

Lipid extraction

Lipids were extracted from the EBC samples by the Bligh-Dyer method [8]. Three ml of chloroform: methanol 1: 2 (v/v) mixture was added to 0.4 ml of EBC in glass tubes and intermittently mixed within 10 min. Then 1.5 ml of distilled water was added. The mixture was intensively stirred and centrifuged at 3000 rpm for 10 min. A chloroform phase was collected and immediately evaporated at rotary evaporator under reduced pressure. Dry residue on the walls of the cone was dissolved in 3 ml of ethanol and stored in a refrigerator until assay.

Absorbance spectra of lipid extracts registration

The absorbance spectra of the lipid extracts from EBC were recorded on a spectrophotometer UNICO 284 by differential scheme against ethanol in the range of 190-400 nm in wavelength scan measurement mode and fixed points measurement (206, 233 and 278 nm) mode. We use UV-Vis Analyst software to control the spectrophotometer, represent and store the results of measurements.

Colorimetric measurements

Individual lipids in the lipid extract from the combined EBC sample were determined by traditional colorimetric methods: phospholipids by inorganic phosphorus of mineralized lipids with Fisce-Subbarow reaction [10], total cholesterol by Zlatkis [11] and acylglycerides by Fletcher [12] methods.

Reagents

Analytical grade cholesterol and tripalmitin were obtained from Serva (Germany), egg-yolk phosphatidylcholine (PC) from Sigma (USA) and soy-bean polyen PC (EPL-substance) from Natterman (Germany).

RESULTS AND DISCUSSION

Absorbance spectra of lipid extracts

Figure 1 shows an absorbance spectrum of the lipid extract from EBC of asthma patient containing oxidized lipids. At the spectrum there are two peaks of absorbance at 207 and 291 nm and the shoulder on the main peak nearly 229 nm. These results corresponds to the generally accepted ideas that the appearance of the conjugated double bonds in the unsaturated fatty acid chain results in a shift of the maximum of absorbance toward longer wavelengths. Conjugated dienes show a maximum of absorbance at 232-233 nm while conjugated trienes and ketodienes at 278 nm [5]. The spectrum of absorbance of the oxidized polyen soy-bean phosphatidylcholine (EPL-substance stored in the freezer for more than 30 years) proves this idea and shows three distinct peaks of absorbance at 206, 232 and 278 nm (Fig. 2). The main peak of absorbance at 207 nm apparently corresponds to the unoxidized lipid. Because of this it is not clear why in the original method of assay of lipid peroxidation products in EBC [5,6] a content of

unoxidized lipid is estimated by the absorbance at 220 nm. According to our results the absorbance at 220 nm is more than 2 times lower compared with the absorbance at 207 nm – 0.66 and 1.49 units of absorbance, respectively (Fig.1). Our experience shows that the maximum of this peak varies in different spectra of EBC lipid extract within 201-207 nm and because of this it is difficult to choose particular wavelength, which will be characteristic for the unoxidized lipid. As can be seen from figure 3 the maxima of absorbance of lipid extracts from three EBC samples corresponds to 203, 206 and 207 nm. It is very likely that the shift of this maximum toward longer wavelengths is associated with the oxidation of lipids. Due to having to select a wavelength characteristic for unoxidized lipid we voluntarily decided that it would be 206 nm.

