



INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

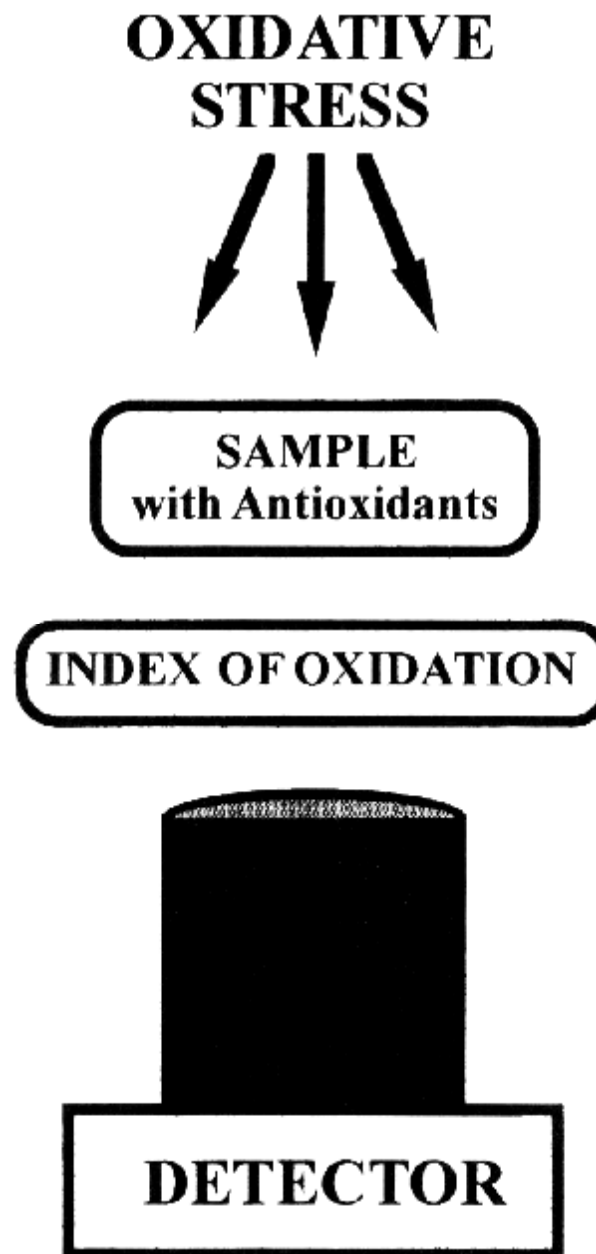
Metody stanovení antioxidační aktivity

Obecné metody hodnocení antioxidační aktivity a antioxidačního stavu biologických systémů, stanovení biomarkerů oxidačního stresu

PharmDr. Ján Vančo, Ph.D.
Ústav chemických léčiv, FaF VFU Brno

Metody hodnocení celkové antioxidační kapacity

- mají využití při nspecifickém popisu stavu antioxidačních ochranných systémů
- jsou využitelné jak pro čisté látky, tak pro směsi a biologické vzorky, např. plasma
- pro lokalizaci oxidačního stresu v organismu je možné využít přímé i nepřímé metody detekce volných radikálů



Přímé metody lokalizace oxidačního stresu v organismu

- nejperspektivnějším se ukazuje využití neinvazivní techniky 3D-ESR (EPRI)

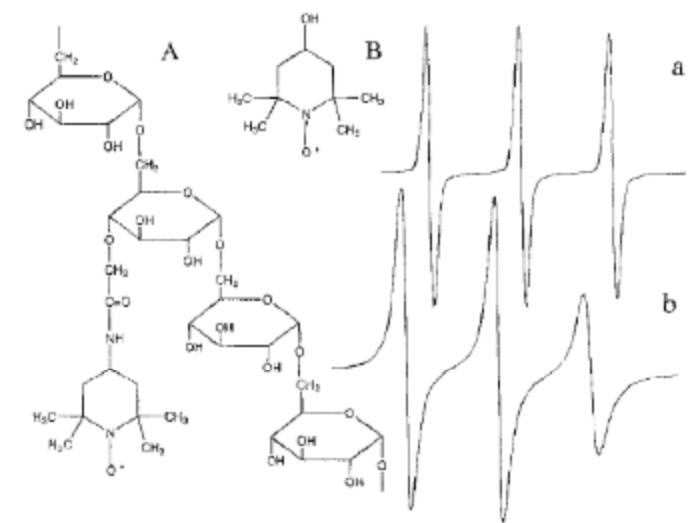


FIG. 1. Molecular structures and ESR spectra of TEMPO-DX and TEMPOL. (A) N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)acetamidyl-dextran (TEMPO-DX); (a) ESR spectrum of a 2% aqueous solution of (A). (B) 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL); (b) ESR spectrum of a 1×10^{-5} M aqueous solution of (B).

Přímé metody lokalizace oxidačního stresu v organismu

- interpretace výsledků měření

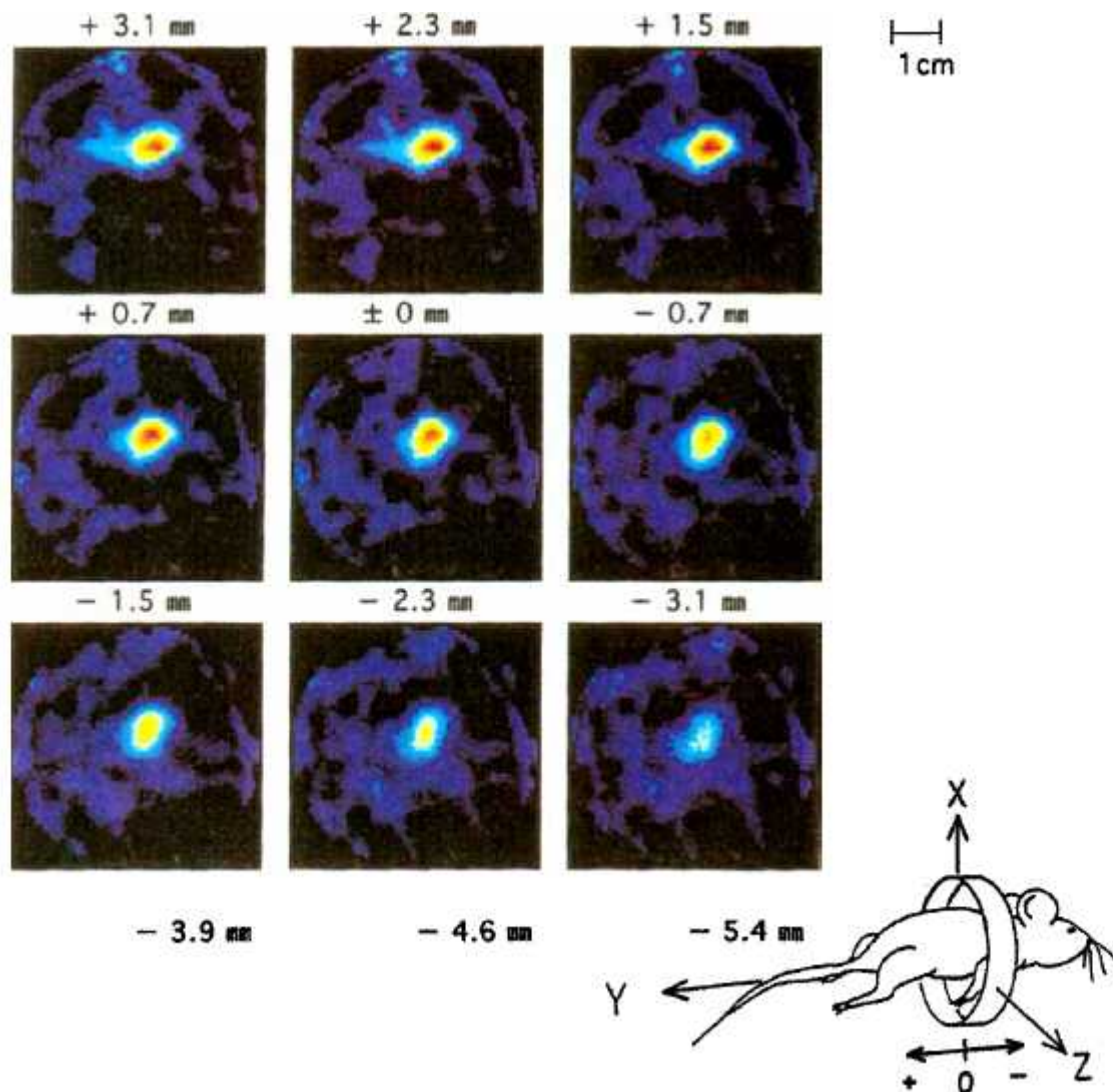
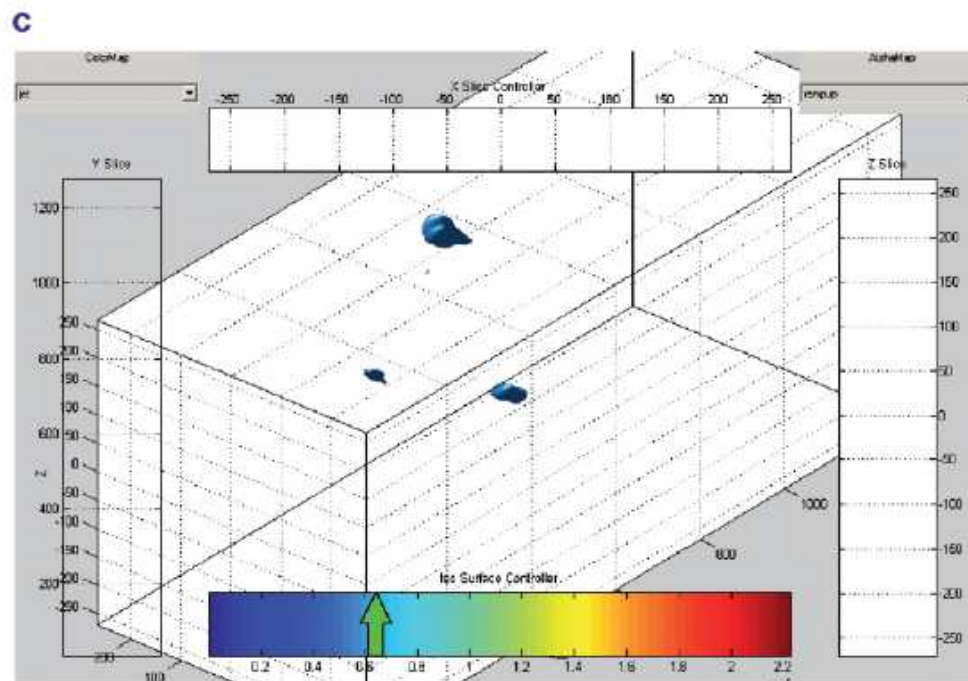
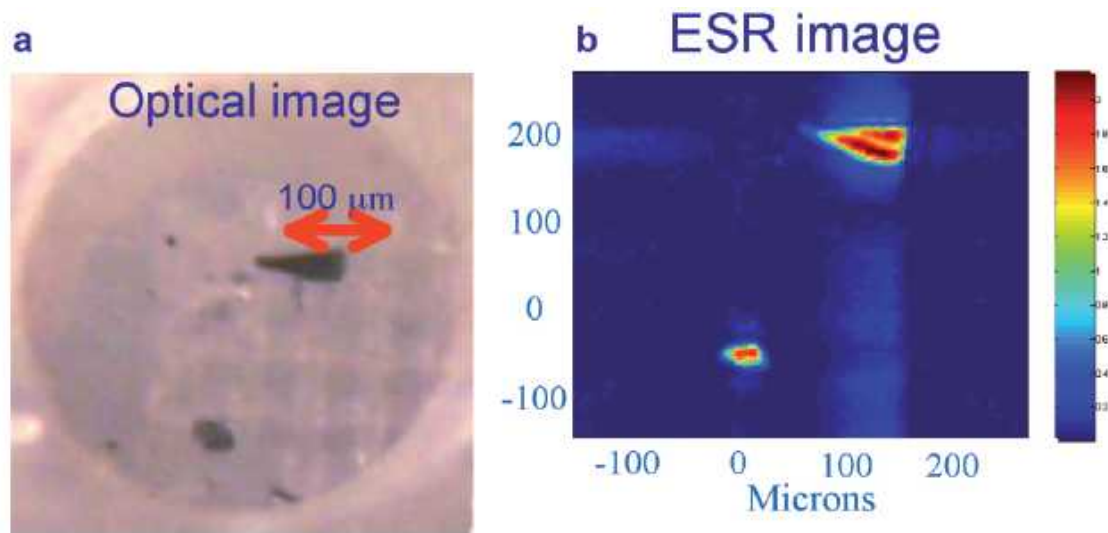


