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Ozone application for the people health state monitoring by the total unsaturation index determination

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ABSTRACT

There exists evidence about the influence of oxidative stress in certain pathologic states, especially chronic diseases. Measurements of lipid peroxide levels and the levels of their by-products are routinely used as indices of oxidative stress; however, until now, analytic assays used to determine lipid peroxidation have limitations regarding sensitivity, specificity, and timing of analysis. In the present brief review, we introduce the method of the double bond index (DB-index), a promising method of evaluation for lipid metabolism disturbance and lipid peroxidation measurement. DB-index calculation is based on the patented Total Unsaturation Analyzer (TUA) which instrumentation is fundament on the organic unsaturated compounds property of integrate ozone in the double bond site (one ozone molecule by each double bond) in a fast way. By this technique it is possible to determine in a short time (1-3 minutes) and with high precision (± 1%) the lipids total unsaturation in plasma and in cellular membranes. Some notable cases where DB-index has been established as diagnostic and prognostic criteria in different diseases and possibilities of present and future applications in the clinical analysis are discussed.

Key words: Total lipids unsaturation, DB-index, oxidative stress, health state monitoring.

RESUMEN

Es evidente la influencia del estrés oxidativo en ciertos estados patológicos, especialmente en enfermedades crónicas. La medición de los niveles de lipoperoxidación, así como de sus subproductos, son usados de forma rutinaria como índices de estrés oxidativo; sin embargo, hasta ahora los métodos analíticos empleados en la determinación de la lipoperoxidación presentan limitaciones asociadas a la sensibilidad, especificidad y tiempo total de análisis. En la presente breve reseña, se describe el método del índice de dobles enlaces (DB-index), un método prometedor para la evaluación de trastornos en el metabolismo de los lípidos y la medición de la lipoperoxidación. El cálculo del DB-index se basa en el analizador de insaturación total (TUA patentado), cuya instrumentación se fundamenta en la propiedad de los compuestos orgánicos insaturados de integrar rápidamente ozono en el sitio de la doble ligadura (una molécula de ozono por cada doble ligadura). Mediante esta técnica, es posible determinar en un corto tiempo (1-3 minutos) y con alta precisión (± 1%) la insaturación total de los lípidos en plasma y en membranas celulares. Se discuten algunos casos notables donde el DB-index se ha establecido como criterio de diagnóstico y pronóstico en diferentes enfermedades, así como posibles aplicaciones presentes y futuras en el análisis clínico.

***Palabras clave:** Insaturación total de los lípidos, DB-index, estrés oxidativo, monitoreo del estado de salud.*

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INTRODUCTION

Oxidative stress is generally described as a situation of imbalance in which excessive levels of oxygen free radicals or reactive oxygen species are present in the body in the face of inadequate availability of the relevant neutralizing substances, referred to as antioxidants, which destroys these harmful products of metabolic process.[1,2]

Oxidative stress has been implicated in the pathology of several human diseases, including cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases.[3-5] Consequently, several methods have been developed to monitor *in vivo* oxidative stress, for instance: direct quantification of reactive species by electron spin resonance[6] and indirect methods such as determination of antioxidants and total antioxidant capacity (TRAP)[7,8] and detection of oxidized biological markers.[9,10] Among biomarkers we can find: products of lipoperoxidation (malondialdehyde, 4-hydroxynonenal, isoprostanes, oxidized LDL), protein oxidation (hydroxyl and carbonyls), and measurements of DNA damage (high-performance liquid chromatography, gas chromatography). However, until now each of these assays has limitations regarding sensitivity, specificity, and timing of analysis. In the present brief review, we introduce the method of the double bond index (DB-index) determination based on the patented total unsaturation analyzer (TUA) equipment[11] analog of the double bonds analyzer (DBA).[12] By this technique it is possible to determine in a short time (1-3 minutes) and with high precision (± 1%) the lipids total unsaturation in plasma and in cellular membranes.[13,14] The estimation of the total unsaturation (TU) of plasma (DBpl-index) and cell membrane (DBcell-index) lipids has became a promising method of evaluation for lipid metabolism disturbance and lipid peroxidation (LPO). The TU determination is carried out by ozonation of lipid fractions of plasma and cells. This method consists to the measuring of ozone absorbed by lipid double bonds (remaining LOP substrate). Remarks about advantages and technical characteristics of the method are included. Some notable cases where DB-index has been established as diagnostic and prognostic criteria in different diseases (15-20) and its implementation and possibilities of present and future applications in the clinical analysis for the investigation and commercial fields are discussed.[21]