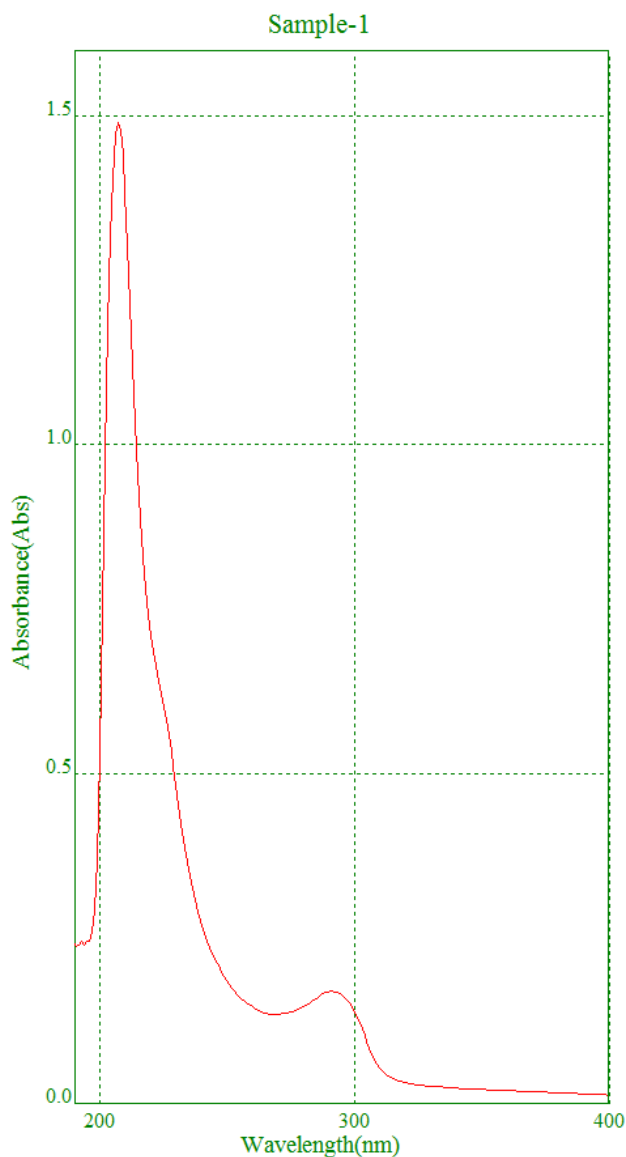


Figure 1. Absorbance spectrum of the lipid extract from EBC of asthma patient

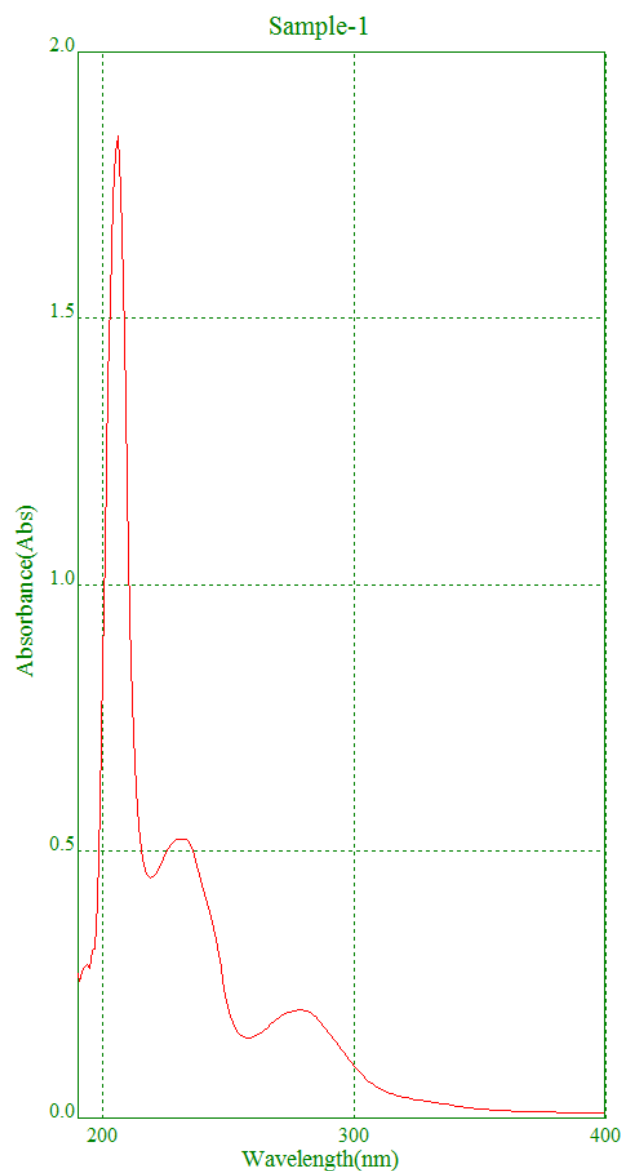


Figure 2. Absorbance spectrum of the oxidized soy-bean PC (FPT.-substance)