FIG. 3. 3D *in vivo* image of the kidney. Each frame reflects part of the 3D ESR tomogram of a 0.77-mm slice of the kidney region. The center of the loop-gap resonator is marked at 0 mm; the other frames are marked at the distance (in mm) from the center along the y-direction of the resonator as shown in the illustration.

Přímé metody lokalizace oxidačního stresu v organismu



Příklady využití EPRI – detekce RONS při ischemicko-reperfusním poškození tkáně

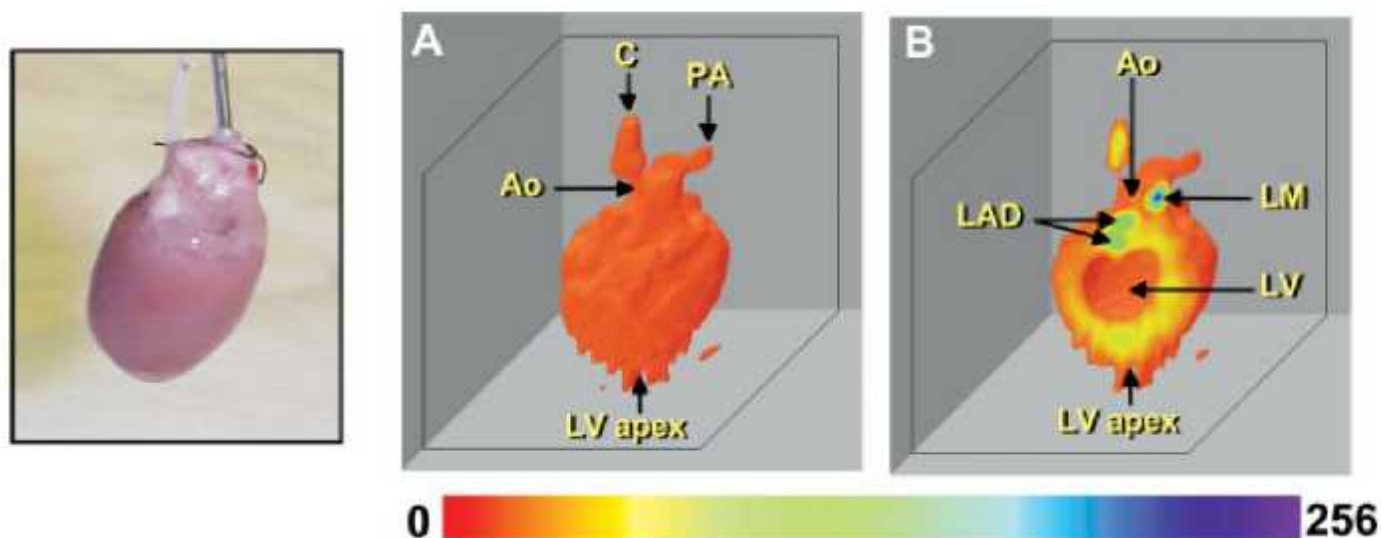


Plate 1. Three-dimensional image of rat heart infused with glucose char suspension. (A) Full view of the heart; (B) a longitudinal cutout showing the internal structure of the heart; photograph of an isolated perfused rat heart is shown on the left. Legends: C, cannula; Ao, aortic root; PA, pulmonary artery; LM, left main coronary artery; LAD, left anterior descending artery; LV, left ventricular cavity. The void seen in the LV cavity is due to the inflated balloon. Image acquisition parameters: projections, 1024; magnetic field gradient, 50.0 G/cm; acquisition time, 78 min. [Reprinted from Kuppusamy P, Wang P, Zweier JL. Three dimensional spatial EPR imaging of the rat heart. *Magn Reson. Med.* Copyright © 1995 John Wiley and Sons, Ltd. This material is used by permission of John Wiley & Sons, Inc.]²⁰

Příklady využití EPRI – průchod značeného léčiva kůží

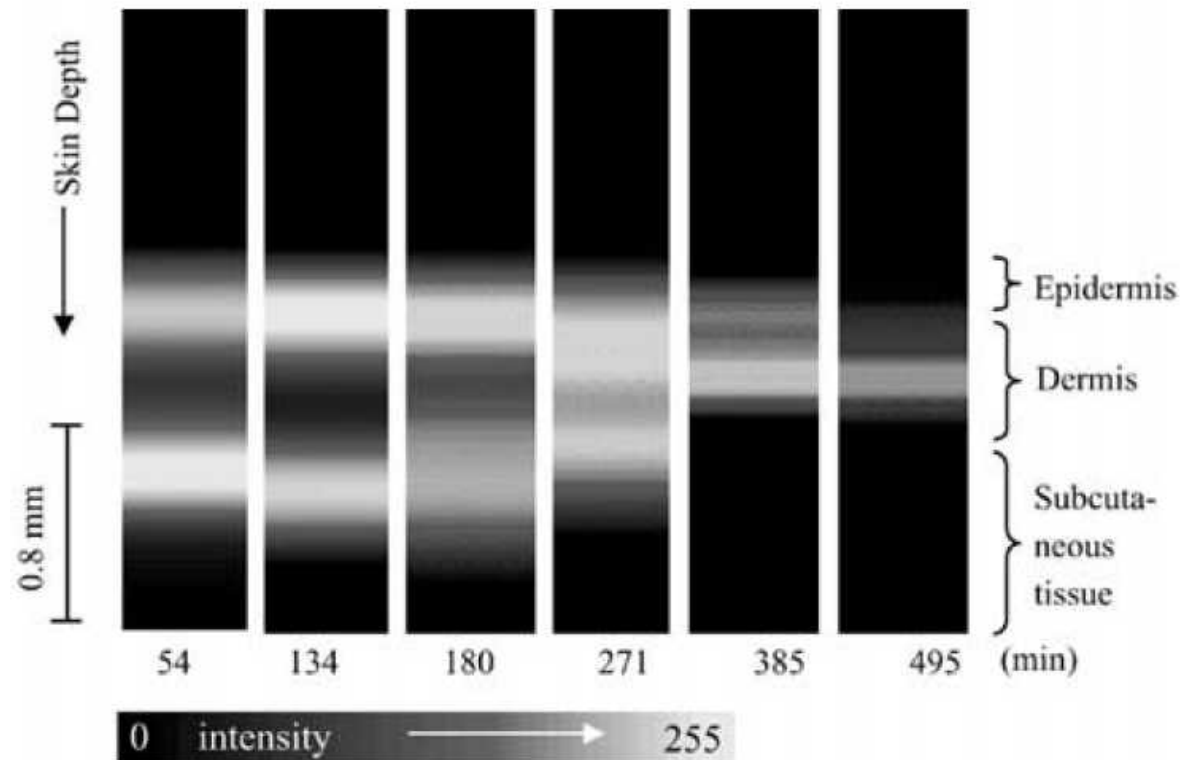
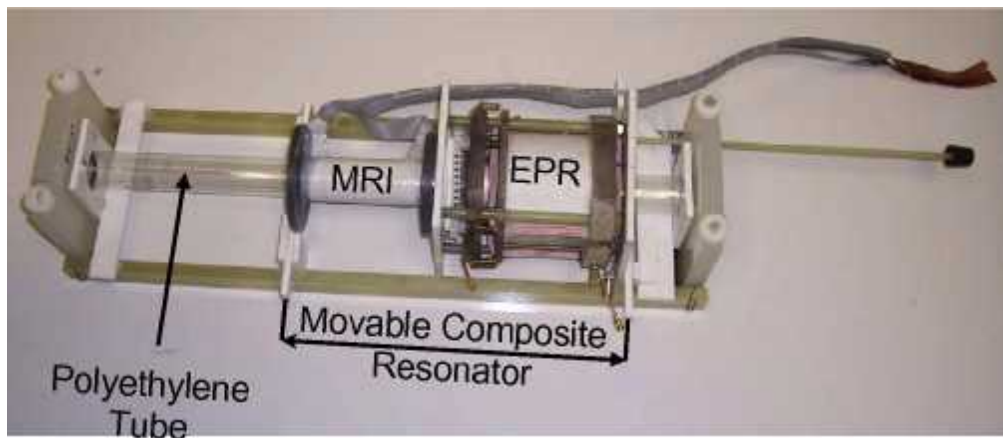
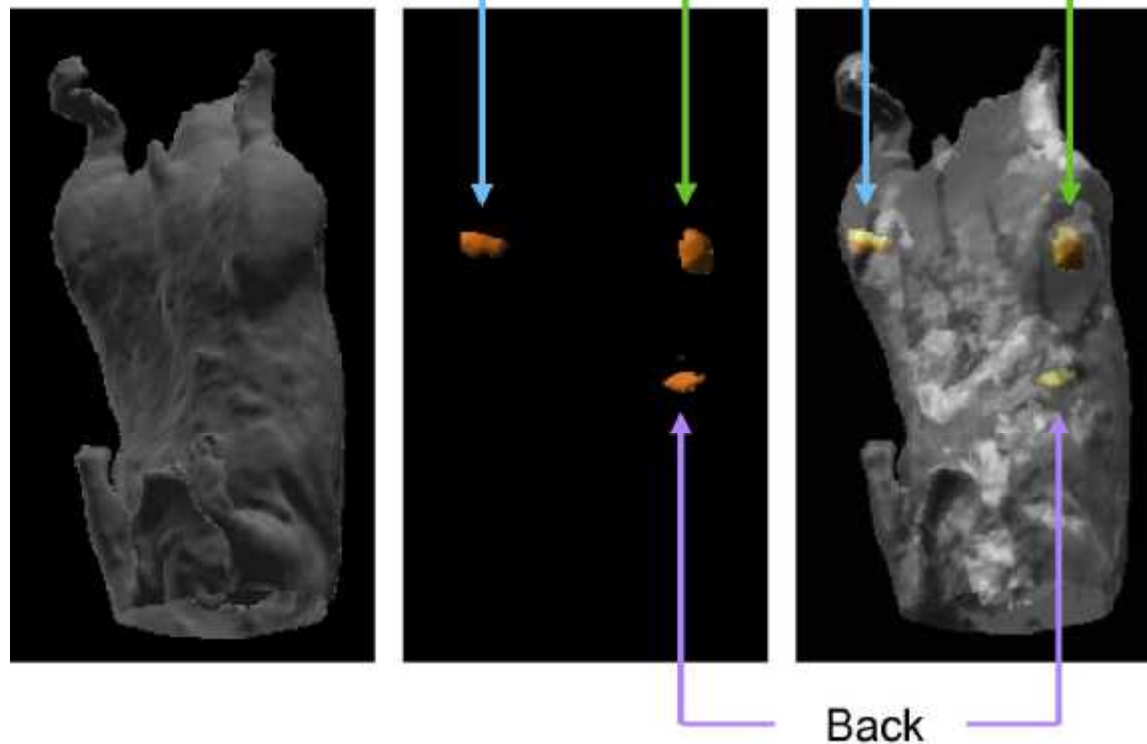
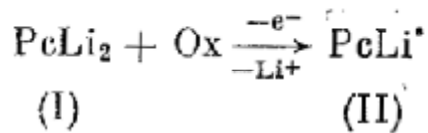
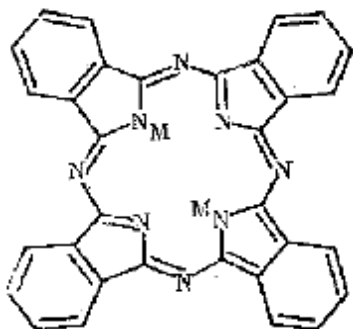