OZONE IN THE DEVELOPMENT OF CLINICAL ANALYSIS TECHNIQUES (THE DB-INDEX DETERMINATION)

Background

Ozone is recognized as a strong oxidant, aspect that has widely considered in the development of environmental remediation techniques since the first half of the last century,[22] more recently, many professionals on medicine and human healthcare have took advantage of this characteristic of ozone and have contributed with their theoretical and practical knowledge in the advance of the so called "ozonotherapy",[23] which has been employed in the treatment and control of many infectious and metabolic diseases.[24-26] However, the exact mechanism of this therapy is not known at all, and it still being a challenge for the scientist in the area.[27-29] Alternatively ozonation of some cellular and non cellular components of mammal's blood (including human beam) have been realized by some scientists in order to reach two main objectives:

- To study the mechanism between ozone and intra and inter cellular components.[30-32]
- To develop a new branch of study employing ozone in the clinical analysis and not only as therapy approach.

Regarding with last point of view we can find the new technique to determine lipids total unsaturation (TU) in plasma and cellular membranes by ozonation; whose fundamentals were developed since the second middle of last century by Russian researches. TU is represented by a concise parameter called Double Bond Index (DB-Index). This DB-index is strongly correlated with the stress oxidative level, since lipids involve in its determination correspond to remaining substrate not affected by oxidative stress mechanism. As it is known, the level of the TU for practically healthy peoples does not depend of the age or sex of the person and its value is altered in the presence of some disease.[17,33] For this reason the DB-Index determination provides a methodological technique for the differential and prospective clinical analysis, providing multiple possibilities in the control

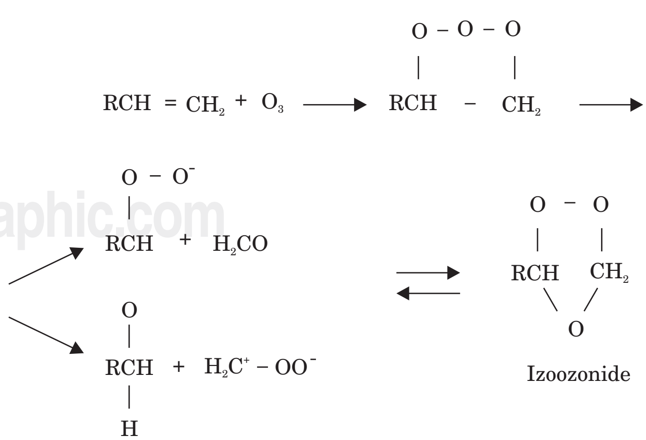

Figure 1. Main scheme of ozone reaction with double bonds of lipids.

of the therapeutic treatments.[15-21] Of course a personal doses strategy in differences diseases and pathologies as well as in ozonotherapy could also be studied by this approach.

Double Bond Index (DB-index) fundamentals

High selectivity of the ozone reaction for the identification of double bonds has been proven according with the ozonation kinetic constants.[34,35] *Figure 1* depicts the mechanism of ozone with unsaturated double bond compounds.

Based on these facts special equipment denominated total unsaturation analyzer (TUA) has been created *(Figure 2)*. The TUA based its operation on the property of organic unsaturated compound of integrate ozone in the double bond site (one ozone molecule by each double bond) in a fast way.

The general TUA operation involves next steps:

1. Oxygen gas is transformed into ozone by a photovoltaic reactor.
2. Controlled ozone concentration is obtained in order to carry out the reaction with a sample of lipids extracted from plasma or cells by a simple modified Folch method.[14]
3. The measure of non reacting ozone concentration (gas-phase) is realized by the principle of UV detection,[36] values are registered and plotted in a computer, and the obtained curve is called ozonogram. *Figure 3* depicts an ozonogram for lipids of plasma obtained from healthy Latin individual.
4. Total area of the obtained ozonogram proportionality corresponds to the double bond quantity in the sample. Calculation of DB-index will be described below.

Technical specifications of TUA are showed in *table I*.

