Chapter 6

Automation of Methods for Determination of Lipid Peroxidation

Jiri Sochor, Branislav Ruttkay-Nedecky, Petr Babula, Vojtech Adam, Jaromir Hubalek and Rene Kizek

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/45945

1. Introduction

Free radicals are atoms or molecules having one (or rarely more) free electron(s). These compounds may attack most of the (bio)molecules in organisms, which leads to the oxidative stress, which belongs to the causes of pathological processes in organisms [1-6]. Oxidative stress occurs in a situation, when the imbalance between the production of free radicals and effectiveness of antioxidant defence system occurs in a healthy organism. Determination of antioxidant activity or eventually markers directly connected with this variable is one way how to monitor the damage of organisms by these compounds [7-14]. The negative effect of free oxygen radicals consists in the lipid peroxidation. This type of peroxidation is a chemical process, in which unsaturated fatty acids of lipids are damaged by free radicals and oxygen under lipoperoxides formation. Lipoperoxides are unstable and decompose to form a wide range of compounds including reactive carbonyl compounds, especially certain aldehydes (malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE)) [15-22] that damage cells by the binding the free amino groups of amino acids of proteins. Consequently, the proteins' aggregates become less susceptible to proteolytic degradation [23-25]. In tissues, the accumulation of age pigment spots appears. In addition, free radicals effects are connected with a formation of atherosclerotic lesions. In body fluids (blood, urine) the increased levels of peroxidation end-products (MDA, 4-HNE, isoprostanes) are present [26,27]. The lipid peroxidation by free radicals occurs in three stages: initiation, propagation and termination [2,26]. Reaction (1) represents initiation, in which a fatty acid molecule of lipid is attacked by free radicals leading to a detachment of the hydrogen atom under fatty acid radical formation. In its structure, a rearrangement of the double bond to form conjugated diene occurs. This diene structure subsequently reacts with oxygen molecule to form a lipoperoxyl radical, which leads to the initiation of the second phase called propagation (2). In another part of the promotion, lipoperoxyl radical further reacts

with another molecule of fatty acid, from which a hydrogen atom is detached under formation of lipid hydroperoxide from original molecule (3). After pairing of all radicals, the last stage of the reaction called termination occurs. In addition to the above-mentioned chemical non-enzymatic peroxidation, enzymatic lipid peroxidation that is catalysed by the enzymes cyclooxygenase and lipoxygenase takes place. [26,28]. Both enzymes are involved in the formation of eicosanoids, which represent a group of biologically active lipid compounds derived from unsaturated fatty acids containing 20 carbon atoms. Cyclooxygenase is involved in the genesis of prostaglandins [29].

$$(1)\ LH + R^{\bullet} \rightarrow L^{\bullet} + RH \quad (2)\ L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (3)\ LOO^{\bullet} + L'H \rightarrow L'^{\bullet} + LOOH$$

Scheme 1. The scheme of lipid peroxidation. Initiation (1), the first part of the propagation (2), the second part of propagation (3).

For the monitoring of lipid peroxidation, spectrophotometric [30,31], chromatographic [32] and immunochemical [33] methods can be used. The analysis itself may be based on the analysis of the primary products of lipid peroxidation as conjugated dienes [34] and lipid hydroperoxides [35], or secondary products, such as malondialdehyde [36], alkanes [37] or isoprostanes [32,38-40]. Chromatographic methods represent the special group of methods, which are mostly based on the decrease of unsaturated fatty acids' concentration [41]. The scope of this review was to summarize the photometric analyses of lipid peroxidation. Less common method - FOX (ferrous oxidation in xylenol orange) was suggested to be automated.

1.1. Spectrophotometric methods in lipid peroxidation analysis

Spectrophotometric methods for the analysis of lipid peroxidation (see Table 1) are well reproducible and low cost. They usually consist of several steps that can be automated without much difficulty. Determination of conjugated dienes and TBARS belong to the one of the oldest and mostlz used methods for their rapidity and simplicity. On the other hand, they are criticized for their non-specificity [42,43]. Lipid hydroperoxides may be determined by the iodometric method and FOX test [44].

Determined analyte	Method	Type of analysed sample	Reference
Conjugated dienes	The structures of conjugated dienes absorb in the UV spectrum of 230-235 nm	Serum lipoproteins, tissue lipids	[34,45]
TBARS/MDA	TBA complex with MDA, Measurement at 532 nm	plasma, urine, tissues (liver), Cell lysates	[36,46-53]
Lipid hydroperoxides	Iodometric method	plasma, plant tissues	[44,54]
	FOX test	plasma, serum lipoproteins, both animal and plant tissues	[35,44,55,56]

Table 1. Summary of spectrophotometric methods used in lipid peroxidation determination.
FOX –ferrous oxidation in xylenol orange, MDA – malondialdehyde, TBARS – thiobarbituric acid reactive substances

1.2. Conjugated dienes

The structures of conjugated dienes (Fig. 1) with alternating double and single bonds between carbon atoms (-C=C-C=C-) absorb wavelengths of 230-235 nm in the UV region. Therefore, it is possible to use UV absorption spectrometry for their determination [41,42]. The method is used for determination of a non-specific lipid peroxidation caused by free radicals in biological samples, and is successfully used in the study of peroxidation in isolated lipoprotein fractions (LDL lipoproteins) [45]. However, its use in the direct analysis of plasma is controversial because of the presence of interfering substances, such as heme proteins, purines or pyrimidines in the UV region measurement [42,57].

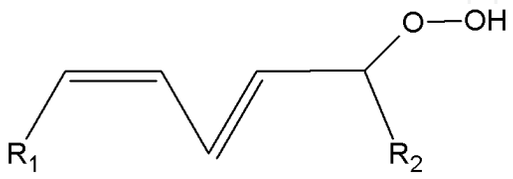


Figure 1. Structural formula of conjugated diene arising from the fatty acids by the free radicals effects during lipid peroxidation.

Increased sensitivity of the method can be achieved by an extraction of lipids into organic solvents in combination HPLC with UV detection [34,58]. However, the result of application the method to lipid extracts from human body fluids after HPLC separation was surprising, because the majority of pre-treated lipid fraction absorbs at wavelengths typical for conjugated dienes consisting of conjugated linoleic acid isomer (*cis*-9, *trans*-11-octadecadienoic acid) [59]. The main sources of conjugated isomer of linoleic acid (CLA) are dairy products and ruminant meat, especially beef [60]. They come into human serum and tissues probably from the diet [61], but can be also produced by bacteria [62,63]. Therefore, formation of large amounts of CLA by free radicals seems unlikely. In addition, the presence of CLA was not detected in the plasma of animals suffering from oxidative stress. *In vivo* induction of lipid peroxidation in rats treated with phenylhydrazin trichlorbrommethan did not cause an increase of CLA plasma values [64]. In the case of the use this method, it is necessary to take into account the above-mentioned shortcomings in the analysis of biological fluids or tissues.

1.3. TBARS, TBA-MDA adducts

TBARS (TBA-MDA) (**T**hio**b**arbituric **A**cid **R**eactive **S**ubstances) is the most widely used method for determination of lipid peroxidation method, especially due to its simplicity and cheapness. As the name of this method implies, it is based on the ability of malondialdehyde, which is one of the secondary products of lipid peroxidation, to react with thiobarbituric acid (TBA) [65]. The principle of this method consists in the reaction of MDA with thiobarbituric acid in acidic conditions and at a higher temperature to form a pink MDA-$(TBA)_2$ complex (Fig. 2), which can be quantified spectrophotometrically at 532 nm [17,66-70]. TBARS method measures the amount of MDA generated during lipid peroxidation, however, other aldehydes generated during lipid peroxidation, which also

absorb at 532 nm, may react with TBA [71]. The results of the assay are expressed in μmol of MDA equivalents. TBARS method can be also used in the case of defined membrane systems, such as microsomes and liposomes, but its application in biological fluids and tissue extracts appears to be problematic [72-74]. The first problem is based on the fact that MDA can be formed by the decomposition of lipid peroxides under heating of the sample with TBA. This decomposition is accelerated by traces of iron in the reagents and is inhibited by the use of chelating agents [42]. At the decomposition of lipid peroxides in the analysis, the originating radicals can amplify the entire process and the amount of MDA could be overestimated [74]. To prevent the decomposition of lipid peroxides during the analysis, inhibitor of the lipid peroxidation called butylated hydroxytoluene is added to the sample [42]. One of the other problems of the TBARS method application has been found in the analysis of biological fluids. In this case, some substances, such as bile pigments and glycoproteins provide a false positive reaction with TBA [71,75]. Unspecificity TBARS test problems can be partially overcome by the using of HPLC techniques for the separation of "authentic", original MDA-$(TBA)_2$ adduct from other chromogens absorbing at 532 nm [76]. Nevertheless, this approach cannot solve all problems. In addition, next molecules, such as aldehydes originated from lipid peroxidation, can form with TBA a original MDA-TBA_2 adduct, which has been demonstrated in the deoxyribose [77]. Using of different techniques in the determination of lipid peroxides in plasma or serum of healthy people (spectrophotometric versus HPLC method) leads to significantly different results. When using spectrophotometric techniques, the content of TBARS in plasma (serum) reached values from 0.9 to 42.7 $\mu mol \cdot L^{-1}$ of MDA equivalents, when HPLC technique was used, the content of TBARS in human plasma (serum) reached values of 0.6 – 1.4 $\mu mol \cdot L^{-1}$ of MDA equivalents [78-84]. This was probably caused by the using different methods for modifying the preparation of plasma (serum) sample. Method for the non-specific index of lipid peroxidation determination in isolated purified lipid fractions seems to be most useful [42].