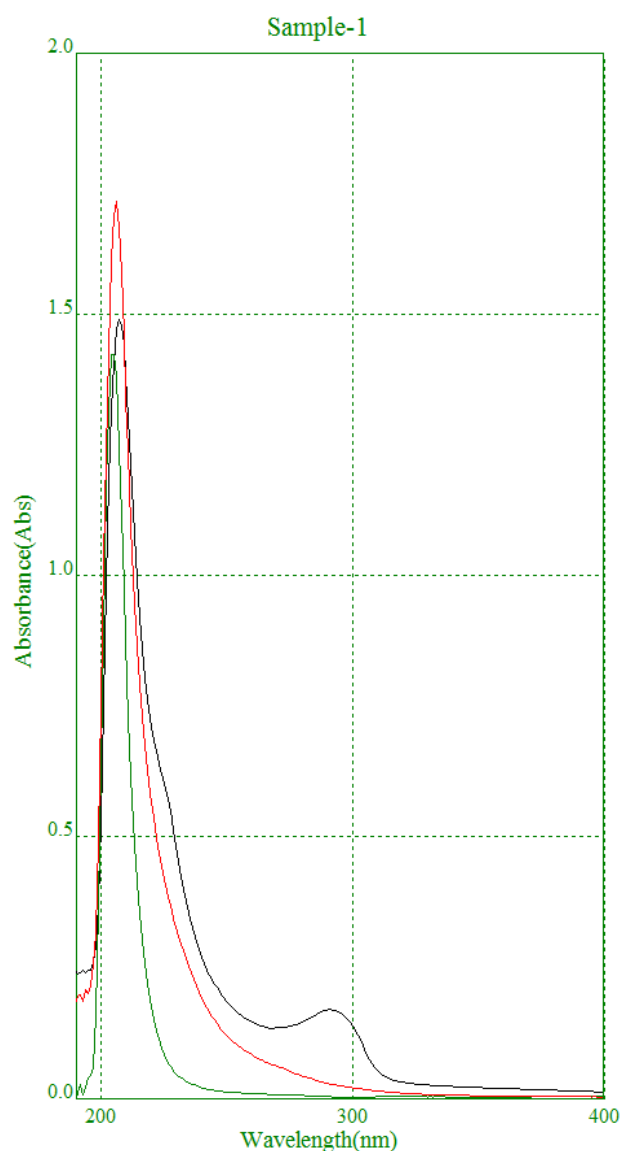


Figure 3. Absorbance spectra of lipid extracts from EBC of healthy persons (green and red) and asthma patient (black)

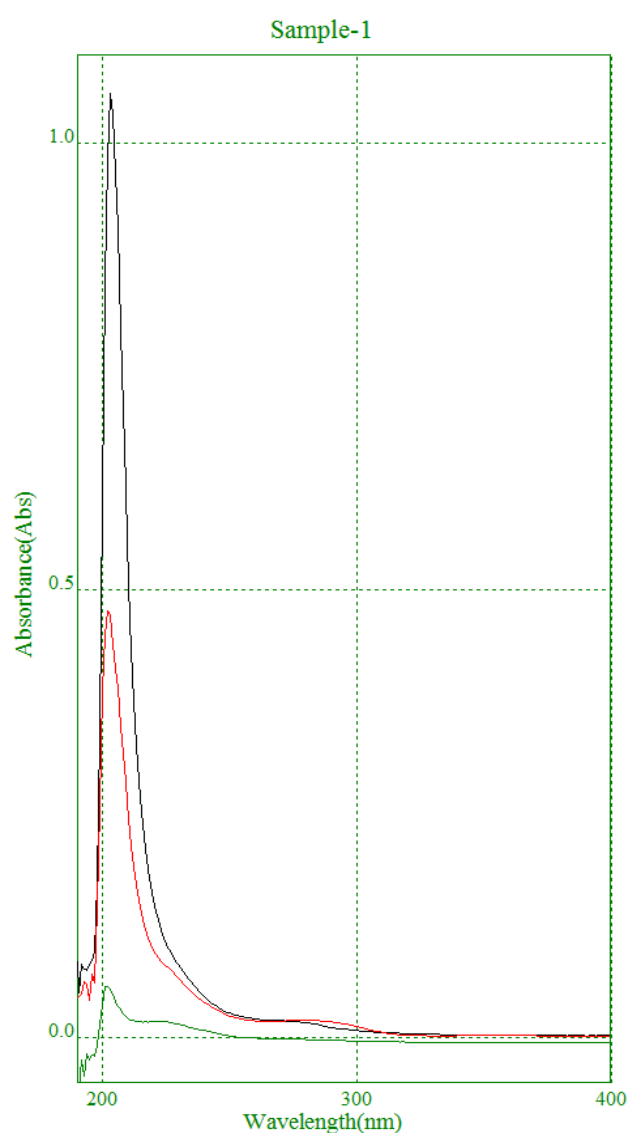


Figure 4. Absorbance spectra of egg-yolk PC (black), cholesterol (red) and tripalmitin (green)