Double Bond Index (DB-index) calculation

Calculation of DB-Index is derived from the expression (I):

$$\text{DB} - \text{index} = \frac{C_{st} \times V_{st} \times S_s \times V_{sol}}{S_{st} \times V_s \times V_{pi} \times K} \text{ (c.u)} \qquad \text{(I)}$$

Where:

$\boldsymbol{C_{st}}$ is concentration of the standard solution (mol/mL), $\boldsymbol{V_{st}}$, $\boldsymbol{V_s}$ are the volumes of the standard sample and the analyzed sample, respectively (mL), $\boldsymbol{S_{st}}$, $\boldsymbol{S_s}$ are the areas of the standard ozonograms and the analyzed sample (c.u.), $\boldsymbol{V_{sol}}$ is the solution volume of the analyzed sample (mL), $\boldsymbol{V_{pi}}$ is the blood plasma volume of the analyzed sample (mL), $\boldsymbol{K}$ is the calculated coefficient equal to 10^{-7} (mL/mol), (c,u) means "conditional unit" (1 c.u.$=1\times10^{-5}$ mol d.b./mL).

For instance, some DB-index values for healthy European and Latin-American population are presented in the *tables II and III*, respectively:

The presented values play the role of a standard in the discrimination process between healthy and unhealthy population for each characterized group; DB-index increasing and diminution can be observed in the presence of

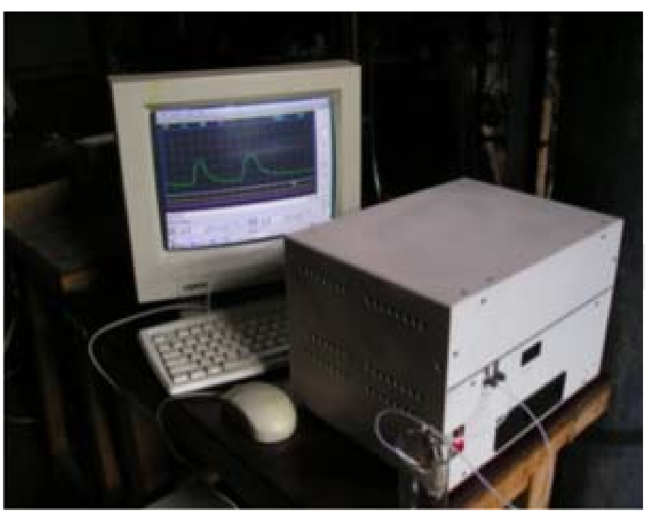

Figure 2. Total unsaturation analyzer (TUA).

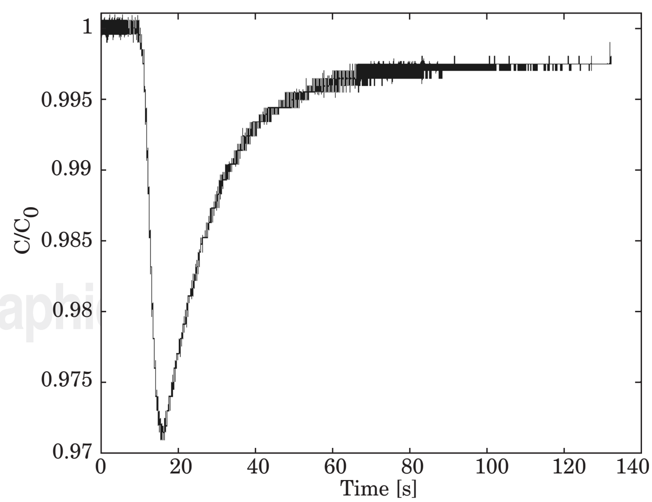

Figure 3. Ozonogram for lipids of plasma obtained from healthy latin individual. C/C_0 corresponds to normalized ozone concentration.

some disease and pathology. The value of this difference respecting to the normal level has proven a strongly relation with the disease severity.[15-21]

Characteristics of the lipids TU determination

The determination of the lipids TU using the TUA can be resumed as follows:

- It is a micro-method, it means only 50 micro liters or 200,000 cells are needed for one analysis
- It is a quick method 2-30 seconds
- High sensibility *(Table I)*
- High precision *(Table I)*
- Reproducibility *(Table I)*

Furthermore, DB-index calculation provides:

1. Possible criterion in the risk groups selection or identification.
2. Predictive information of the health state before disease manifestation.
3. Determination way for the latency periods and collateral effects.
4. Possibility of verification and correction of the therapeutic treatment.