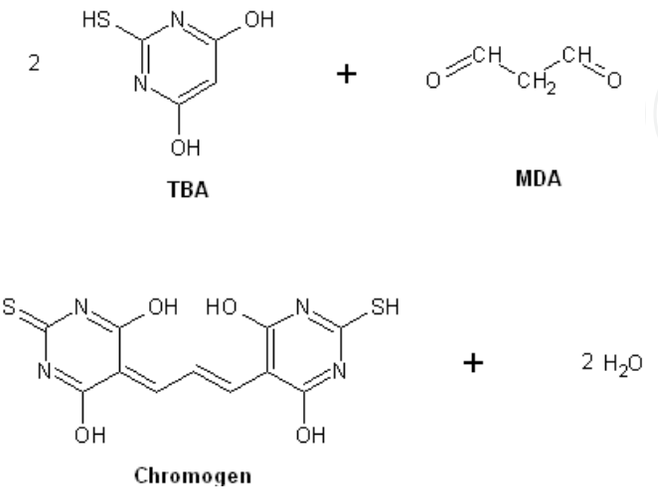


Figure 2. Chromophore produced by a condensation of MDA with TBA

1.4. Lipid hydroperoxides

1.4.1. Iodometric method

Iodometric method for lipid hydroperoxides determination is one of the oldest methods and is still used to determine lipid peroxide number [42,85]. Principle of this method is based on the ability of lipid hydroperoxides to oxidize iodide (I^-) to iodine (I_2), which further reacts with unreacted iodide (I^-) to triiodide anion (I_3^-) [86] and can be determined spectrophotometrically at 290 or 360 nm [87]. Modification of the iodometric method using commercially available reagent used for the determination of cholesterol can also be used to determine lipid (hydro)peroxides spectrophotometrically at 365 nm [54]. The method can be applied to extracts of biological samples without present the oxidizing agents. The possible interfering factors are especially the presence of oxygen, hydrogen peroxide and protein peroxides, which are able to oxidize iodide. Oxygen interference can be avoided by the using the anaerobic cuvettes and cadmium ions, which form a complex with unreacted iodide [86]. Values of lipid hydroperoxides in human plasma determined by iodometry are about 4 $\mu mol.L^{-1}$ [88,89].

1.4.2. Ferrous oxidation in xylenol orange

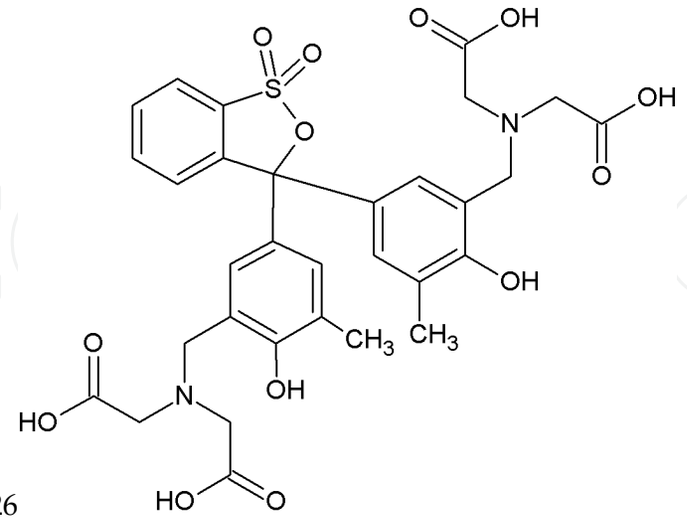
Total hydroperoxides can be determined using the oxidation of ferrous ions in the test with xylenol orange (FOX). The principle of the FOX method is based on the oxidation of ferrous ions to ferric by the hydroperoxide activity in the acidic environment [90-94]. The exact mechanism of the sequence of radical reactions is not known, but the mechanism has been designed by Gupta et al. [95] and is shown in reactions 1-4 (equation 2) [96]. The increase in the concentration of ferric ion is then detected using xylenol orange (Fig. 3), which forms a blue-violet complex with ferric ion (equation 2, reaction 5) with an absorption maximum at 560 nm [35]. However, the experimentally determined stoichiometry of 3 moles of Fe^{3+}-xylenol orange produced from 1 mol of peroxide [96,97] cannot be explained by the mechanism proposed by Gupta [95].

$$(1)\ Fe^{2+} + LOOH + H^+ \rightarrow Fe^{3+} + H_2O + LO^{\bullet}$$
$$(2)\ LO^{\bullet} + \text{xylenol orange} + H^+ \rightarrow LOH + \text{xylenol orange}^{\bullet}$$
$$(3)\ \text{Xylenol orange}^{\bullet} + Fe^{2+} \rightarrow \text{xylenol orange} + Fe^{3+}$$
$$(4)\ LO^{\bullet} + Fe^{2+} + H^+ \rightarrow Fe^{3+} + LOH$$
$$(5)\ Fe^{3+} + \text{xylenol orange} \rightarrow \text{blue} - \text{violet complex}\ (560\ \text{nm})$$

Scheme 2. Equation of mechanism sequence of radical reactions.

Gay et al. [90] have found during comparison of the reactions of different peroxides with FOX reagents that the stoichiometry of the reaction ranged from 2.2 (H_2O_2) to 5.3 moles (Cu-OOH, *t*-BuOOH) Fe^{3+}-xylenol orange (Fe-XO) generated from 1 mol of peroxide, which was observed due to determination of molar absorption coefficients of Fe-XO complexes. Therefore, it is possible to compare only the results of FOX method analyses, in which the

same type of peroxide was used in calibration. Hydrogen peroxide (H_2O_2) and Cumene hydroperoxide (Cu-OOH) are the most often peroxides used to calibrate the FOX method.



26

Figure 3. Structural formula of xylenol orange

The literature describes two versions of the FOX method called FOX1 and FOX2.. - FOX1 method can be used for the hydroperoxides determination in water phase and FOX2 method is suitable for the hydroperoxides of the lipid phase [30,35,98]. In the FOX1 method, chemicals used for a preparation of reagents (ferrous salt and sulphuric acid) are dissolved in water, whereas in FOX2 method methanol (90 % *v*/*v*) is the solvent [35]. FOX methods are not specific to hydroperoxides, the presence of oxidizing agent(s) in sample leads to the oxidization of ferrous ions to ferric ions. In the case of FOX2, the specificity of the method is achieved by the first FOX2 test performance in the presence of triphenylphosphine (TPP), which selectively reduces hydroperoxides to alcohols. The result of this test is used as a blank. After it, the FOX test without triphenylphosphine is performed and after deduction of blank values, we get the real value of lipid hydroperoxides. Improved specificity of the method using triphenylphosphine was later achieved also in FOX1 test [99]. Peroxidation chain reactions, which might occur during the analysis, are prevented by the addition of butylated hydroxytoluene prevented into the FOX1 agent. Plasma samples collected using ethylenediaminetetraacetic acid (EDTA) or diethylenetriaminepentaacetic acid pentasodium salt abbreviated as DETAPAC (anticoagulants or iron chelating agents) cannot be used due to interference with FOX reagents [30]. FOX1 method has been automated [100].

Measurement of lipid peroxidation in (blood) plasma

Banerjee et al. [99] enhanced sensitivity of FOX1 method by the addition of sorbitol into the FOX1 reagent in accordance with Wolff [98], and concurrently by the stabilization of pH of reagents at the values of 1.7 - 1.8. Improved specificity of method was obtained using triphenylphosphine and butylated hydroxytoluene. A comparison of both FOX1 and FOX2 methods on plasma samples of healthy individuals and diabetic patients was performed, where modified FOX1 method was more sensitive compared to the FOX2 method. Another advantage of the FOX1 method was based on the skip the centrifugation step that is

necessary in FOX2 method. Nourooz-zadeh et al. [55] determined total lipid hydroperoxides in plasma by the use the FOX2 method and subsequently monitored content of lipid hydroperoxides in individual lipoprotein fractions (VLDL, LDL and HDL fractions). Content of total lipid hydroperoxides in plasma was 3.50±2.05 μmol/L. The highest rate of hydroperoxides (67 %) was detected in LDL lipoprotein fractions. Södergren et al. [101] studied the impact of the storage of samples at low temperatures on the total lipid hydroperoxide content by the use the FOX2 method. They were focused on possible reduction of total lipid hydroperoxides content during the storage of samples under these conditions. Researchers found that storage of samples for 6 weeks at -70 °C leads to the 23 % average reduction of hydroperoxides content. The finding that the content of lipid hydroperoxides in short-term stored plasma samples (6 weeks) did not differ from the content of lipid hydroperoxides in the long-term stored samples (60 weeks) was interesting too.