Fig. 6. Image of the time course of spatial distribution of the nitroxide as a function of skin depth. This image was obtained from the spatial profiles. Two bands in the distribution of the label along the skin-depth are seen. The first band appears in the outer 400 μm of the skin, the epidermis region, whereas the second band is centered at a depth of 1000 μm in the subcutaneous region with a thickness about 400 μm . These two bands decay and merge in to a single band with time.

Příklady využití EPRI – neinvazivní oximetrie v orgánech metoda MRI-EPRI co-imaging



Ftalocyanát dilithný (PcLi₂)



Nepřímé metody hodnocení celkové antioxidační aktivity

Table 11.1 Methods of measuring free radical scavenging antioxidant activity

Oxidative stress	Oxidation index	Measurement	Reference
Tissue autoxidation (brain)	Lipid peroxidation	TBARS	Stocks <i>et al.</i> , 1974
Peroxyl radicals (ABAP)	Lipid peroxidation	Oxygen consumption	Wayner <i>et al.</i> , 1987
Peroxyl radicals (ABAP)	Luminol	Luminescence	Metsä-Ketelä, 1991
Peroxyl radicals (AMVN)	Luminol	Luminescence	Metsä-Ketelä and Kirkkola, 1992
H ₂ O ₂ /peroxidase	<i>o</i> -Phenylenediamine	Absorbance 430 nm	Nakamura <i>et al.</i> , 1987
Peroxyl radicals (AAPH)	β -Phycoerythrin	Fluorescence 565 nm	DeLange, 1989
Cu ²⁺ /H ₂ O ₂	Lipid peroxidation	TBARS	Arshad <i>et al.</i> , 1991
Cu ²⁺ /hydroperoxide	<i>cis</i> -Parinaric acid	Fluorescence 413 nm	McKenna <i>et al.</i> , 1991
H ₂ O ₂ /peroxidase/enhancer	Luminol	Luminescence	Whitehead <i>et al.</i> , 1992
Peroxyl radicals (AAPH)	β -Phycoerythrin	Fluorescence 565 nm	Cao <i>et al.</i> , 1993
Ferryl myoglobin	ABTS	Absorbance 734 nm	Miller <i>et al.</i> , 1993
Fe ³⁺	Fe/tripyridyltriazine	Absorbance at 593 nm	Benzie and Strain, 1996

ABTS = 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate)

AAPH = 2,2'-azobis(2-amidinopropane) dihydrochloride

ABAP = 2,2'-azobis(2-amidopropane) hydrochloride

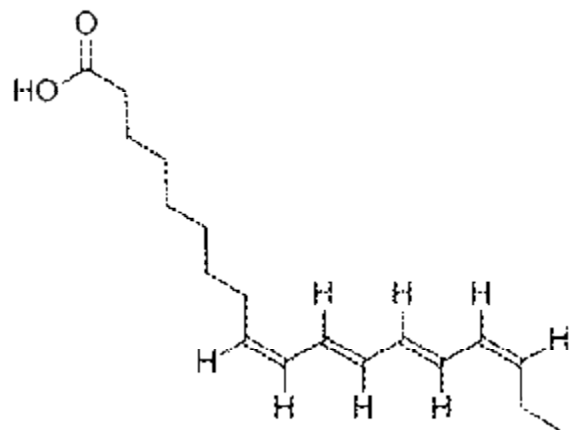
AMVN = 2,2'-azobis(2,4-dimethylvaleronitrile)

TBARS = thiobarbituric acid reactive substances

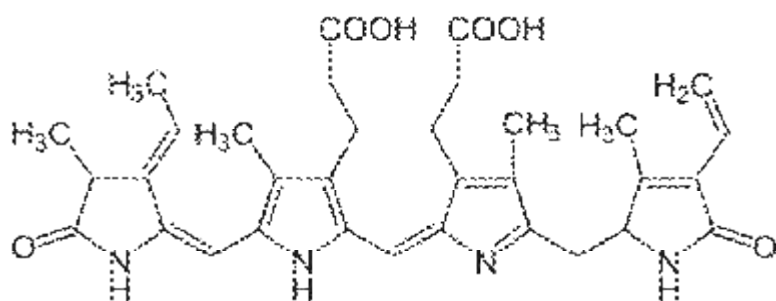
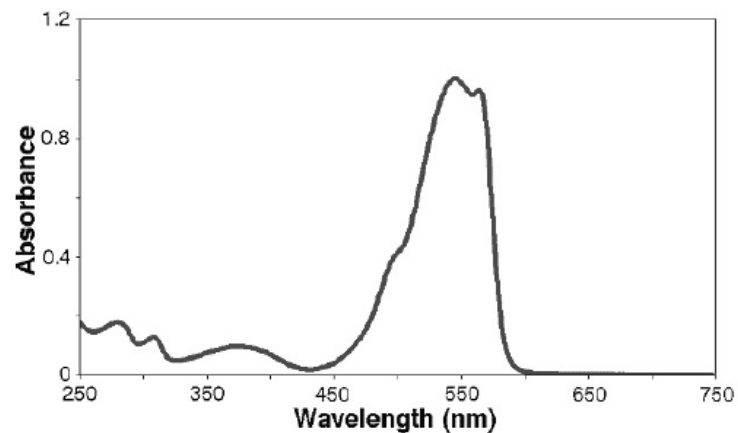
DOPA = 3,4-dihydroxyphenylalanine

AMVN – 2,2'-azobis(2,4-dimethylvaleronitrile).