Table I. TUA technical specifications.

Parameter	Value	Unit
Precision	0.1	%
Ozone sensitivity	10^{-6}	mol/L
Time of analysis	1-2	min
Noise to signal level	0.01	%
Zero drift	0.01	% per hour
Time resolution	2	s
Oxygen (air) flow rate	5-30	cm^3/min
Supply voltage	110	Volts (A .C.)
Overall dimensions	320 × 230 × 200	Mm
Weight	3	kg

SUCCESSFULLY APPLICATION CASES OF THE LIPIDS TOTAL UNSATURATION METHOD

In this section we would like to show some examples of studies where the determination of the total unsaturated lipids has been applied; two aspects have been mainly considered, evaluation of a harmful environmental factor and differential diagnostic in a specific disease.

Use the lipids TU determination for the toxicity level evaluation of a harmful environmental factor in an animal and a human population

Chromium toxic effect monitoring using ozonation method[17]

The hexavalente chromium toxicity *(in vitro)* to plasma, erythrocytes and semen lipids was evaluated in a sample from exposed Mexican population. The ozonation technique is suggested to realize the rapid measurement of the lipid peroxidation (LPO) by means of the double bond indexes (DB-index and DB_{cell}-Index) calculation. Main conclusions of this study are listed as follows:

- The values of the index DB obtained for people in Mexico City are significantly smaller with respect to European ones.
- The DB_{cell}-index for human sperms has been determined showing that these cells are very sensitive to chromium, even to very small doses (2.5 μM).
- The micro dose effect of chromium on the LPO depends on the initial value of DB-indexes.

The obtained experimental results permitted conclude that it is possible to detect the chromium effect on LPO by ozonation. The DB-index ad DB_{cell}-index determination in the plasma, erythrocytes, and sperm can be considered as a measure of this effect.

Table II. DB-index value for European healthy population.

European population			
DB_{Plasma}-index a.u.	DB_{cell}-index c.u.		
250 ± 10	Erythrocytes 50 ± 2	Lymphocytes 60 ± 2	Leucocytes 70 ± 3

Total unsaturation of plasma and cell membrane lipids as a new biochemical test of ionizing radiation aftereffect[37]

Total unsaturation of plasma (DB-index) and cell membrane (DB_{cell}-index) lipids of shallow and big animals was studied by means of the ozonation method. The doses dependence of the DB-index in the acute radiation regime was studied considering groups of Dogs and mice irradiated by different radiation doses (10, 25, 50 and 100 radian) and in long and acute radiation. *Table IV* shows the nominal DB-index values obtained for non irradiated animals.

In the cases of irradiated mice, both two DB-indexes changed in the first 6 hours after radiation. Specifically DB-index was decreased from 7 up to 30%, and DB_{cell}-index for erythrocytes was increased from 30% up to 45% as can be see it in *figure 3*.

In the case of dogs group during first two days after radiation dose the DB-index filled down up to 50% respect to nominal value; however, it was not showed a tendency to normalization *(Figure 4a)*.

The experimental data showed that the relative decreasing of the DB-index which was determined in the first 6 hours after radiation depends on the radiation doses *(Figure 4b)*.

Table III. DB-index value for Mexican healthy population.

Mexican population	
DB_{plasma}-index a.u.	DB_{cell}-index c.u.
160 ± 10	Erythrocytes 100 ± 2

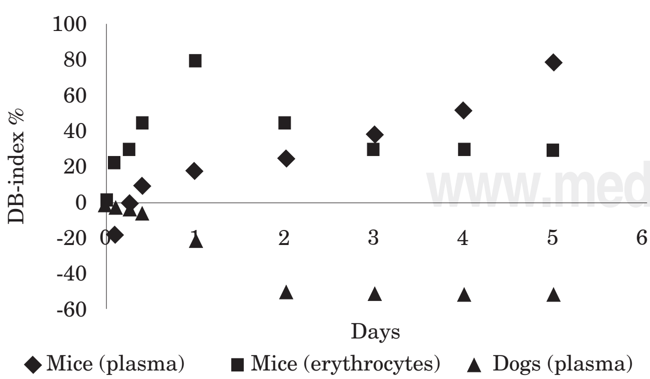

Figure 4a. Dynamics of the DB-index variation of plasma and erythrocytes for mice by the acute regime (25 radians) and for dogs by prolonged irradiation regime (50 radians).