Measurement of lipid peroxidation in animal tissues

Hermes-Lima et al. [96] proposed and elaborated methodology for application of FOX1 test in determination of lipid hydroperoxides in animal tissue extracts. They used methanol extracts of kidney, liver and heart from adult mice (*Mus musculus* Linnaeus), brain and lungs from adult Wistar rats (*Rattus norvegicus* Berkenhout var. *alba*), liver and adipose tissues from adult golden-mantled ground squirrels (*Spermophilus lateralis* Say), and liver and muscle tissues from adult red-eared slider turtles (*Trachemis scripta elegans* Wied-Neuwied). The highest values of lipid hydroperoxide content were detected in mice organs. The contents of lipid peroxides in animal tissues measured by the FOX1 method well correlated with results obtained by the TBARS. Grau et al. [102] adapted the FOX2 method for the determination of lipid hydroperoxides in raw and cooked dark chicken meat. Chickens were fed by a diet with different contents of α-tocopherol and fats from different sources. They determined the absolute values of lipid hydroperoxides in different experimental groups of chickens. Eymard et al. [56] modified the FOX1 method used by Hermes-Lima et al. [96] for the determination of lipid hydroperoxides in small pelagic fish. They used methanol extracts of ground tissues of the Atlantic horse mackerel (*Trachurus trachurus* Linnaeus). The original FOX1 reagent was replaced by the FOX2 reagent used by Wolff et al. [98] with the increased content of methanol to increase a solubility of extracts.

Measurement of lipid peroxidation in plant tissues

De Long et al. [44] applied the FOX2 method in the determination of hydroperoxides in plant tissues. They used ethanol extracts of pericarp of avocado (*Persea americana* P. Mill.), periderm of potatoes (*Solanum tuberosum* L.), leaves of red cabbage (*Brassica oleracea* convar. *capitata* var. *rubra* DC. Ranost), leaves of spinach (*Spinacia oleracea* L.), pericarp of the European Pear (*Pyrus communis* L.) and fruits of red pepper (*Capsicum annuum* L.) for analyses. The effect of UV radiation on lipid peroxidation was monitored. Parts of plants were exposed to UV radiation for 10-12 days prior the extraction due to induction of lipid peroxidation in plants. Lipid hydroperoxides were determined by the FOX2, the TBARS and

the iodometric methods. UV radiation induced an increase in lipid peroxidation values in all samples of different plant tissues determined by the FOX method. The good correlation was found between the FOX and iodometric methods. However, the iodometric method had limitations in the determination of the low concentrations of lipid hydroperoxides. Similar results were obtained by the use the TBARS method. Griffiths et al. [103] applied the FOX2 method in determination of lipid peroxides in different types of plant tissues. They analysed plant tissues, such as extracts of bean hypocotyls (*Phaseolus* sp.) and microsomes, potato leaves (*Solanum tuberosum* L.), flowers of alstromeria (*Alstroemeria* spp.), broccoli (*Brassica oleracea* var. *italica* Plenck) and cells of green algae (*Chlamydomonas* sp.). Lipid hydroperoxide levels ranged from 26 to 602 nmol.g^{-1} of FW. The highest content of lipid hydroperoxides was detected in broccoli and green alga cells in their study.

2. Experimental section

2.1. Instruments

For dilution of stock solutions of standards an epMotion 5075 (Eppendorf, Germany) automated pipetting system was used (Fig. 4). The pipetting provides a robotic arm with adapters (TS 50, TS 300 and TS 1000) and Gripper (TG-T). The empty microtubes are placed in the position B3 (Fig. 4) in adapter Ep0.5/1.5/2 ml. Module Reservoir is located in the position B1, where stock solutions are available. The device is controlled by the epMotion control panel. The tips are located in the A4 (ePtips 50), A3 (ePtips 300) and A2 (ePtips 1000) positions. For preparation of the standards tips of sizes 300 μl and 1000 μl (Eppendorf – Germany) were used. For determination of antioxidant activity, a BS-400 automated spectrophotometer (Mindray, China) was used. It is composed of cuvette space tempered to 37±1 °C, reagent space with a carousel for reagents (tempered to 4±1 °C), sample space with a carousel for preparation of samples and an optical detector. Transfer of samples and reagents is provided by robotic arm equipped with a dosing needle (error of dosage up to 3 % of volume). Cuvette content is mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples. Contamination is reduced due to its rinsing system, including rinsing of the dosing needle as well as the stirrer by MilliQ water. For detection itself, the following range of wave lengths can be used as 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm. In addition, a SPECOL 210 two beam UV-VIS spectrophotometer (Analytik Jena AG, Germany) with cooled semiconductor detector for measurement within range from 190 to 1,100 nm with control by an external PC with the programme WinASPECT was used as the manual instrument in this study. Laboratory scales (Sartorius, Germany) and pipettes (Eppendorf Research, Germany) were used.

2.2. Chemicals

Xylenol orange disodium salt, iron D-gluconate dihydrate, glycerol, *tert*-butylhydroperoxide (t-BHP) 70% in water, sodium chloride, sulphuric acid, formic acid and water ACS reagent were purchased from Sigma Aldrich (USA).

2.3. Preparation of reagents and standards

FOX1 reagents were prepared according Arab et al. [100]. The general acidic reagent (acidic reagent A) final concentrations were 0.9 % NaCl, 40 mM H_2SO_4, 20 mM formic acid and 1.37 M glycerol in ACS water. The pH of the reagent was adjusted to the value of 1.35. The reagent R1 contained 167 μM xylenol orange disodium salt, which was dissolved in acidic reagent A. The reagent R2 contained 833 μM iron D-gluconate dehydrate, which was also dissolved in acidic reagent A. Standards were prepared from the 70% water solution of *tert*-butylhydroperoxide, which was diluted by ACS water to the 20 mM pre-stock solution. From the pre-stock solution, five stock solutions: and 0.2, 3.9, 62.5, 375 and 1,000 μM were prepared daily by dilutions of pre-stock solution with 0.9 % NaCl. For further preparation of 20 standards from five stock solutions, an automated pipetting system epMotion 5075 was used to minimalize possible pipetting errors. The standards had following concentrations: 0.06, 0.12, 0.24, 0.48, 0.97, 1.9, 3.9, 7.8, 15.6, 31.2, 46.8, 62.5, 93.7, 125, 187, 250, 375, 500, 750 and 1000 μM. These standards were used for the preparation of calibration curves in both manual and automatic measurements.

2.4. Working procedure for manual spectrophotometric determination

A volume of 720 μl of the reagent R1 (167 μM xylenol orange in acidic reagent) was pipetted into plastic cuvettes. Subsequently, a volume of 100 μl of the sample was added. Absorbance was measured at λ = 591 nm. After it, a volume of 180 μl of the reagent R2 (833 μM iron D-gluconate in acidic reagent A) was pipetted to a reaction mixture and after 6 minutes of the incubation, absorbance was measured. Final value is calculated from the absorbance value of the mixture of the reagent R1 with sample and from the absorbance value after 6 minutes of incubation of the mixture with the reagent R2. The final concentrations in the cuvette of xylenol orange (R1) and iron D-gluconate (R2) were 120 and 150 μM, respectively.

2.5. Working procedure for automated spectrophotometric determination

A volume of 180 μL of the solution R1 (167 μM xylenol orange in acidic reagent) was pipetted into a plastic cuvette with subsequent addition of a 25 μL of sample. This mixture was incubated for 4.5 minutes. Subsequently, 45 μL of solution R2 (833 μM iron D-gluconate in acidic reagent) was added and the solution was incubated for next 6 minutes. Absorbance was measured at λ = 570 nm. Final value is calculated from the absorbance value of the mixture of reagent R1 with sample before the addition of the reagent 2 and from the absorbance value after 6 minutes of incubation of the mixture with the reagent 2. The final concentrations in the cuvette of xylenol orange (R1) and iron D-gluconate (R2) were 120 and 150 μM, respectively.

3. Results and discussion

Spectrophotometric methods for determination of lipid peroxidation have a relatively simple procedure of a measurement. In addition, they are relatively low-cost with easy

applicability and they do not require specialized equipment or personnel. To maintain the sustainability of these methods, it is necessary to introduce these methods to automated operation, which has not been yet satisfactorily solved. Analyses of samples performed due to intensive work of personnel, which is expensive, slow, and, in addition, the human factor is responsible for a high percentage of errors. Requirement for laboratories, in which a large number of samples is analysed per day, consists in relatively simple and easy to apply method. Our aim was to automate the pre-analytical and analytical phase of the FOX1 method. For specification and comparison of this method, the method based on the use the manual spectrophotometer was also carried out.

3.1. Pre-analytical phase

Pre-analytical processing of biological samples in the laboratory is a necessary and important part of laboratory work. It represents a wide range of manual, often stereotyped operations that do not require special knowledge and skills, but require maintenance of the standard procedure(s) and prevent the possibility of errors connected with this analytical phase. Pre-analytical laboratory process is destined to automation and robotics. Automation and robotics of the pre-analytical phase brings many benefits and advantages to laboratory. It reduces the number of errors, the time necessary for sample manipulation, and the response time. It significantly increases the productivity, cost savings connected with productivity, and minimizes the exposure of personnel with biological material [104].

For automation of pre-analytical phase, the epMotion 5075 automated pipetting system was used. Stock solutions of *tert*-butylhydroperoxide (*t*-BHP) at the concentrations of 1000, 375, 62.5, 3.9 and 0.2 μM prepared in 0.9 % NaCl solution were applied into five vials. Sixth vial contained diluting solution (0.9% NaCl). Twenty empty Eppendorf tubes (1.5 ml) were placed into the metal holder. Scheme of the preparation of standards is shown in Table 2. Pipetting robot first pipetted different volumes of diluting solution (0.9% NaCl) into vials and after it, different volumes of stock solutions of various concentrations of *t*-BHP were pipetted. When pipetting the stock solution into the dilution buffer in micro test tube, robot three times mixed the solution by a pipetting.