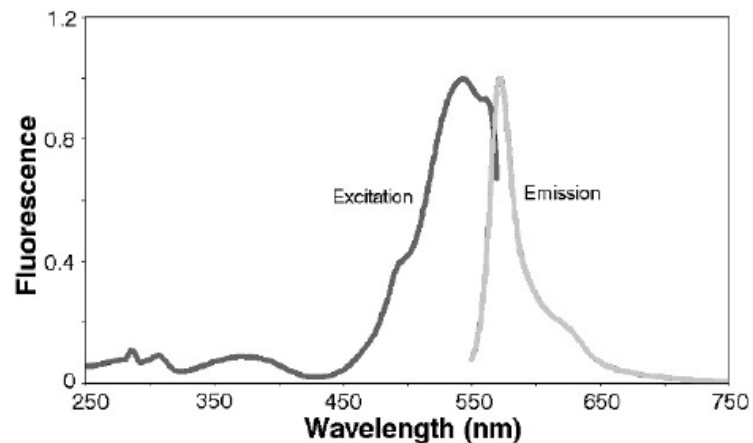
Nepřímé metody hodnocení celkové antioxidační aktivity



kyselina α -parinarová



phycoerythrobilin – typický chromofór v
štruktúre β -phycoerytrinu (Mr=240 kDa)



Nepřímé metody hodnocení celkové antioxidační aktivity

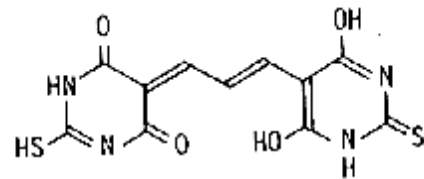


FIG. 1. Structure of the product from the reaction of lipid peroxid

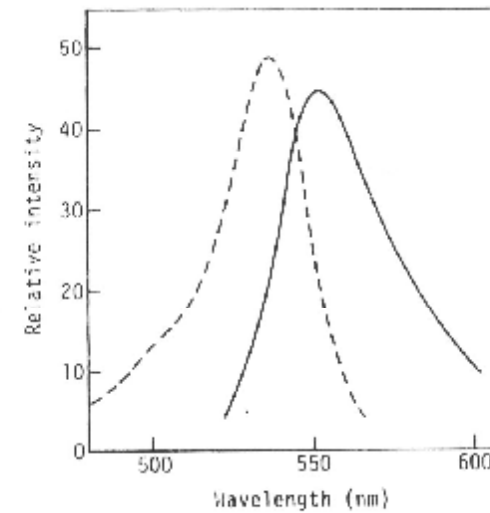
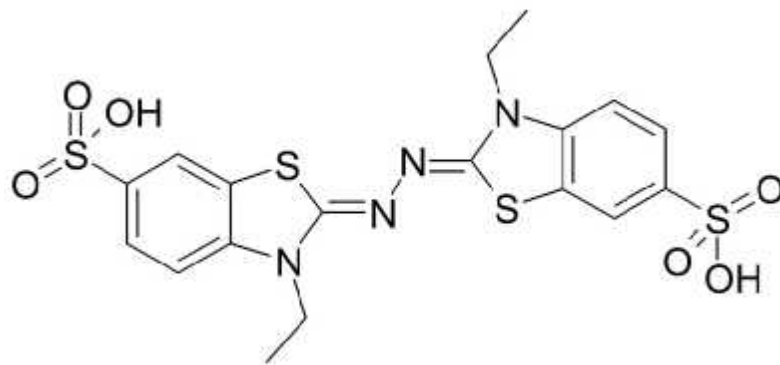


FIG. 2. Excitation and emission spectra of the product from the reaction of lipid peroxides with TBA. Dotted line: excitation spectrum (monitored at 565 nm); solid line: emission spectrum (excited at 515 nm).



ABTS

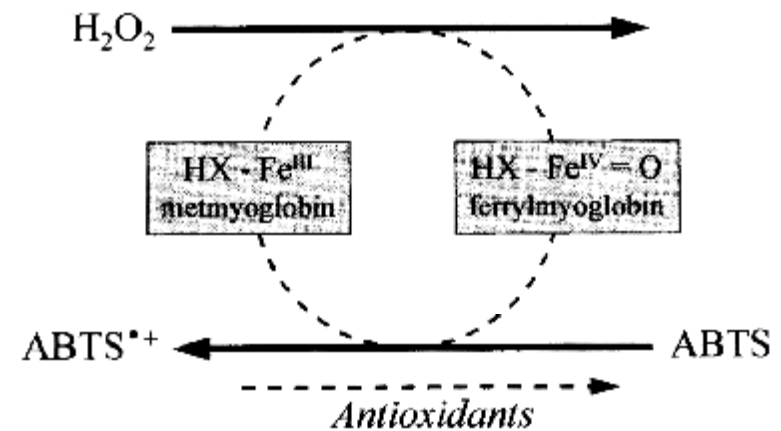
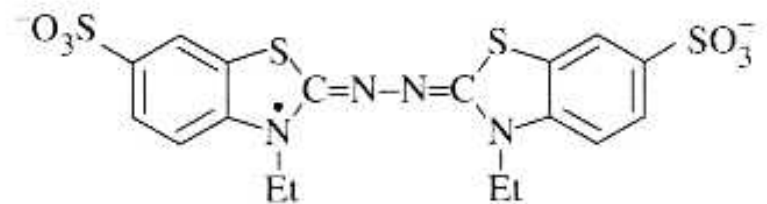
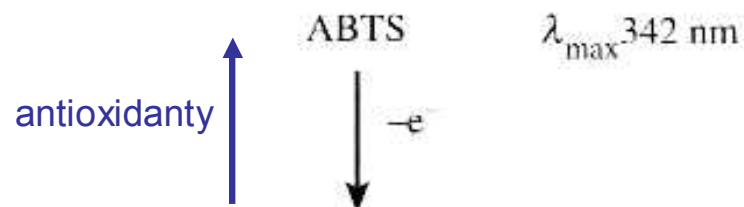
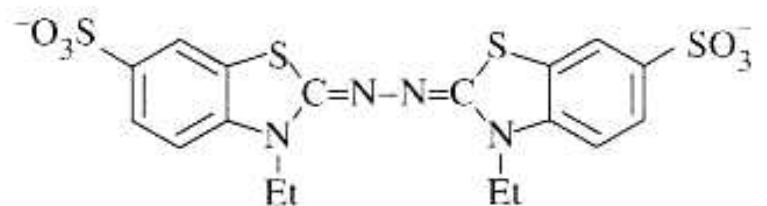
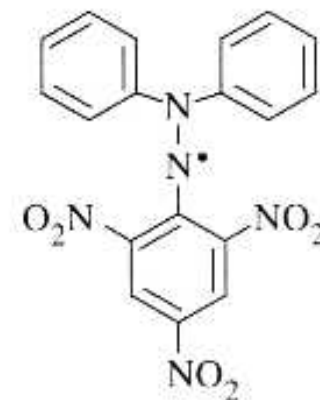


Figure 11.6 The formation of the $ABTS^{\bullet+}$ radical cation. The reaction of metmyoglobin with hydrogen peroxide forms the ferrylmyoglobin radical. This radical abstracts an electron from ABTS to form the blue-green $ABTS^{\bullet+}$ radical cation. The characteristic absorption of the radical cation can be inhibited by antioxidants and other reductants.

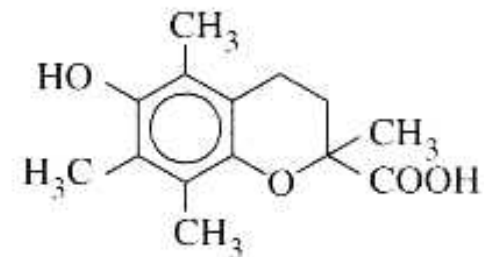
Nepřímé metody hodnocení celkové antioxidační aktivity



ABTS radical cation λ_{\max} 417 nm
(peaks at 645, 734 and 815 nm)



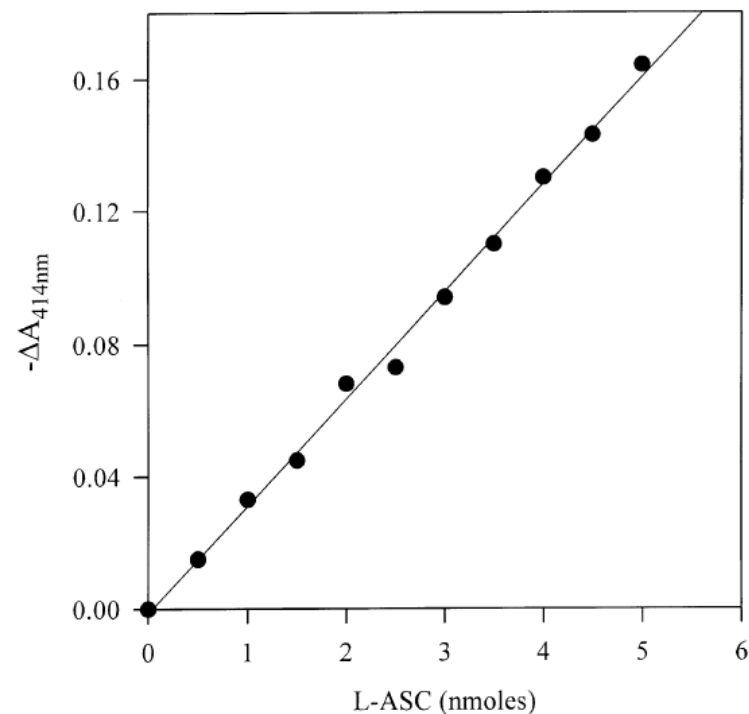
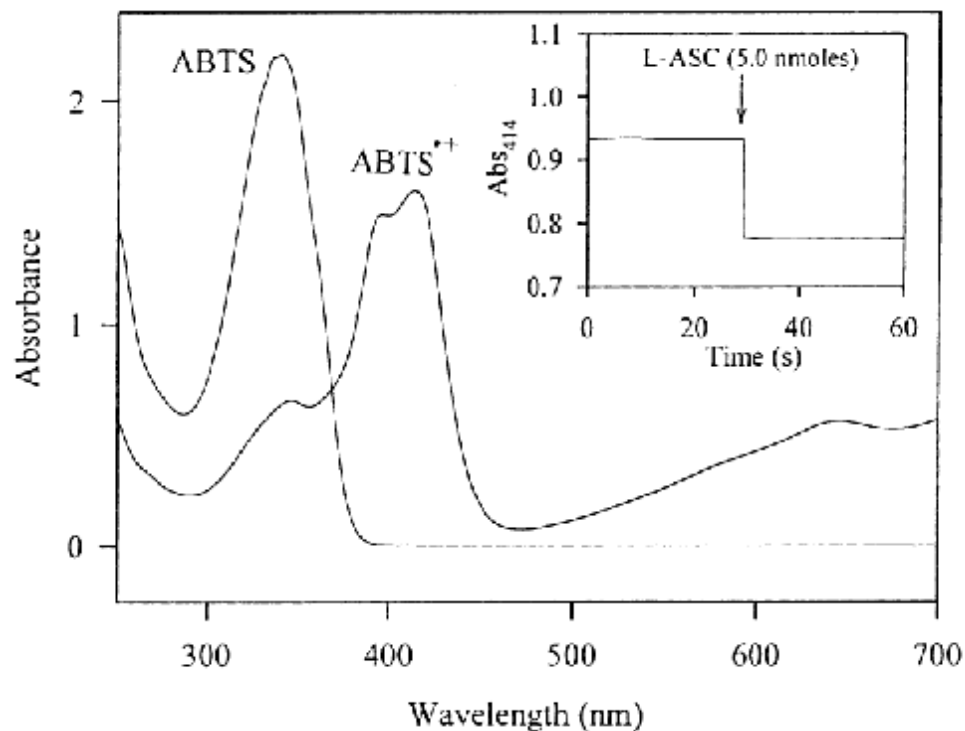
DPPH
(diphenyl-*p*-picrylhydrazyl)