Total unsaturated lipid level as a differential diagnostic and prognostic parameter in a specific disease

Differential diagnostic and prognostic of pneumonia by DB-index determination[13]

Clinical validation of the DB-index for the diagnostic and prognostic of pneumonia in children was developed. DB-index was compared against the LPO level and with the dynamic of the disease in a 15 children sample with pleural pneumonia and purulent lung damage. Patients shown an inverse correlation between DB-index and LPO activity, concluding that the proposed ozonation method can characterize the membrane destruction process in complicated pneumonia forms usually non detectable since the first stage. The sample of patients was divided into three different groups according to their The DB-index determination and their radiological and clinical exams *(Figure 5)*. As could be seen it destructive pneumonia forms present a DB-index diminution respecting to the first day evaluation and slow increasing tendency, in the case of the lees aggressive pneumonia type it was observed a fast growth of the DB-index which corresponds with a positive recuperation among the first 3-7 days.

Quick diagnostic of the lipids metabolism alteration for the evaluation and prognostic of burned patients[15]

The study was realized considering a 100 burned patients sample with different burnt level; evaluation of their evolution at different time was realized. DB-index presented a variation in an interval from 34 to 287 depending of the burnt magnitude. DB-index was correlated with the magnitude of the damage and with the treatment effectiveness. The direct correlation between DB-index and the unsaturated fatty acids concentration is presented in the *table V*.

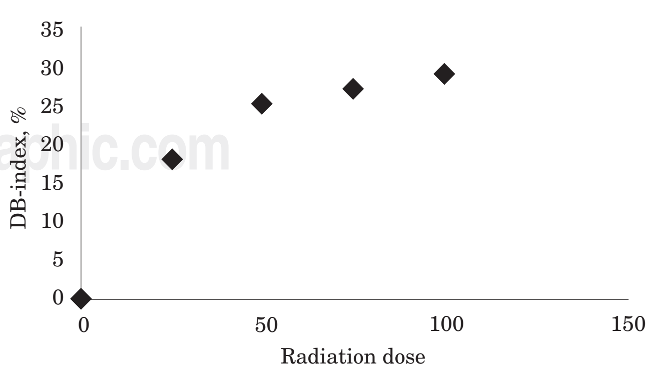

Figure 4b. The dependence of the DB-index on the radiation doses for mice in acute radiation regime.

Under normal conditions the number of DB in lipids of blood plasma varied from 190 x 10^{-8} to 311 x 10^{-8} moles DB/mL, with a mean value of 244 x 10^{-8} moles DB/mL. For a healthy group selected to this test the ozone absorption curves had similar characteristics and, as a rule, they repeated one another *(figure 6)*. No differences were discovered depending on the sex and age of the patients. *figure 7* depicts as an example the results of 8-day monitoring of patient (called G)., aged 26 years with a chemical burn affecting 40% of the body surface and of the IIIA+ B degree, starting from the first few hours after trauma and until discharge from the clinic. The number of DB in the blood plasma lipid of the patient 5 h after burning was 183x10^{-8} moles DB/mL, and on the 2nd day after burning it was 88 x 10^{-8} moles DB/mL. At this time massive suppuration had begun in the patient's wounds, the nitrogen balance was showing negative features, and marked toxemia was present. From the 18th day after burning the number of DB gradually began to increase, and as the burn healed and the patient's condition improved, the number increased steadily until the patient's discharge from hospital (165 x 10^{-8} moles DB/mL).

As a second example the results of investigation of patient (Feminine), aged 21 years, with thermal burns of the III A + B degree affecting 70% of the body surface, are shown in *figure 8*. It is reported that her state on admission was extremely grave because of toxemia and extensive burns: the number of DB on admission was 56 x 10^{-8} moles DB/mL. On the 18th day of her stay in the burns unit, treated by the open method on a "Clinitron" bed (France), the number increased to 116 x 10^{-8} moles DB/mL, and as the wounds healed gradually, the number of DB continued to rise. However, after 40 days the patient's condition deteriorated, healing became sluggish, and signs of burn exhaustion appeared: loss of body weight, loss of appetite, inability to assimilate food (vomiting, retching, and meteorism). The DB level on the 42nd day had fallen from 197 x 10^{-8} to 133 x 10^{-8} moles DB/mL. From that moment the patient began to receive additional feeding through nasal gastric tube, and after 3 weeks the number of DB became normal (236 x 10^{-8} moles DB/mL). This coincided with a considerable improvement in the patient's condition: during this period she gained 7 kg in weight, her appetite improved, pink granulations appeared, and marginal epithelization of the wounds took place. Au-