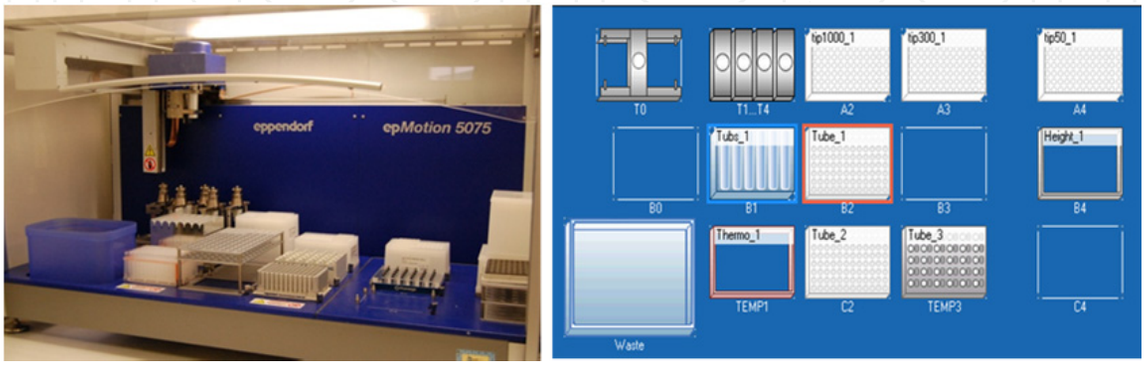


Figure 4. epMotion 5075 automated pipetting system from frontal part.

Tube nb.	Final concentration t-BHP (μM)	Pipetting volume (μl)					
		solution 0.9% NaCl	solution 1 1000 μM t-BHP	solution 2 375 μM t-BHP	solution 3 62.5 μM t-BHP	solution 4 3.906 μM t-BHP	solution 5 0.244 μM t-BHP
1	1000	-	1000	-	-	-	-
2	750.0	250	750	-	-	-	-
3	500.0	500	500	-	-	-	-
4	375.0	-	-	1000	-	-	-
5	250.0	750	250	-	-	-	-
6	187.5	500	-	500	-	-	-
7	125.0	875	125	-	-	-	-
8	93.75	750	-	250	-	-	-
9	62.50	-	-	-	1000	-	-
10	46.87	875	-	125	-	-	-
11	31.25	500	-	-	500	-	-
12	15.62	750	-	-	250	-	-
13	7.812	875	-	-	125	-	-
14	3.906	-	-	-	-	1000	-
15	1.953	500	-	-	-	500	-
16	0.977	750	-	-	-	250	-
17	0.488	875	-	-	-	125	-
18	0.244	-	-	-	-	-	1000
19	0.122	500	-	-	-	-	500
20	0.061	750	-	-	-	-	250

Table 2. Volume of the solution in the preparation of standards using epMotion 5075 automated pipetting system.

Using the epMotion 5075 automated pipetting system, work time of 20 minutes was saved (time, when laboratory staff was not needed). The only time-demanding operation consisted in replenishment of vials and initiation of the program. Potential errors that arise due to human activity were avoided. Accuracy of a pipetting was verified by weighing, the average error was approximately 1.8 %.

3.2. Analytical phase

Our goal was to introduce the FOX1 method to an automated operation and improve both analysis itself and conditions of analysis. The experiment was carried out using *tert*-butylhydroperoxide standard prepared at the concentrations from 0.06 to 1000 μM. Furthermore, the spectral curves of generated chromatic complexes were observed and the concentration dependence on temperature and time were determined. In addition, reaction kinetics during the reaction was established.

3.2.1. Monitoring the spectral courses at different concentrations and times

Spectral changes in the *t*-BHP concentration range from 0.06 to 1000 μM (Figures 5A and 4B) were observed. Two peaks at the wavelengths of 444 and 591 nm were detected in the formed complex at the recommended temperature of interaction of 37 °C.

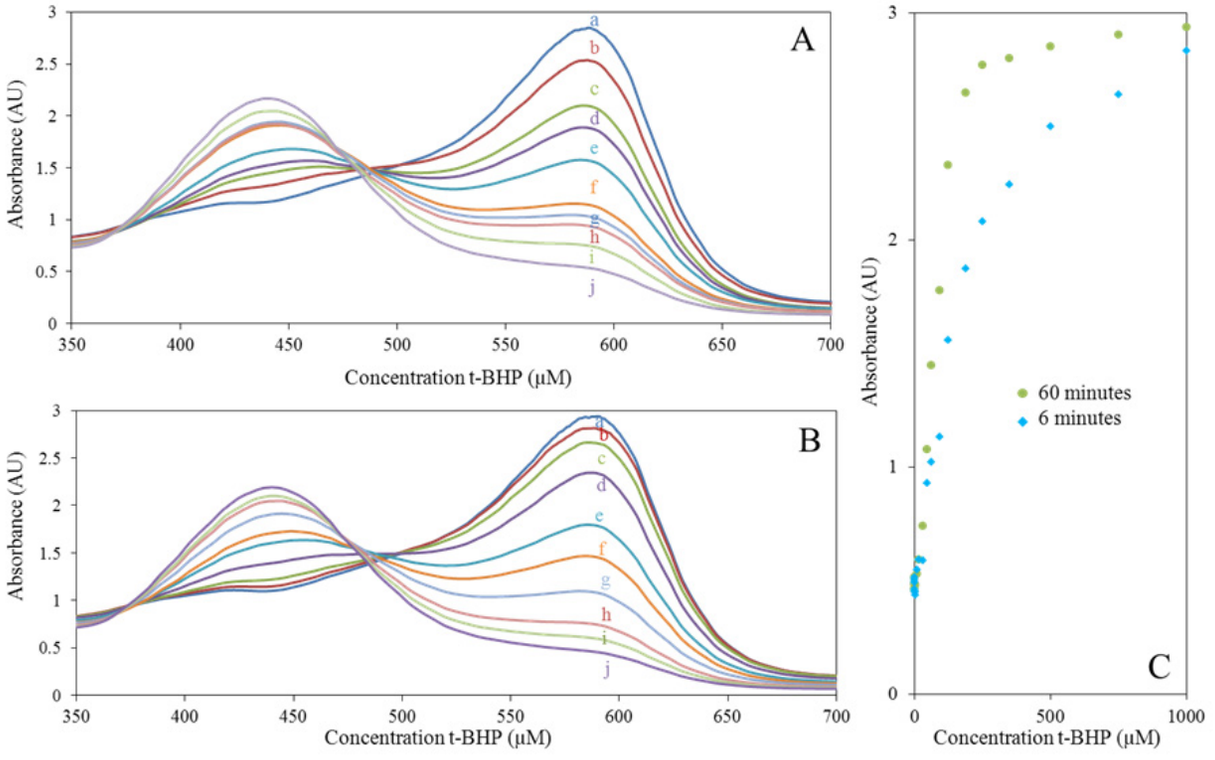


Figure 5. Courses of spectra of *t*-BHP in the concentrations from 0.06 to 1000 μM - **a)** 1000, **b)** 500, **c)** 250, **d)** 125, **e)** 62.50, **f)** 31., **g)** 7.8, **h)** 1.9, **i)** 0.4, **j)** 0.06 in the time of 6 **(A)** and 60 **(B)** minutes. **(C)** Comparison of values of absorption maximum at the wavelength of 591 nm and a time period of 6 and 60 minutes. The courses were measured in the interval form 350 to 700 nm using the SPECORD 210 apparatus. All analyses were carried out in triplicates.

Absorption maximum at low concentrations (up to the concentration of 0.122 μM) was at 444 nm, and with the increasing concentrations (higher than 0.122 μM) the absorption maximum was sifted and observed at 591 nm. Interaction of sample and reagents proceeded in six minutes, after this time, absorbance could be measured and the final value of lipid peroxidation calculated. We wanted to determine the changes in the absorbance during one hour. Comparison of absorbance values at the time of 6 and 60 min at λ = 591 nm is shown in Figure 5C. Absorbance values during the monitoring decreased for about 13 % on an average. When interlaying the trends points in the linear concentration part from 0.12 to 125 μM, the determination factor decreased from 0.996 (for the 6-minute reaction time) to 0.987 (for the 60-minute reaction time). This fact can be explained by unequal reaction kinetics during the analysis (see the reaction kinetics, Chapter 3.2.3) and oxidation of the sample during the analysis.

3.2.2. Monitoring the reaction under different temperature conditions

Dependences of representative concentration (62.5 μM) on the temperature conditions (17, 27, 37 and 47 °C) and the time from 0 to 30 minutes and absorption maximum of 591 nm is shown in Figure 6. The absorbance increased with the increasing temperature; after 6 minutes of reaction, the difference of absorbance value between the lowest (17 °C) and the highest (47 °C) temperature was about 0.64 AU. In other words, the value of absorbance at 47 °C was higher for 71 % compared to the absorbance determined at 17 °C. The highest values of absorbance and concurrently the most prominent difference was detected at 47 °C, therefore, this temperature was the most suitable for our purposes. On the other hand, this temperature may lead to degradation of biological samples. Due to this fact, the temperature of interaction of 37 °C was selected for further analyses.