Trolox

Nepřímé metody hodnocení celkové antioxidační aktivity

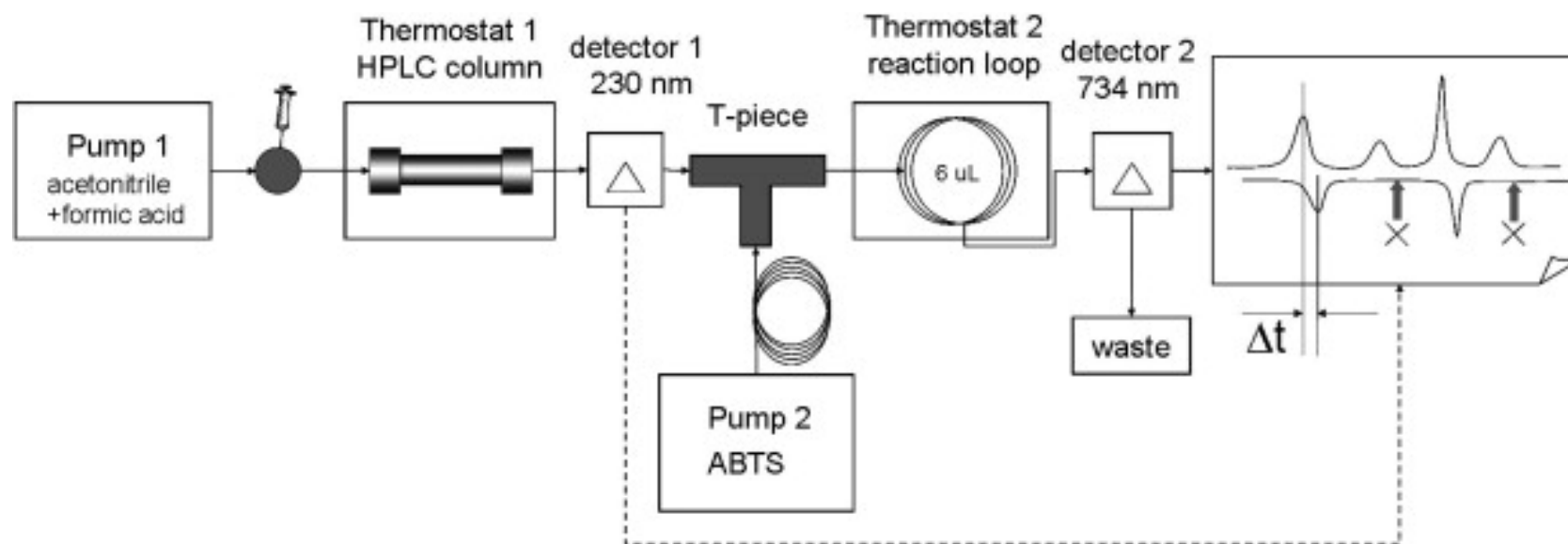
Metoda zhášení ABTS – principy, použití



Zhodnocení výsledků – pro větší skupinu látek je možné srovnávat %poklesu absorbance při stejné koncentraci, srovnání směrnic přímky, propočet na **ekvivalent standardního antioxidantu**

Nepřímé metody hodnocení celkové antioxidační aktivity

Metoda zhášení ABTS – screening antioxidačních schopností směsí (on-line)



- výhody – RP-HPLC analýza vhodná pro identifikaci složek směsí
- post-kolonovou derivatizací je umožněná on-line identifikace aktivních látek

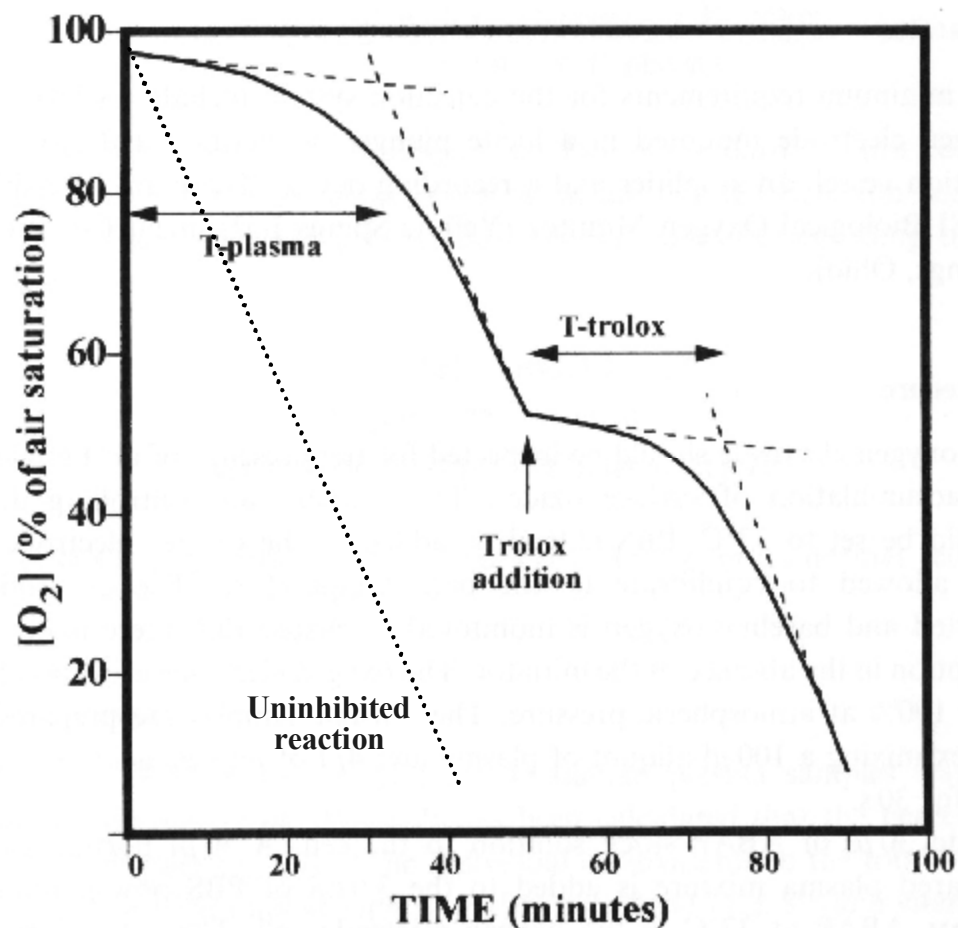
Zhodnocení výsledků – antioxidační profil směsi a konkrétního antioxidantu ve směsi, propočet na **ekvivalent standardního antioxidantu (kyseliny gallové)**

Ref.: Pazourek J., Václavík J., Žemlička M. *Food Chem.* 125, 785-790 (2011).

Nepřímé metody hodnocení celkové antioxidační aktivity

TRAP assay – Total peroxide radical-trapping antioxidant parameter

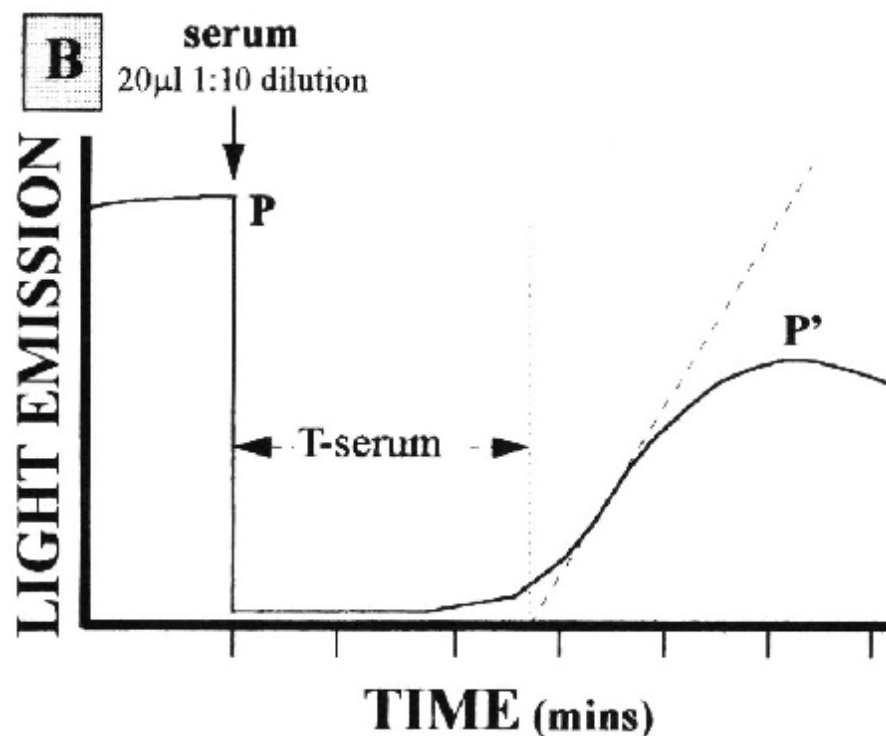
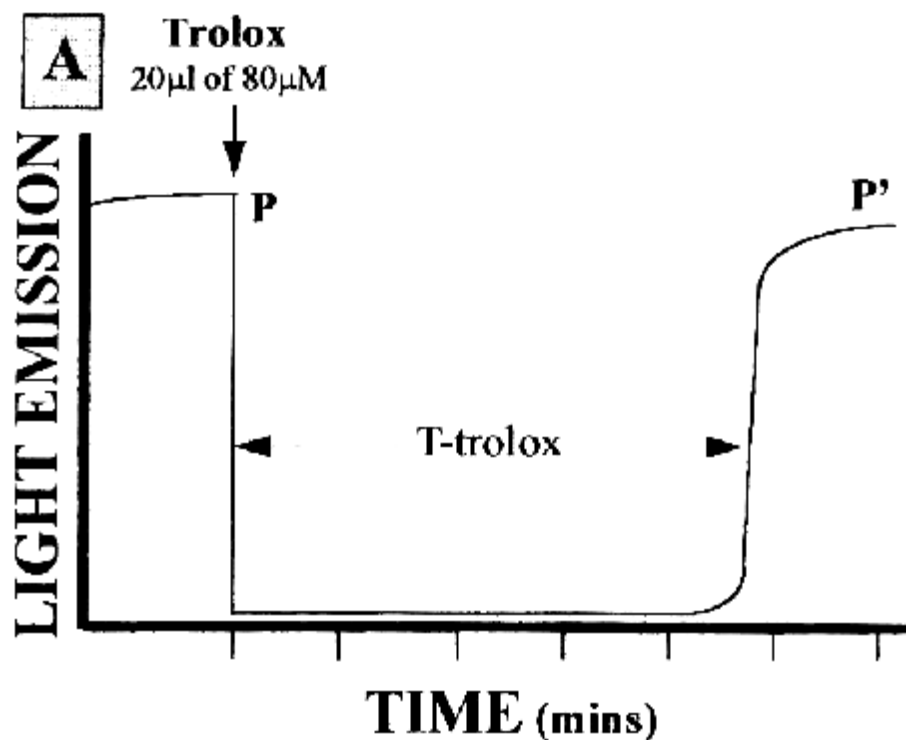
- jedna z nejčastěji používaných metod
- jako zdroje volných peroxylových radikálů se používají AAPH, ABAP, AMVN, které se samovolně rozkládají za vzniku radikálu
- jako substrát se nejčastěji používá kyselina linoleová, jako kalibrant Trolox
- vyhodnocuje se čas potřebný na dosažení maximální spotřeby kyslíku v roztoku