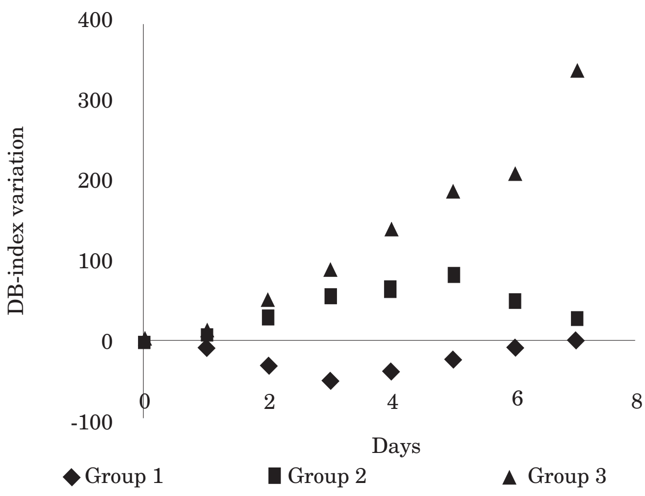

Figure 5. DB-index variation respecting to the first day: Group 1- purulent lung destruction, Group 2 - multisegmentarium serosum pleuritis, Group 3- multisegmentary pneumonia.

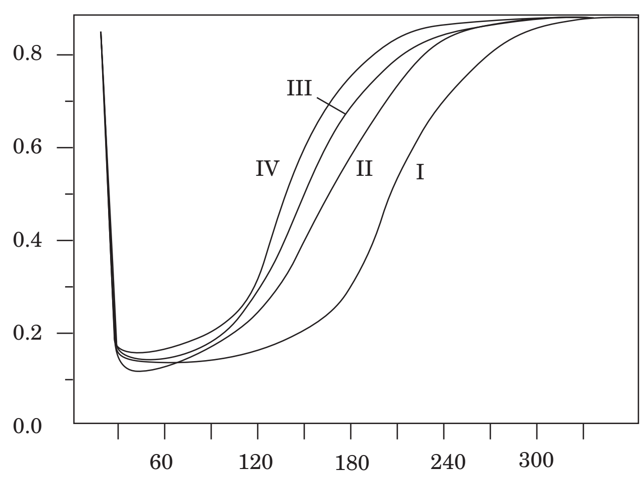

Figure 6. Ozonograms of blood plasma lipids of healthy patients. (Abscissa: time, s; ordinate: ozone absorption (in moles DB/mL x 10^{-8}).

Table IV. Nominal values of the DB-index and the DB_{cell}-index for different animals.

Animals	DB-index c.u. Plasma	Erythrocytes	DB_{cell}-index mole d.b/10^6 cells Leucocytes	Lymphocytes
Dogs	200 ± 10	41 ± 3	(0.64 ± 0.03) x 10^{-5}	-
Mice	57 ± 3	43 ± 3	-	(0.12 ± 0.03) x 10^{-7}

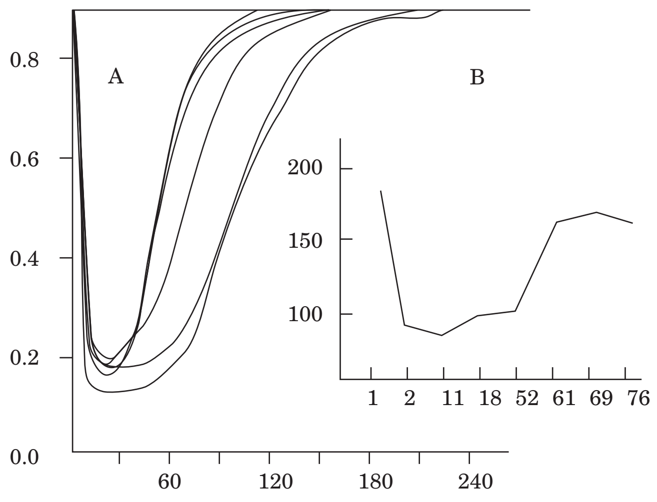

Figure 7. Ozonograms of blood plasma lipid of burned patient (A). (Abscissa and ordinate are as in *Figure 6*). DB variation in blood plasma lipids depending on time after trauma (B). (Abscissa: is time after trauma (days); ordinate: are moles DB/mL x 10^{-8}).