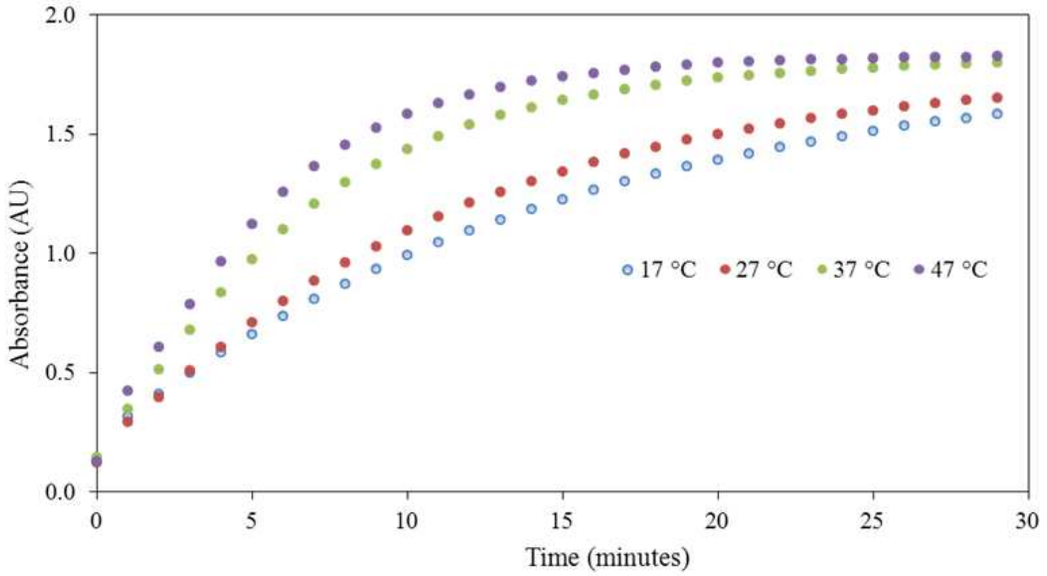


Figure 6. Dependences of representative concentration (62.5 μM) of applied *t*-BHP on temperature conditions (17, 27, 37 and 47 °C) and the time of interaction. Detected at 591 nm, interval of record is 1 minute, interval period 0 - 30 minutes. All analyses were carried out in triplicates.

3.2.3. Determination of reaction kinetics

Reaction kinetics at the temperature of 37 °C in the shortest time intervals in all concentrations (0.06 – 1000 μM) was monitored. Automated analyser BS-400 was used for this purpose. All samples could be studied at all once. This is not possible using the manual spectrophotometer, thus, use the automated analyser represents one of the most important steps in the analysis automation.

The curves were used for the calculating the reaction rate constants indicating the course and conception of the impact of the effect of *t*-BHP concentration on the reaction rate. The constant was calculated as the change in the absorbance per time unit (second, minute) according to the equation x = A/t, where x is the rate constant, A the value of absorbance after 6 minutes and t time for which the rate constant was related (second, minute). The effect of each of concentrations on the change in absorbance value was determined.

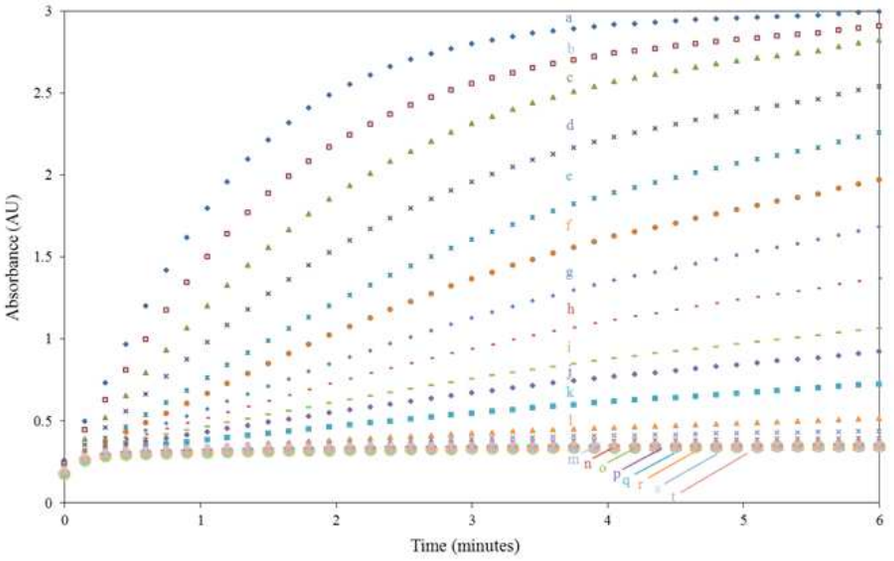


Figure 7. Monitoring of reaction curves of *t*-TBH in the concentrations from 0.06 to 1000 μM - **a)** 1000, **b)** 750, **c)** 500, **d)** 375, **e)** 250, **f)** 187, **g)** 125 **h)** 94, **i)** 63, **j)** 47., **k)** 31, **l)** 15.6, **m)** 7.8, **n)** 3.9, **o)** 1.9, **p)** 0.9, **q)** 0.4, **r)** 0.2, **s)** 0.1, and **t)** 0.06 μM in the time interval from 0 to 6 minutes. All analyses were carried out in triplicates.

Concentration	Logarithmic equation	Change in absorbance per second	Change in absorbance per minute	Change in abs. per minute recalculated to 1 μM t-BHP
1000	y = 3.7532ln(x) - 5.899	0.02304	1.383	0.0013
750.0	y = 3.6495ln(x) - 5.544	0.02211	1.345	0.0017
500.0	y = 3.4895ln(x) - 5.241	0.02168	1.301	0.0028
375.0	y = 3.1895ln(x) - 4.872	0.01987	1.258	0.0036
250.0	y = 3.2076ln(x) - 4.677	0.01853	1.112	0.0044
187.5	y = 2.7574ln(x) - 4.375	0.01534	0.924	0.0052
125.0	y = 2.2477ln(x) - 3.945	0.01298	0.779	0.0062
93.75	y = 1.7316ln(x) - 2.968	0.01000	0.600	0.0060
62.50	y = 1.2213ln(x) - 1.998	0.00705	0.423	0.0068
46.87	y = 1.0049ln(x) - 1.596	0.00580	0.348	0.0070
31.25	y = 0.7102ln(x) - 1.054	0.00410	0.246	0.0079
15.62	y = 0.3846ln(x) - 0.445	0.00222	0.133	0.0085
7.812	y = 0.2525ln(x) - 0.183	0.00146	0.088	0.0112
3.906	y = 0.1765ln(x) - 0.037	0.00102	0.061	0.0157
1.953	y = 0.1303ln(x) + 0.033	0.00075	0.045	0.0231
0.976	y = 0.1177ln(x) + 0.073	0.00068	0.041	0.0418
0.488	y = 0.1031ln(x) + 0.089	0.00060	0.036	0.0732
0.244	y = 0.0965ln(x) + 0.101	0.00057	0.034	0.1370
0.122	y = 0.0926ln(x) + 0.105	0.00055	0.033	0.2629
0.061	y = 0.0957ln(x) + 0.131	0.00053	0.032	0.5434

Table 3. Mathematical formularization of the course of reaction curves for *t*-TBH in the concentration range from 0.06 to 1000 μM by the use the logarithmic equation. Reaction rate constant is expressed as a

change in absorbance per second, and per minute. In addition, change in absorbance per minute recalculated to 1 μM t-BHP is introduced.

3.2.4. Dependence on concentration

By the using manual spectrophotometer and automated analyser, the dependence of *t*-TBH concentration (0.06 – 1000 μM) on the changes of coloured complex was determined. The calibration curves were calculated from final values.

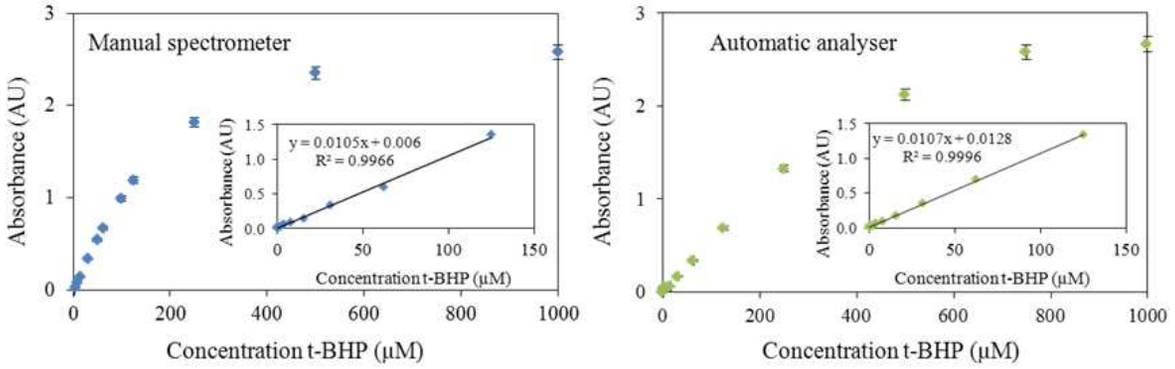


Figure 8. Dependence of absorbance on applied *t*-BHP concentration measured by manual spectrophotometer SPECOL 210 and automated analyser BS-400. All analyses were carried out in triplicates. For other experimental detail, see Fig. 7.