Nepřímé metody hodnocení celkové antioxidační aktivity

ECL assay – Enhanced chemiluminescence method

- chemiluminescence je kontinuálně produkována v systému HRP-H₂O₂-luminol
- látky schopné zhaset radikály způsobí pokles nebo úplné vymizení luminiscence na čas, který je závislý na jejich antioxidační aktivitě



Další metody hodnocení celkové antioxidační aktivity

FRAP assay

- je založena na měření schopnosti testovaných látek poskytovat elektrony a redukovat činidlo (železitý komplex tripyridyltriazinu) na intenzivně modře zbarvený železnatý komplex (abs. maximum při 593 nm)

OPD assay

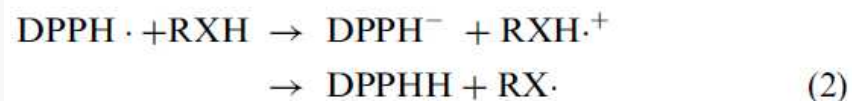
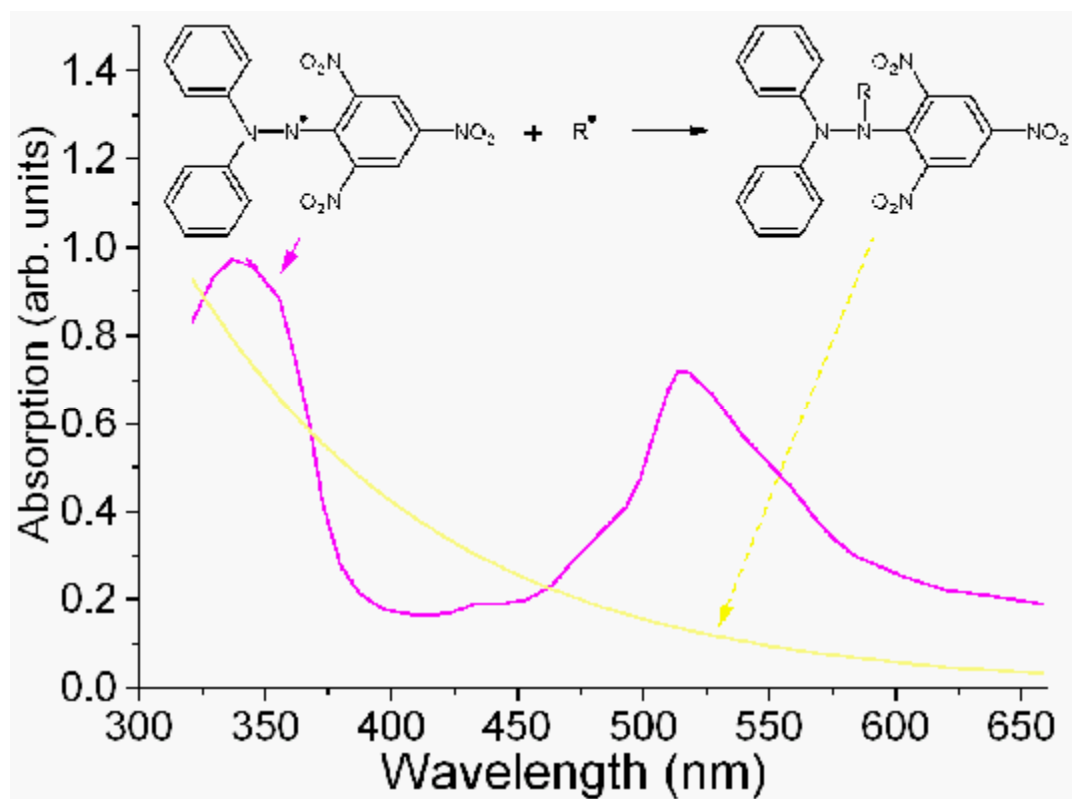
- činidlo, o-fenylendiamin je substrátem peroxidas a jejich působením se mění na barevnou radikálovou formu (obdoba semichinonu), který vykazuje absorpci při 430 nm

Plasma peroxidation potential assay

- je založena na sledování produkce TBARS po přidání systému generujícího hydroxylový radikál Fentonovou reakcí ke vzorku plasmy

Další metody hodnocení celkové antioxidační aktivity

Metoda zhášení DPPH – princip, použití



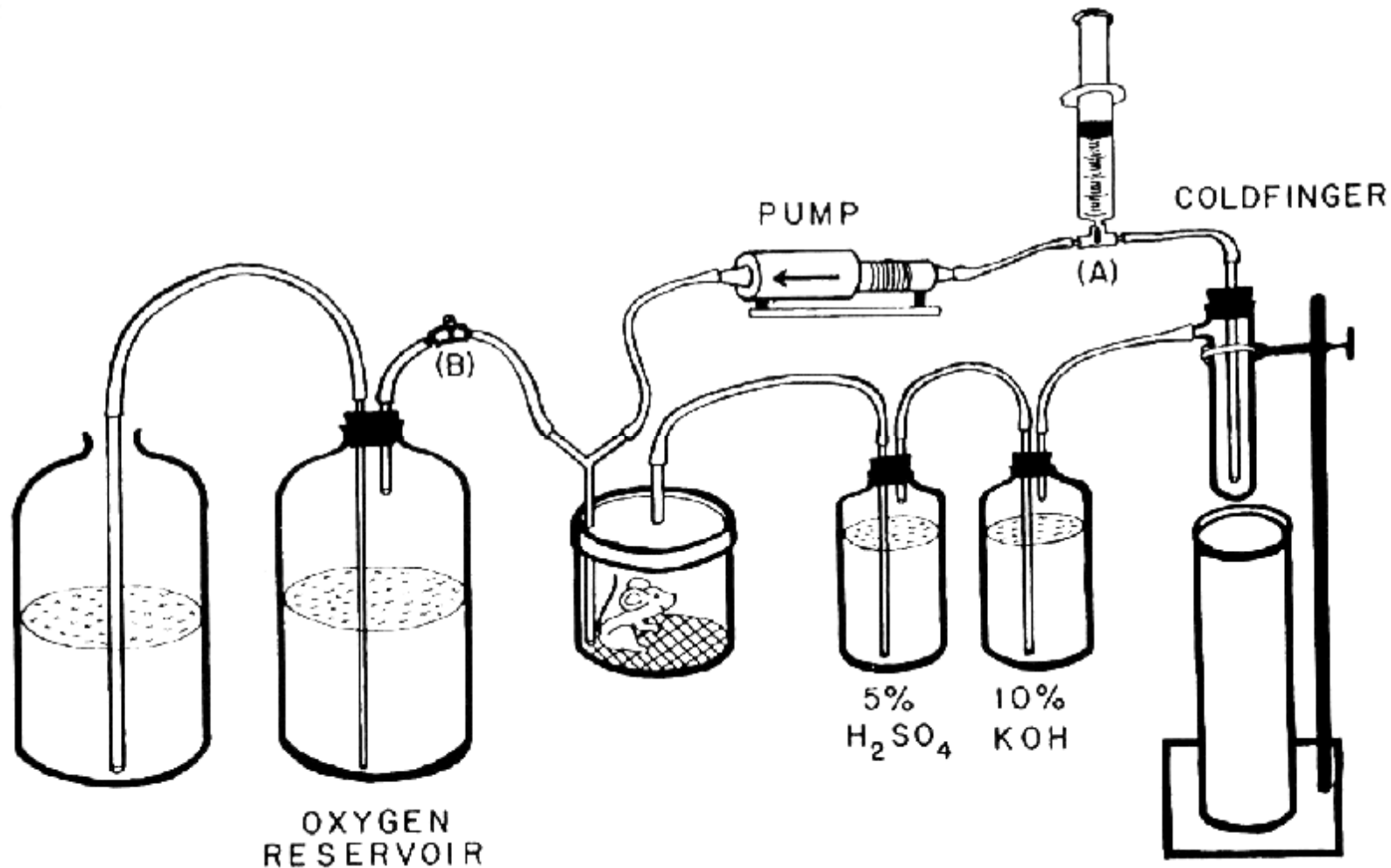
- spektrofotometrická metoda (520 nm)
- velice výhodná pro rychlý screening
- účinné látky obvykle obsahují lehkobstrahovatelný vodík

Zhodnocení výsledků – pro větší skupinu látek možné srovnat %poklesu absorbance při stejné koncentraci, srovnání kinetických parametrů, propočít na **ekvivalent standardního antioxidantu** (L-ASC)