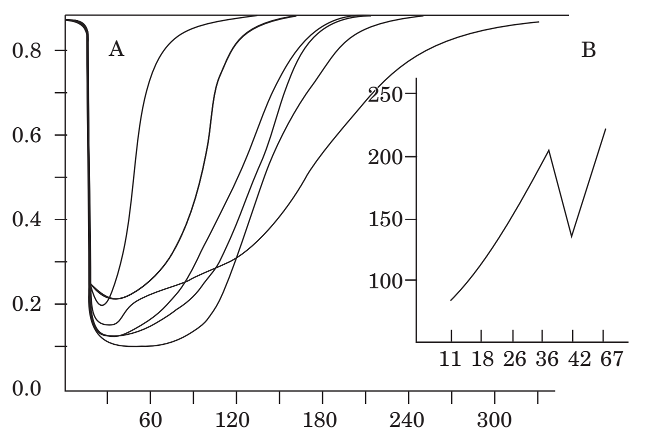

Figure 8. Ozonograms of blood plasma lipid of burned patient F. (A). (Abscissa and ordinate are as in *figure 6*). DB variation in blood plasma lipids depending on time after trauma (B). (Abscissa: is time after trauma (days); ordinate: are moles DB /mL x 10^{-8}).

thors conclude that response of a change in the DB-index preceded clinical manifestation of the corresponding change in the patient's condition. So, the ozonation method in burned patients poses enormous possibilities in the prognostic evaluation, as could be seen.

Distribution of the total unsaturation in lipid components of plasma, as a new differential diagnostic method in clinical analysis[14]

In that study children suffering from Insulin-Dependent Diabetes Mellitus (IDDM) were examined for TU of blood plasma lipids and for unsaturation in individual fractions of blood plasma lipids. The objective was to find a biochemical criterion for metabolic disorders and in IDDM, based on the DB-index calculation. Twenty children were examined during the compensation stage (group 1), and twelve during decompensation with ketoacidosis (group 2). According with the authors DB-index depends on the compensation degree, as well as duration of the disease. Total unsaturation distribution in plasma lipid fractions was found to be significantly decreased when compared to those in healthy controls *(Table VI)*. The DB-index dynamic observation in children with IDDM could be used to determine the adequate treatment formulation including the insulin dosage correction, hypolipidemic and membranostabilizing medicine introduction.

CONCLUSIONS

DB- index method is an excellent option to evaluating lipid metabolism disturbance and lipid peroxidation (LPO) without limitation involved in other assays considered in the oxidative stress measurement. As can be appreciated from the discussed cases, DB-index method could be included in the evaluation of harmful environmental factors and as a parameter for differential diagnostic in a specific disease. Furthermore, prognostic evaluation from DB-index values has been successfully proved. Many other disciplines can take advantage of these facts, implementing clinical protocols to determining the DB-index behavior under patient medical control in some specific disease.

Table V. Variation of the DB-index and the unsaturated fatty acids concentration.

Unsaturated fatty acids mM/L	Time after trauma (days) 5	10	30	37
Oleic (C 18:1)	1.47	0.10	5.09	5.97
Linoleic (C 18:2)	0.68	0.49	3.83	3.96
Arachidonic (C 20:4)	0.09	0.04	0.96	3.26
DB-index	94	35	119	165

Table VI. The total unsaturation distribution in plasma lipid fractions in children groups of different stage.

Children groups	DB_{liq}-index	DB-index rel %			
		PhL	FCh	NEFA	TG
Control	260 ± 10	13.9 ± 5.0	10.0 ± 2.3	43.4 ± 7.1	17.5 ± 1.9
First	164.3 ± 11	13.8 ± 3.2	14.3 ± 1.9	40.5 ± 7.4	14.6 ± 1.9
Second	150.5 ± 11	17.1 ± 2.8	14.7 ± 1.9	23.7 ± 6.1	22.5 ± 12

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