The analysis of 60 samples (20 samples in a standard three repetitions) took using the BS-400 automated analyser only 24 minutes. The analysis of 60 samples including delays for the pipetting, mixing and displacement of samples using the manual spectrophotometer took about 7 hours (6 minutes per sample + one minute of delay, 60 × 6 minutes of sample analysis). By using the fully automated analyser, results were obtained in more than 17 times less time compared to manual spectrophotometer. Shortening of the time of analysis contributes especially to higher quality of results due to reduction of possibility of chemical modification including degradation of the measured samples. This fact resulted in the preparation of calibration curves, where the determination factor for the calibration curve obtained using the automatic analyser was $R^2 = 0.9996$, while the determination factor for the results from manual spectrophotometer was $R^2 = 0.9966$. In addition, a limit of detection (LOD) and limit of quantification (LOQ) were determined. In the case of both automated and manual analyses, the LOD was determined as LOD = 0.06 μM of *t*-BHP, limit of quantification (LOQ) was also determined as LOQ = 0.2 μM of *t*-BHP (see Table 3). All measurements of all concentrations of *t*-BHP (concentration range from 0.06 to 1000 μM) were carried out in 3 repetitions and repeatability (RSD) was determined. In the case of automated method, the repeatability was RSD = 2.6 % compared to manual spectrophotometer, where RSD = 3.8 %.

Technical development is responsible for a tendency to increase the speed of analysis and analytical process itself. Automatic analysers allow analysing more samples at the same time, reducing the time required to analyse one sample and errors caused by incorrect

pipetting and manipulation with sample, and generally provide higher data quality compared to manual analysis. Due to automation, the risk of sample confusion is significantly reduced. In addition, the whole process is much faster, the consumption of reagents and demands of personnel staff are reduced. The aim of automation is to eliminate stereotypical incompetent operation, eliminate the possibility of error, and to accelerate operations under significant increase of capacity while maintaining the precise performance of all necessary operations. The disadvantage, however, consists in still high acquisition costs and the need for compete service [105,106].

Apparatus	Wavelength (nm)	LOD	LOQ	Measuring range (μM)	Calibration equation	Confidence coefficient (R^2)	RSD	Time analysis of 60 samples (min)
SPECOL	591	0.06	0.2	0.012 - 125	y=0.0105x +0.006	0.9969	3.8	420
BS-400	570	0.06	0.2	0.012 - 125	y=0.0107x +0.0128	0.9996	2.6	24

Table 4. Analytic parameters for the FOX1 method for *t*-BHP analysis using manual SPECOL and automated BS-400 analysers.

4. Conclusion

This chapter brought a comprehensive overview of photometric methods used in the study of lipid peroxidation. Main attention was devoted to the detection of lipid peroxidation by using the less common FOX1 method. The proposal to automation the pre-analytical and analytical phases of the sample was introduced. In addition, conditions and parameters influencing the photometric reaction were studied and described. The comparison of results obtained using the manual and automated apparatuses (manual/automated operation) is introduced and discussed.

Author details

Jiri Sochor, Branislav Ruttkay-Nedecky, Vojtech Adam, Jaromir Hubalek and Rene Kizek*
Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Brno, Czech Republic, European Union
Department of Microelectronics, Faculty of Electrical Engineering and Communication, Brno University of Technology, Brno, Czech Republic, European Union

Petr Babula
Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic, European Union

* Corresponding Author

Acknowledgement

This project was supported by SIX research centre CZ.1.05/2.1.00/03.0072. The authors wish to express their thanks to Lukáš Nejdl for excellent technical assistance.

5. References

[1] Grinna LS (1977) Age-related changes in lipids of microsomal and mitochondrial-membranes of rat-liver and kidney Mech. Ageing Dev. 6: 197-205.

[2] De Zwart LL, Meerman JHN, Commandeur JNM, Vermeulen NPE (1999) Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic. Biol. Med. 26: 202-226.

[3] Knight JA (1995) Diseases related to oxygen-derived free-radicals. Ann. Clin. Lab. Sci. 25: 111-121.

[4] Gutteridge JMC (1993) Free-radicals in disease processes - A compilation of cause and consequence. Free Rad. Res. Commun. 19: 141-158.

[5] Evans PH (1993) Free-radicals in brain metabolism and pathology. Br. Med. Bull. 49: 577-587.

[6] Adams JD, Odunze IN (1991) Oxygen free-radicals and Parkinsons-disease. Free Radic. Biol. Med. 10: 161-169.

[7] Pohanka M, Novotny L, Zdarova-Karasova J, Bandouchova H, Zemek F, Hrabinova M, Misik J, Kuca K, Bajgar J, Zitka O, Cernei N, Kizek R, Pikula J (2011) Asoxime (HI-6) impact on dogs after one and tenfold therapeutic doses: Assessment of adverse effects, distribution, and oxidative stress. Environ. Toxicol. Pharmacol. 32: 75-81.

[8] Diopan V, Babula P, Shestivska V, Adam V, Zemlicka M, Dvorska M, Hubalek J, Trnkova L, Havel L, Kizek R (2008) Electrochemical and spectrometric study of antioxidant activity of pomiferin, isopomiferin, osajin and catalposide. J. Pharm. Biomed. Anal. 48: 127-133.

[9] Babula P, Kohoutkova V, Opatrilova R, Dankova I, Masarik M, Kizek R (2010) Pharmaceutical importance of zinc and metallothionein in cell signalling. Chim. Oggi-Chem. Today. 28: 18-21.

[10] Jurikova T, Rop O, Mlcek J, Sochor J, Balla S, Szekeres L, Hegedusova A, Hubalek J, Adam V, Kizek R (2012) Phenolic Profile of Edible Honeysuckle Berries (Genus Lonicera) and Their Biological Effects. Molecules. 17: 61-79.

[11] Sochor J, Skutkova H, Babula P, Zitka O, Cernei N, Rop O, Krska B, Adam V, Provaznik I, Kizek R (2011) Mathematical evaluation of the amino acid and polyphenol content and antioxidant activities of fruits from different apricot cultivars. Molecules. 16: 7428-7457.

[12] Sochor J, Zitka O, Skutkova H, Pavlik D, Babula P, Krska B, Horna A, Adam V, Provaznik I, Kizek R (2010) Content of phenolic compounds and antioxidant capacity in fruits of apricot genotypes. Molecules. 15: 6285-6305.

[13] Rop O, Sochor J, Jurikova T, Zitka O, Skutkova H, Mlcek J, Salas P, Krska B, Babula P, Adam V, Kramarova D, Beklova M, Provaznik I, Kizek R (2011) Effect of five different

stages of ripening on chemical compounds in Medlar (Mespilus germanica L.). Molecules. 16: 74-91.

[14] Rop O, Jurikova T, Sochor J, Mlcek J, Kramarova D (2011) Antioxidant capacity, scavenging radical activity and selected chemical composition of native apple cultivars from Central Europe. J. Food Qual. 34: 187-194.

[15] Balestrieri ML, Dicitore A, Benevento R, Di Maio M, Santoriello A, Canonico S, Giordano A, Stiuso P (2012) Interplay between membrane lipid peroxidation, transglutaminase activity, and Cyclooxygenase 2 expression in the tissue adjoining to breast cancer. J. Cell. Physiol. 227: 1577-1582.

[16] Cai F, Dupertuis YM, Pichard C (2012) Role of polyunsaturated fatty acids and lipid peroxidation on colorectal cancer risk and treatments. Curr. Opin. Clin. Nutr. Metab. Care. 15: 99-106.

[17] Zalejska-Fiolka J, Wielkoszynski T, Kasperczyk S, Kasperczyk A, Birkner E (2012) Effects of Oxidized Cooking Oil and alpha-Lipoic Acid on Blood Antioxidants: Enzyme Activities and Lipid Peroxidation in Rats Fed a High-Fat Diet. Biol. Trace Elem. Res. 145: 217-221.

[18] Flohr L, Fuzinatto CF, Melegari SP, Matias WG (2012) Effects of exposure to soluble fraction of industrial solid waste on lipid peroxidation and DNA methylation in erythrocytes of Oreochromis niloticus, as assessed by quantification of MDA and m(5)dC rates. Ecotox. Environ. Safe. 76: 63-70.

[19] Janowska B, Kurpios-Piec D, Prorok P, Szparecki G, Komisarski M, Kowalczyk P, Janion C, Tudek B (2012) Role of damage-specific DNA polymerases in M13 phage mutagenesis induced by a major lipid peroxidation product trans-4-hydroxy-2-nonenal. Mutat. Res.-Fundam. Mol. Mech. Mutagen. 729: 41-51.

[20] Demir E, Kaya B, Soriano C, Creus A, Marcos R (2011) Genotoxic analysis of four lipid-peroxidation products in the mouse lymphoma assay. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 726: 98-103.

[21] Guo LL, Chen ZY, Cox BE, Gragg S, Zhang YQ, Amarnath V, van Lenten B, Epand R, Davies SS (2011) Lipid Peroxidation Generates Aldehyde-Modified Phosphatidylethanolamines That Induce Inflammation. Free Radic. Biol. Med. 51: S103-S104.

[22] Guo J, Prokai L (2011) To tag or not to tag: A comparative evaluation of immunoaffinity-labeling and tandem mass spectrometry for the identification and localization of posttranslational protein carbonylation by 4-hydroxy-2-nonenal, an end-product of lipid peroxidation. J. Proteomics. 74: 2360-2369.

[23] Esterbauer H, Schaur RJ, Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11: 81-128.

[24] Benedetti A, Comporti M, Esterbauer H (1980) Identification of 4-hydroxynoneal as a cyto-toxic product originating from the peroxidation of liver microsomal lipids. Biochim. Biophys. Acta. 620: 281-296.