Thus, to assess the degree of lipid peroxidation in EBC samples we recommend to use 5 indices: $A_{206\text{nm}}$ - unoxidized lipids, $A_{233\text{nm}}$ - conjugated dienes, $A_{278\text{nm}}$ - conjugated trienes and ketodienes, A_{233}/A_{206} - a ratio of absorbance of diene conjugates to absorbance of unoxidized lipids, and A_{278}/A_{206} - a ratio of absorbance of conjugated trienes and ketodienes to absorbance of unoxidized lipids. Absorbance at 400 nm usually corresponds to the baseline and it looks impossible to estimate the content of the Schiff bases in EBC lipids by measuring absorbance spectra. The content of diene conjugates in EBC may be calculated in nmoles/ml taking into account dilution during extraction of EBC and the molar extinction coefficient for the conjugated dienes $0.22 \cdot 10^6 \text{ M}^{-1} \text{ cm}^{-1}$. It is important to stress that the content of lipids in the samples of EBC varies drastically (Fig.3) as it follows from the values of absorbance at 206 nm, which may vary within 0.1-2.0 in the analyzed EBC samples. It looks promising to use $A_{206\text{nm}}$ to evaluate the lipid content in EBC samples and investigate the diagnostic value of this index.

Which lipids do contribute to the absorbance spectra of EBC lipid extracts? To answer this question we registered absorbance spectra of phospholipids (black), cholesterol (red) and tripalmitin (green) (Fig. 4). The spectra of 5 μg of egg-yolk PC and 10 μg of cholesterol are similar but the absorbance of PC is more that 2-times higher despite the content of PC in the cell of the spectrophotometer is 2-time lower. The absorbance of 15 μg of tripalmitin is negligible. Thus it is phospholipids and partly cholesterol, which may contribute to the absorbance spectra of EBC lipid extracts. This conclusion has been proved by the measurement of the content of phospholipids, acylglycerides and cholesterol in the extracts of EBC.

Lipids of EBC

The content of nonvolatile compounds and in particular lipids in EBC is very low and it may be measured quantitatively in the samples of EBC only by the sensitive methods of analysis, such as HPLC-MS, and so on [13]. To measure the content of phospholipids, acylglycerides and cholesterol in EBC by colorimetric methods we had combined a few dozen of EBC samples and extracted lipids by the Bligh-Dyer method from 140 ml of combined EBC. The chloroform phase of the extract was evaporated. The dry residue on the walls of the cone was weighted on an Explorer Pro Balance (OHAUS) and dissolved in 2 ml of ethanol. Thus EBC lipids were concentrated in the obtained ethanol solution 70-times in relation to the original EBC samples. Such approach allowed us to determine the content of phospholipids, acylglycerides and cholesterol in the lipid extract by colorimetric methods and to compare the content of these lipids in EBC and blood plasma of healthy persons. Results are presented in the table 1.

Table 1 Lipid content in the obtained lipid extract, EBC and blood plasma

Fluid	Total lipids	Phospholipids	Total Cholesterol	Acylglycerides
Lipid extract* (µg)	7500****	343	378	2380
EBC** (µg/ml)	50	2.45	2.7	17
Blood plasma*** (µg/ml)	3500-8000	1505-2045	1480-3470	885-2040
Blood plasma/EBC****	70-160	600-830	550-1300	50-120

* - total content of lipids in the obtained lipid extract from 140 ml of EBC; ** - lipid content in EBC was calculated by dividing their total content in the lipid extract on the volume of EBC taken on the extraction (140ml); *** - the content of lipids in blood plasma was calculated from the standard values for healthy adults: total lipids – 3.5-8.0 g/l; phospholipids – 2.0-4.7 mmoles/l, total cholesterol - 3.9-5.3 mmoles/l and triglycerides - 1.0-2.3 mmoles/l [13]; **** - the ratio of lipid content in blood plasma to EBC; ***** - the weight of the dry residue.

The content of phospholipids and cholesterol in EBC is very low and consists of 2.45 and 2.7 µg/ml, respectively. The content of acylglycerides in EBC is slightly higher (17 µg/ml) but not enough for the sufficient contribution of acylglycerides to the absorbance spectra of the lipid extracts from EBC. It is interesting to note that the total measured content of phospholipids, cholesterol and acylglycerides in the lipid extract makes up less than 50% from the weight of the dry residue. A detailed study of the lipid composition of EBC is necessary. The total cholesterol and phospholipid contents in EBC are 3 orders lower than in blood plasma and the acylglyceride content in EBC is 2 orders lower. Such comparison clearly indicates that conventional laboratory methods of analysis are unacceptable for the analysis of EBC and a lot of published results on the content of lipids, proteins, enzymes and other nonvolatile compounds in EBC should be treated with a great caution [14]. In contrast to these methods the registration of UV-absorbance spectra of lipid extracts from EBC represents noninvasive sensitive method which allows to measure quantitatively the extent of oxidation of airways lipids.