Další metody hodnocení celkové antioxidační aktivity

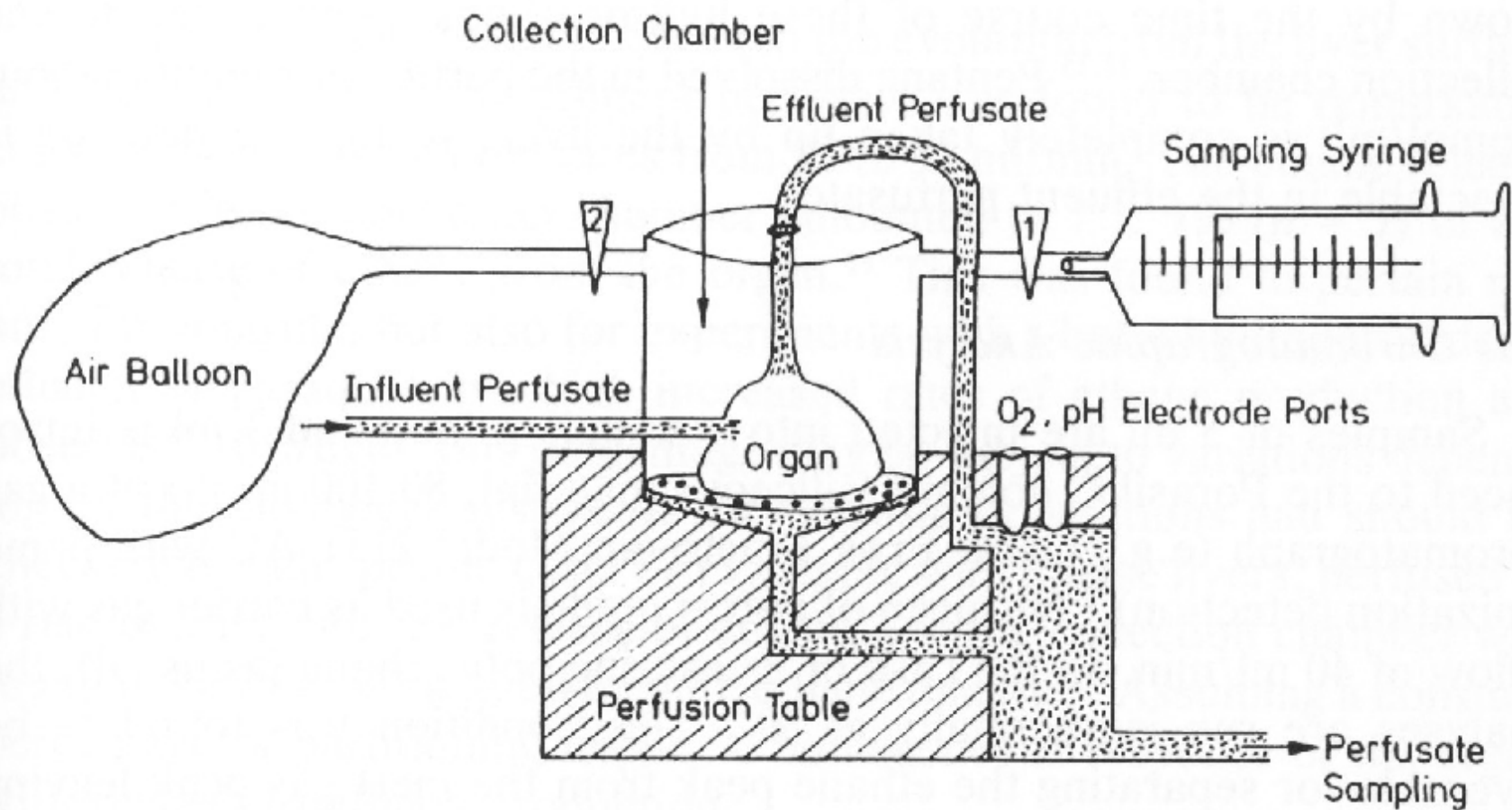
Neinvazivní metoda analýzy plynů ve vydechovaném vzduchu

- je založena na měření koncentrace alkanů a alkenů, vznikajících zejména degradací lipidů pomocí GC-MS, metoda je využitelná i pro perfundované izolované orgány



Další metody hodnocení celkové antioxidační aktivity

Neinvazivní metoda analýzy plynů nad perfundovaným orgánem



Nepřímé metody specifického hodnocení antioxidační aktivity

Hydroxylový radikál

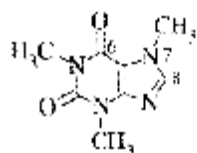
Table 5.3. Some methods for detection of hydroxyl radicals

Method	Principle of method	Comments
Hydroxylation of terephthalic acid	Non-fluorescent; hydroxylated by OH [•] to fluorescent product.	Has not been widely used in biological systems (<i>Life Sci.</i> 56, PL89 (1995)).
Bleaching of <i>p</i> -nitrosodimethylaniline (PNDA)	PNDA reacts rapidly with OH [•] but not with O ₂ ^{•-} or singlet O ₂ . Reaction is accompanied by bleaching of the yellow colour (<i>Eur. J. Biochem.</i> 95, 621 (1975)).	PNDA is bleached non-specifically by many biological systems; confirmatory evidence for role of OH [•] required.
Conversion of methional (CH ₃ SCH ₂ CH ₂ CHO) and related compounds (methionine or 2-keto-4-methylthiobutanoic acid, CH ₃ SCH ₂ CH ₂ C(=O)COOH) into ethylene gas (H ₂ C=CH ₂)	Measurement of ethylene by GC	Not specific for OH [•] ; oxidized by RO ₂ [•] , decomposing ONOO ⁻ and some peroxidase enzymes; confirmatory evidence for role of OH [•] required.
<u>Tryptophan method</u>	Reaction of OH [•] with tryptophan produces a characteristic set of products (<i>Bull. Eur. Physiopath. Resp.</i> 17, 31 (1981)).	Tryptophan also reacts with singlet O ₂ but the products are different (Chapter 2).
Coumarin fluorescence	Coumarin-3-carboxylic acid (CCA) is hydroxylated at position 7 to a fluorescent product.	CCA has been covalently linked to various biomolecules and fluorescence changes used to measure OH [•] generation in their vicinity (<i>Int. J. Rad. Biol.</i> 63, 445 (1993)). For example, OH [•] generation by copper ions bound to DNA could not be decreased by adding DMSO, methanol or ethanol but could be by histidine (which chelates copper). This is typical of a 'site-specific' reaction (Fig. 5.7) (<i>Free Rad. Biol. Med.</i> 18, 669 (1995)).

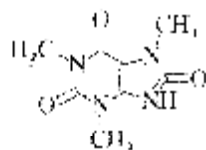
Nepřímé metody specifického hodnocení antioxidační aktivity

Hydroxylový radikál

Conversion of caffeine to 8-oxocaffeine



Caffeine



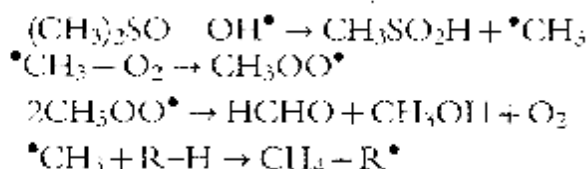
8-Oxocaffeine (1,3,7-trimethylxanthine-8-oxide)

Caffeine is hydroxylated at position 8; product analysed by HPLC with electrochemical detection.

Analogous to analysis of 8-OH-deoxyguanosine in DNA. Formation of 8-oxocaffeine from endogenous caffeine has been used to measure free-radical generation during roasting and brewing of coffee (*J. Agr. Food. Chem.* **43**, 1332 (1995)).

Dimethylsulphoxide (DMSO) method

OH[•] radicals react with DMSO, generating, among other products, methane gas, measured by gas-liquid chromatography (*Biochemistry* **20**, 6006 (1981)), or formaldehyde, measured colorimetrically.



Not specific for OH[•], e.g. oxidized by decomposing ONOO⁻. Confirmatory evidence for role of OH[•] required. Babbs *et al.* (*Free Rad. Biol. Med.* **6**, 493 (1989)) have suggested that oxidation of DMSO to CH₃SO₂H, methanesulphonic acid (measured colorimetrically or by HPLC) is a means of detecting OH[•] *in vivo*. Another approach is to trap the CH₃[•] radicals (*Anal. Chem.*, **69**, 4295 (1997)).

Sensitive method (*Biochem. J.* **243**, 709 (1987)); confirmatory evidence for role of OH[•] required

Electrochemical detection can allow high sensitivity (e.g. *Anal. Biochem.* **196**, 111 (1991)).

Benzoate fluorescence

Reaction of benzoic acid with OH[•] gives 3- and 4-hydroxybenzoates, which are fluorescent at 407 nm when excited at 305 nm.