[25] Esterbauer H, Comporti M, Benedetti A (1980) Biochemical effects of 4-hydroxyalkenals, in particular 4-hydroxynonenal produced by microsomal lipid-peroxidation. J. Am. Oil Chem. Soc. 57: A144-A145.
[26] Gutteridge JMC (1995) Lipid-peroxidation and antioxidants as biomarkers of tissue-damage Clin. Chem. 41: 1819-1828.
[27] Tanrikulu AC, Abakay A, Evliyaoglu O, Palanci Y (2011) Coenzyme Q10, Copper, Zinc, and Lipid Peroxidation Levels in Serum of Patients with Chronic Obstructive Pulmonary Disease. Biol. Trace Elem. Res. 143: 659-667.
[28] Viita H, Pacholska A, Ahmad F, Tietavainen J, Naarala J, Hyvarinen A, Wirth T, Yla-Herttuala S (2012) 15-Lipoxygenase-1 Induces Lipid Peroxidation and Apoptosis, and Improves Survival in Rat Malignant Glioma. In Vivo. 26: 1-8.
[29] Kuehl FA, Egan RW (1980) Prostaglandins, arachidonic-acid, and inflammation. Science. 210: 978-984.
[30] Nourooz-Zadeh J Ferrous ion oxidation in presence of xylenol orange for detection of lipid hydroperoxides in plasma, in: L. Packer (Ed.), Oxidants and Antioxidants, Pt B, 1999, pp. 58-62.
[31] Rop O, Mlcek J, Jurikova T, Valsikova M, Sochor J, Reznicek V, Kramarova D (2010) Phenolic content, antioxidant capacity, radical oxygen species scavenging and lipid peroxidation inhibiting activities of extracts of five black chokeberry (Aronia melanocarpa (Michx.) Elliot) cultivars. J. Med. Plants Res. 4: 2431-2437.
[32] Nouroozzadeh J, Gopaul NK, Barrow S, Mallet AI, Anggard EE (1995) Analysis of F-2-isoprostanes as indicators of nonenzymatic lipid-peroxidation in-vivo by gas-chromatography mass-spectrometry - Development of a solid-phase extraction procedure. J. Chromatogr. B-Biomed. App. 667: 199-208.
[33] Bachi A, Zuccato E, Baraldi M, Fanelli R, Chiabrando C (1996) Measurement of urinary 8-epi-prostaglandin F-2 alpha, a novel index of lipid peroxidation in vivo, by immunoaffinity extraction gas chromatography mass spectrometry. Basal levels in smokers and nonsmokers. Free Radic. Biol. Med. 20: 619-624.
[34] Iversen SA, Cawood P, Dormandy TL (1985) A method for the measurement of a diene-conjugated derivative of linoleic-acid, 18-2(9,11), in serum phospholipid, and possible origins. Ann. Clin. Biochem. 22: 137-140.
[35] Nouroozzadeh J, Tajaddinisarmadi J, Wolff SP (1994) Measurement of plasma hydroperoxide concentrations by the ferrous oxidation xylenol orange assay in conjunction with triphenylphosphine. Anal. Biochem. 220: 403-409.
[36] Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M (1993) A comparative-evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological-materials. Free Radic. Biol. Med. 15: 353-363.
[37] Burk RF, Ludden TM (1989) Exhaled alkanes as indexes of invivo lipid-peroxidation. Biochem. Pharmacol. 38: 1029-1032.
[38] Nikolaidis MG, Kyparos A, Vrabas IS (2011) F(2)-isoprostane formation, measurement and interpretation: The role of exercise. Prog. Lipid Res. 50: 89-103.

[39] Janicka M, Kot-Wasik A, Kot J, Namiesnik J (2010) Isoprostanes-Biomarkers of Lipid Peroxidation: Their Utility in Evaluating Oxidative Stress and Analysis. Int. J. Mol. Sci. 11: 4631-4659.

[40] Soffler C, Campbell VL, Hassel DM (2010) Measurement of urinary F(2)-isoprostanes as markers of in vivo lipid peroxidation: a comparison of enzyme immunoassays with gas chromatography mass spectrometry in domestic animal species. J. Vet. Diagn. Invest. 22: 200-209.

[41] Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid-peroxidation in biological-systems. Trends Biochem.Sci. 15: 129-135.

[42] Halliwell B, Chirico S (1993) Lipid-peroxidation - its mechanism, measurement, and significance Am. J. Clin. Nutr. 57: S715-S725.

[43] Devasagayam TPA, Boloor KK, Ramasarma T (2003) Methods for estimating lipid peroxidation: An analysis of merits and demerits. Indian J. Biochem. Biophys. 40: 300-308.

[44] DeLong JM, Prange RK, Hodges DM, Forney CF, Bishop MC, Quilliam M (2002) Using a modified ferrous oxidation-xylenol orange (FOX) assay for detection of lipid hydroperoxides in plant tissue. J. Agric. Food Chem. 50: 248-254.

[45] Esterbauer H, Striegl G, Puhl H, Rotheneder M (1989) Continuous monitoring of invitro oxidation of human low-density lipoprotein. Free Rad. Res. Commun. 6: 67-75.

[46] Yagi K (1984) Assay for blood-plasma or serum Methods Enzymol. 105: 328-331.

[47] Hong R, Kang TY, Michels CA, Gadura N (2012) Membrane Lipid Peroxidation in Copper Alloy-Mediated Contact Killing of Escherichia coli. Appl. Environ. Microbiol. 78: 1776-1784.

[48] Martins DB, Mazzanti CM, Franca RT, Pagnoncelli M, Costa MM, de Souza EM, Goncalves J, Spanevello R, Schmatz R, da Costa P, Mazzanti A, Beckmann DV, Cecim MD, Schetinger MR, Lopes STD (2012) 17-beta estradiol in the acetylcholinesterase activity and lipid peroxidation in the brain and blood of ovariectomized adult and middle-aged rats. Life Sciences. 90: 351-359.

[49] Kwok CT, van de Merwe JP, Chiu JMY, Wu RSS (2012) Antioxidant responses and lipid peroxidation in gills and hepatopancreas of the mussel Perna viridis upon exposure to the red-tide organism Chattonella marina and hydrogen peroxide. Harmful Algae. 13: 40-46.

[50] Ahmad MK, Syma S, Mahmood R (2011) Cr(VI) Induces Lipid Peroxidation, Protein Oxidation and Alters the Activities of Antioxidant Enzymes in Human Erythrocytes. Biol. Trace Elem. Res. 144: 426-435.

[51] Naziroglu M, Akkus S, Celik H (2011) Levels of lipid peroxidation and antioxidant vitamins in plasma and erythrocytes of patients with ankylosing spondylitis. Clin. Biochem. 44: 1412-1415.

[52] Pimentel VC, Pinheiro FV, Kaefer M, Moresco RN, Moretto MB (2011) Assessment of uric acid and lipid peroxidation in serum and urine after hypoxia-ischemia neonatal in rats. Neurol. Sci. 32: 59-65.

[53] Tonin AA, Thome GR, Calgaroto N, Baldissarelli J, Azevedo MI, Escobar TP, dos Santos LG, Da Silva AS, Badke MRT, Schetinger MR, Mazzanti CM, Lopes STD (2011) Lipid

Peroxidation and Antioxidant Enzymes Activity of Wistar Rats Experimentally Infected with Leptospira interrogans. Acta Sci. Vet. 39: 966-976.

[54] Elsaadani M, Esterbauer H, Elsayed M, Goher M, Nassar AY, Jurgens G (1989) A spectrophotometric assay for lipid peroxides in serum-lipoproteins using a commercially available reagent. J. Lipid Res. 30: 627-630.

[55] NouroozZadeh J, TajaddiniSarmadi J, Ling KLE, Wolff SP (1996) Low-density lipoprotein is the major carrier of lipid hydroperoxides in plasma - Relevance to determination of total plasma lipid hydroperoxide concentrations. Biochem. J. 313: 781-786.

[56] Eymard S, Genot C (2003) A modified xylenol orange method to evaluate formation of lipid hydroperoxides during storage and processing of small pelagic fish. Eur. J. Lipid Sci. Technol. 105: 497-501.

[57] Corongiu FP, Banni S (1994) Detection of conjugated dienes by 2nd-derivative ultraviolet spectrophotometry. Oxygen Radicals in Biological Systems, Pt C. 233: 303-310.

[58] Banni S, Day BW, Evans RW, Corongiu FP, Lombardi B (1995) Detection of conjugated diene isomers of linoleic-acid in liver lipids of rats fed a choline-devoid diet indicates that the diet does not cause lipoperoxidation. J. Nutr. Biochem. 6: 281-289.

[59] Dormandy TL, Wickens DG (1987) The experimental and clinical pathology of diene conjugation. Chem. Phys. Lipids. 45: 353-364.

[60] Chin SF, Storkson JM, Liu W, Pariza MW (1991) Dietary sources of the anticarcinogen CLA (conjugated dienoic derivatives of linoleic-acid). Faseb J. 5: A1444-A1444.

[61] Britton M, Fong C, Wickens D, Yudkin J (1992) Diet as a source of phospholipid esterified 9,11-octadecadienoic acid in humans. Clin. Sci. 83: 97-101.

[62] Fairbank J, Ridgway L, Griffin J, Wickens D, Singer A, Dormandy TL (1988) Octadeca-9-11-dienoic acid in diagnosis of cervical intraepithelial neoplasia. Lancet. 2: 329-329.