Oxidative modification of lipids of airways in asthma patients

Lipids of EBC obtained from asthma patients (Fig. 3, black curve) usually are more oxidized than lipids from healthy persons (Fig. 3, green and red curves). We used the method described above for the study of the role of oxidative modification of airway lipids in the formation of airways hyperresponsiveness in asthma patients with the help of the bronchial provocation test by inhalation of ultrasonically nebulized distilled water (a hyposmolar stimulus). Figure 5 shows the absorbance spectra of the lipid extracts of EBC obtained before (red) and after inhalation of distilled water (black) by asthma patient with airways hyperresponsiveness.

The maximum of absorbance of the lipid extracts from both EBC samples was observed at 201 nm, indicating low extent of lipid oxidation. However, the test results in the decrease of the lipid content in EBC possibly due to the bronchial constriction as it may be concluded from the decrease of $A_{201\text{nm}}$ from 0.290 to 0.230 and $A_{206\text{nm}}$ from 0.204 to 0.163 and in the increased oxidation of EBC lipids. The values of indices of lipid oxidation in the lipid extracts from EBC samples obtained before and after the hyposmolar bronchial provocation test consist of $A_{233\text{nm}}$ - 0.033 and 0.038, $A_{278\text{nm}}$ - 0.006 and 0.010, A_{233}/A_{206} - 0.161 and 0.233 and A_{278}/A_{206} - 0.029 and 0.049, respectively. In general, the use of the described method for the study of oxidative modification of airway lipids in healthy people and asthma patients shows the increased oxidation of lipid in airways of asthma patients. This increase expressed largely in asthmatics with airways hyperresponsiveness [9].

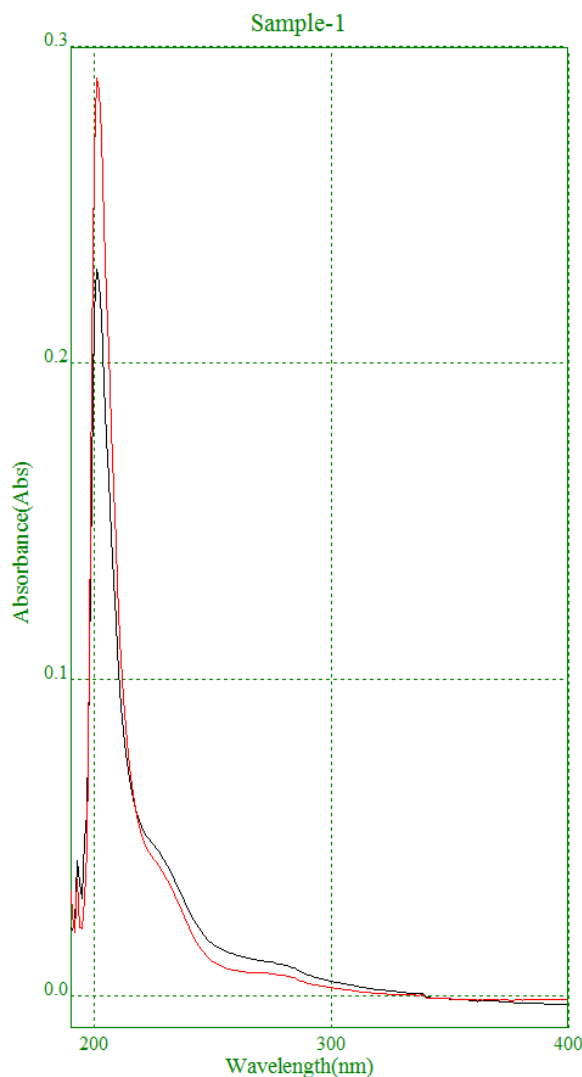


Figure 5. Absorbance spectra of lipid extracts from EBC of asthma patient before (red) and after (black) bronchial provocation test

CONCLUSION

We suggest a modified noninvasive sensitive method for study of the oxidative modification of lipids in airways. The modification includes extraction of lipids from EBC by Bligh-Dyer method and registration of UV-absorbance spectra of the obtained lipid extracts. To assess the degree of lipid oxidation in EBC samples we recommend to use 5 indices: $A_{206\text{nm}}$ - unoxidized lipids, $A_{233\text{nm}}$ - conjugated dienes, $A_{278\text{nm}}$ - conjugated trienes and ketodienes, A_{233}/A_{206} - a ratio of absorbance of diene conjugates to absorbance of unoxidized lipids, and A_{278}/A_{206} - a ratio of absorbance of conjugated trienes and ketodienes to absorbance of unoxidized lipids. The described method shows that lipids of EBC obtained from asthma patients are more oxidized compared with healthy people and this is expressed in larger extent in asthmatics with hyperresponsiveness to a hyposmolar stimulus.

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