[63] Jack CIA, Ridgway E, Jackson MJ, Hind CRK (1994) Serum octadeca-9,11 dienoic acid - an assay of free-radical activity or a result of bacterial production. Clin. Chim. Acta. 224: 139-146.

[64] Thompson S, Smith MT (1985) Measurement of the diene conjugated form of linoleic-acid in plasma by high-performance liquid-chromatography - A questionable noninvasive assay of free-radical activity Chem.-Biol. Interact. 55: 357-366.

[65] Janero DR (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indexes of lipid-peroxidation and peroxidative tissue-injury. Free Radic. Biol. Med. 9: 515-540.

[66] Yu TC, Sinnhuber RO (1957) 2-Thiobarbituric acid method for the measurement of rancidity in fishery products. Food Technol. 11: 104-108.

[67] Mihara M, Uchiyama M (1981) Evaluation of thiobarbituric acid (TBA) value as an index of lipid-peroxidation in CCl4-intoxicated rat-liver. Yakugaku Zasshi-J. Pharm. Soc. Jpn. 101: 221-226.

[68] Mihara M, Uchiyama M (1983) Properties of thiobarbituric acid-reactive materials obtained from lipid peroxide and tissue homogenate. Chem. Pharm. Bull. 31: 605-611.

[69] Mohebbi-Fani M, Mirzaei A, Nazifi S, Shabbooie Z (2012) Changes of vitamins A, E, and C and lipid peroxidation status of breeding and pregnant sheep during dry seasons on medium-to-low quality forages. Trop. Anim. Health Prod. 44: 259-265.
[70] Keles H, Ince S, Kucukkurt I, Tatli, II, Akkol EK, Kahraman C, Demirel HH (2012) The effects of Feijoa sellowiana fruits on the antioxidant defense system, lipid peroxidation, and tissue morphology in rats. Pharm. Biol. 50: 318-325.
[71] Kosugi H, Kato T, Kikugawa K (1987) Formation of yellow, orange, and red pigments in the reaction of alk-2-enals with 2-thiobarbituric acid. Anal. Biochem. 165: 456-464.
[72] Frankel EN (1991) Recent advances in lipid oxidation. J. Sci. Food Agric. 54: 495-511.
[73] Bonnestaourel D, Guerin MC, Torreilles J (1992) Is malonaldehyde a valuable indicator of lipid-peroxidation Biochem. Pharmacol. 44: 985-988.
[74] Lapenna D, Cuccurullo F (1993) TBA test and free MDA assay in evaluation of lipid-peroxidation and oxidative stress in tissue systems Am. J. Physiol. 265: H1030-H1031.
[75] Gutteridge JMC, Tickner TR (1978) Thiobarbituric acid reactivity of bile-pigments. Biochem. Med. 19: 127-132.
[76] Largilliere C, Melancon SB (1988) Free malondialdehyde determination in human-plasma by high-performance liquid-chromatography. Anal. Biochem. 170: 123-126.
[77] Halliwell B, Gutteridge JMC, Aruoma OI (1987) The deoxyribose method - a simple test-tube assay for determination of rate constants for reactions of hydroxyl radicals. Anal. Biochem. 165: 215-219.
[78] Wade CR, Vanrij AM (1989) Plasma malondialdehyde, lipid peroxides, and the thiobarbituric acid reaction. Clin. Chem. 35: 336-336.
[79] Wade CR, Jackson PG, Vanrij AM (1985) Quantitation of malondialdehyde (MDA) in plasma, by ion-pairing reverse phase high-performance liquid-chromatography. Biochem. Med. 33: 291-296.
[80] Wade CR, Vanrij AM (1988) Plasma thiobarbituric acid reactivity - Reaction conditions and the role of iron, antioxidants and lipid peroxy-radicals on the quantitation of plasma-lipid peroxides. Life Sciences. 43: 1085-1093.
[81] Wong SHY, Knight JA, Hopfer SM, Zaharia O, Leach CN, Sunderman FW (1987) Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde thiobarbituric acid adduct. Clin. Chem. 33: 214-220.
[82] Santos MT, Valles J, Aznar J, Vilches J (1980) Determination of plasma malondialdehyde-like material and its clinical-application in stroke patients. J. Clin. Pathol. 33: 973-976.
[83] Mezes M, Bartosiewicz G (1983) Investigations on vitamin-E and lipid peroxide status in rheumatic diseases. Clin. Rheumatol. 2: 259-263.
[84] Kedziora J, Bartosz G, Gromadzinska J, Sklodowska M, Wesowicz W, Scianowski J (1986) Lipid peroxides in blood-plasma and enzymatic antioxidative defense of erythrocytes in Downs-syndrome. Clin. Chim. Acta. 154: 191-194.
[85] Takagi T, Mitsuno Y, Masumura M (1978) Determination of peroxide value by colorimetric iodine method with protection of iodide as cadmium complex. Lipids. 13: 147-151.

[86] Jessup W, Dean RT, Gebicki JM (1994) Iodometric determination of hydroperoxides in lipids and proteins. Oxygen Radicals in Biological Systems, Pt C. 233: 289-303.
[87] Hicks M, Gebicki JM (1979) Spectrophotometric method for the determination of lipid hydroperoxides. Anal. Biochem. 99: 249-253.
[88] Cramer GL, Miller JF, Pendleton RB, Lands WEM (1991) Iodometric measurement of lipid hydroperoxides in human plasma. Anal. Biochem. 193: 204-211.
[89] Chajes V, Sattler W, Stultschnig M, Kostner GM (1996) Photometric evaluation of lipid peroxidation products in human plasma and copper oxidized low density lipoproteins: Correlation of different oxidation parameters. Atherosclerosis. 121: 193-203.
[90] Gay C, Collins J, Gebicki JM (1999) Hydroperoxide assay with the ferric-xylenol orange complex. Anal. Biochem. 273: 149-155.
[91] Griffiths G, Leverentz M, Silkowski H, Gill N, Sanchez-Serrano JJ (2000) Lipid hydroperoxides in plants. Biochem. Soc. Trans. 28: 837-839.
[92] Nouroozzadeh J, Tajaddinisarmadi J, Birlouezaragon I, Wolff SP (1995) Measurement of hydroperoxides in edible oils using the ferrous oxidation in xylenol orange assay J. Agric. Food Chem. 43: 17-21.
[93] Burat KM, Bozkurt O (1996) Improvement of calibration curve for determining peroxide values of food lipids by the modified ferrous oxidation-xylenol orange method. J. AOAC Int. 79: 995-997.
[94] Bou R, Codony R, Tres A, Decker EA, Guardicila F (2008) Determination of hydroperoxides in foods and biological samples by the ferrous oxidation-xylenol orange method: A review of the factors that influence the method's performance. Anal. Biochem. 377: 1-15.
[95] Gupta BL (1973) Microdetermination techniques for H2O2 in irradiated solutions. Microchem J. 18: 363-374.
[96] Hermeslima M, Willmore WG, Storey KB (1995) Quantification of lipid-peroxidation in tissue-extracts based on Fe(III)xylenol orange complex-formation. Free Radic. Biol. Med. 19: 271-280.
[97] Jiang ZY, Hunt JV, Wolff SP (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density-lipoprotein Anal. Biochem. 202: 384-389.
[98] Wolff SP Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides, in: L. Packer (Ed.), Oxygen Radicals in Biological Systems, Pt C, 1994, pp. 182-189.
[99] Banerjee D, Madhusoodanan UK, Sharanabasappa M, Ghosh S, Jacob J (2003) Measurement of plasma hydroperoxide concentration by FOX-1 assay in conjunction with triphenylphosphine. Clin. Chim. Acta. 337: 147-152.
[100] Arab K, Steghens JP (2004) Plasma lipid hydroperoxides measurement by an automated xylenol orange method. Anal. Biochem. 325: 158-163.
[101] Sodergren E, Nourooz-Zadeh J, Berglund L, Vessby B (1998) Re-evaluation of the ferrous oxidation in xylenol orange assay for the measurement of plasma lipid hydroperoxides. J. Biochem. Biophys. Methods. 37: 137-146.

[102] Grau A, Codony R, Rafecas M, Barroeta AC, Guardiola F (2000) Lipid hydroperoxide determination in dark chicken meat through a ferrous oxidation-xylenol orange method. J. Agric. Food Chem. 48: 4136-4143.

[103] Griffiths G, Leverentz M, Silkowski H, Gill N, Sanchez-Serrano JJ (2000) Lipid hydroperoxide levels in plant tissues. J. Exp. Bot. 51: 1363-1370.

[104] Huska D, Adam V, Babula P, Trnkova L, Hubalek J, Zehnalek J, Havel L, Kizek R (2011) Microfluidic robotic device coupled with electrochemical sensor field for handling of paramagnetic micro-particles as a tool for determination of plant mRNA. Microchim. Acta. 173: 189-197.

[105] Sochor J, Salas P, Zehnalek J, Krska B, Adam V, Havel L, Kizek R (2010) An assay for spectrometric determination of antioxidant activity of a biological extract. Lis. Cukrov. Repar. 126: 416-417.

[106] Sochor J, Ryvolova M, Krystofova O, Salas P, Hubalek J, Adam V, Trnkova L, Havel L, Beklova M, Zehnalek J, Provaznik I, Kizek R (2010) Fully automated spectrometric protocols for determination of antioxidant activity: Advantages and disadvantages. Molecules. 15: 8618-8640.