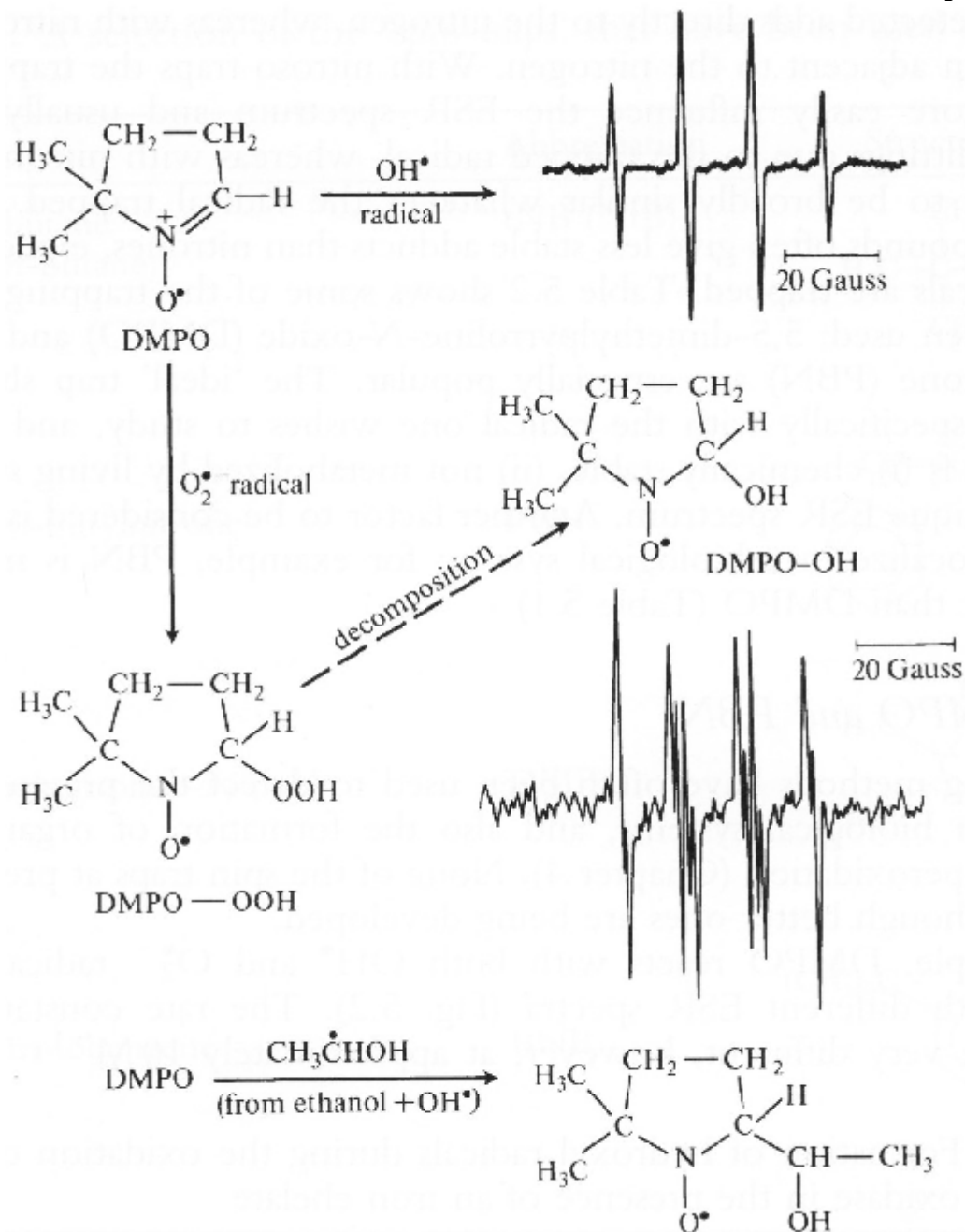
Spin trapping/HPLC

A combination of the principles of spin trapping and aromatic hydroxylation; HPLC is used to separate radical adducts of a spin trap, such as DMPO.

Nepřímé metody specifického hodnocení antioxidační aktivity

Hydroxylový radikál

- detekce spin-trapového produktu reakce DMPO (5,5-dimethylpyrrolin-N-oxid) pomocí ESR
- vyhodnocení – kvalitativní nebo kvantitativní na základě intenzity signálu
- vhodné jako relativní metoda



Přímé a nepřímé metody hodnocení antioxidační aktivity

Oxid dusnatý

Table 5.4. Some methods for detection of nitric oxide

Methods	Principle of method	Comments
A. Direct methods		
Light emission	<p>Reaction of NO[•] with O₃ produces light, via excited-state nitrogen dioxide</p> $\text{NO} + \text{O}_3 \rightarrow \text{NO}_2^* \rightarrow \text{NO}_2 + h\nu$ <p style="text-align: center;">(excited)</p>	Highly sensitive (nM range). Measures only gas-phase NO [•] . Potential interference by other light-emitting systems. NO [•] must be displaced from the biological material into the gas phase for analysis, e.g. by flushing with N ₂ , sometimes the transfer is not quantitative.
<u>NO[•] electrodes</u>	<p>Several types. In porphyrinic sensors NO[•] binds to a Ni²⁺-porphyrin adsorbed on to an anode and is oxidized electrochemically:</p> $\text{Ni(P)} + \text{NO}^{\bullet} \rightarrow \text{Ni(P)NO} \rightarrow \text{Ni(P)NO}^+ + e^-$ <p>Electron flow is proportional to [NO[•]]. Another method is adaptation of the Clark O₂ electrode, in which O₂ diffuses through a membrane and is reduced. NO[•] can be detected by changing the relative potentials of the silver and platinum electrodes.</p>	Used to study NO [•] exhalation in humans, and demonstrate elevated levels in asthma (<i>Thorax</i> 51 , 233 (1996)), ~10 nM sensitivity. Easy to use, but several reports of chemical interference with the electrodes. Clark probe is slower to respond to changes in NO [•] than the porphyrinic probes and less sensitive. Insertion of a porphyrinic probe into a hand vein has been used to detect NO [•] <i>in vivo</i> (<i>Lancet</i> 346 , 153 (1995)).
Haemoglobin trapping	NO [•] reacts with oxyhaemoglobin, eventually converting it to methaemoglobin, and the ΔA is measured.	Sensitivity: ~μM. Simple to perform. Interference can occur with other redox agents. ONOO ⁻ leads to the same oxidation; NO ₂ ⁻ (much more slowly) oxidizes oxyhaemoglobin to methaemoglobin. Myoglobin can also be used.

Nepřímé metody specifického hodnocení antioxidační aktivity

Oxid dusnatý

ESR spin-trapping

Various traps are available, including

- haemoglobin (ESR changes an alternative to ΔA measurement)
- other haem proteins (Fe^{2+} state)
- Fe^{2+} -dithiocarbamates
- Fe^{2+} -thiosulphate

NO^\bullet is a good ligand to Fe^{2+} ions and the nitrosyl complexes produced have characteristic ESR spectra (e.g. HbNO , $\text{NoFe}[\text{DTC}]_2$, $\text{Fe}[\text{S}_2\text{O}_3]_2[\text{NO}]_2$).

- ribonucleotide reductase (removal of the tyrosyl radical at the active site by NO^\bullet abolishes the ESR signal). Depends on availability of enzyme, thus not widely used.

Attempts to use DNBNS and DMPO to trap NO^\bullet have given equivocal results. More success has been obtained with *nitronyl nitroxides*, a group of organic compounds containing both nitron and nitroxide groups. Nitronyl nitroxide reacts with NO^\bullet to give an imino nitroxide, detectable by ESR (Fig. 5.9) since it has a very different ESR spectrum. NNO has also been used to antagonize the action of NO^\bullet in biological systems. Chelotropic agents such as 7,7,8,8-tetra-methyl-*o*-quinodimethane, have also been used to detect NO^\bullet . They bind NO^\bullet to generate a nitroxide radical, detectable by ESR (*J. Am. Chem. Soc.* **116**, 2767 (1994)).

Formation of ESR-detectable

nitrosylhaemoglobin in blood and tissues is often used as an index of NO^\bullet production, e.g. in ischaemia-reperfusion of organs and in animals with septic shock. Injected Fe^{2+} dithiocarbamates have also been used to detect NO^\bullet in animals with septic shock (*FEBS Lett.* **345**, 120 (1994)). Nitrothiolate ligand signals, probably involving non-haem iron-sulphur proteins have been detected by ESR in NO^\bullet -exposed cells and tissues.

Reduction of nitroxides to ESR-silent species by ascorbate and GSII (Fig. 5.3) can be a problem in biological systems. Superoxide might also reduce some of these species.

Nepřímé metody specifického hodnocení antioxidační aktivity

Oxid dusnatý

Table 5.4. (Continued)

Methods	Principle of method	Comments
B. Indirect methods <u>NO₂⁻ measurement</u>	Griess reaction: NO ₂ ⁻ reacts with sulphanilamide in an acidic solution of N-(1-naphthyl)ethylenediamine to give a coloured azo product (ΔA at 548 nm) Fluorimetry: reaction of NO ₂ ⁻ with 2,3-diaminonaphthalene forms a fluorescent product, 1H-naphthotriazole.	Sensitivity: μM . Easy assays. NO ₂ ⁻ <i>in vivo</i> is rapidly oxidized to NO ₃ ⁻ , which appears to be stable in body fluids. NO ₃ ⁻ can be re-reduced for the assay using nitrate reductase enzymes or chemical reducing agents. NO ₃ ⁻ /NO ₂ ⁻ can also come from diet. ⁷¹ Thus in one human study basal plasma NO ₃ ⁻ was $29 \pm 1 \mu\text{M}$ and rose to $205 \mu\text{M}$ 2 h after intake of NO ₃ ⁻ -rich food. NO ₃ ⁻ in diet can be reduced to NO ₂ ⁻ in the gut. In saliva, NO ₃ ⁻ is converted to NO ₂ ⁻ by oral bacteria. The ranges of NO ₂ ⁻ and NO ₃ ⁻ in plasma samples are quoted as 1.3–13 μM and 4.0–45.3 μM in healthy people (<i>Clin. Chem.</i> 41 , 892 (1995)). Inhaled NO [•] is largely converted to NO ₃ ⁻ in humans.
Measurement of other oxidation products	Based on formation of more reactive species on the pathway from NO [•] to NO ₂ ⁻ in presence of O ₂ . Thus oxidizing NO [•] oxidizes ferrocyanide to ferricyanide (ΔA at 420 nm) and the colourless dye ABTS to the coloured ABTS ⁺ (ΔA at 660 nm). Nitrosation of sulphanilamide generates a coloured azo dye.	All simple spectrophotometric methods. Potential interference from other RNS, e.g. ONOO ⁻ or inhaled NO ₂ [•] .

Nepřímé metody specifického hodnocení antioxidační aktivity

Oxid dusnatý

Use of NOS inhibitors

Can be 'general' inhibitors of all NOS isoforms or selective for a particular type. The former are generally analogues of the NOS substrate L-arginine, such as **N-monomethyl-L-arginine** (L-NMMA) or **N-nitro-L-arginine methyl ester** (L-NAME). Essential controls include showing that D-isomers do not inhibit and that inhibition is reversed by adding excess L-arginine. More selective inhibitors include *N*-iminoethyl L ornithine (eNOS), aminoguanidine (iNOS) and 7-nitro-indazole (nNOS).

Subject to suitable controls, can provide evidence that NO[•] (or species derived from it) are involved. However, NO[•] can arise by reactions not involving NOS, e.g. decomposition of NO₂⁻ by acid in the stomach.

Adapted from Table 1 on p. 278 of *Analysis of Free Radical Reactions in Biological Systems* (Favier *et al.*, eds), Birkhauser-Verlag, Basle, 1995 by courtesy of Dr M. Fontecave and the publishers. Also see *Methods Enzymol.* **268** (1996).

Nepřímé metody specifického hodnocení antioxidační aktivity

Superoxid, metody stanovení SOD-aktivity

- zdroje: chemické – KO_2 , $\text{N}(\text{Bu})_4\text{O}_2$, crown étery, UV-fotolýza ethanolických roztoků, elektrochemická produkce, autooxidace flavinů, epinefrinu, pyrogalolu a NADH oxidace fenazin methosulfátem, biologické – aktivované neutrofily
- detekční systémy
 - přímé – pulsní radiolýza, rapid-freeze EPR, stoped flow-spectroscopy, polarografické techniky, ^{19}F -NMR, cyklická voltametrie
 - nepřímé – redukce cytochromu c, redukce tetrazoliových barviv, redukce nitromethanu, spektrální analýza produktů autooxidace epinefrinu, pyrogalolu, 6-hydroxydopaminu, chemiluminiscence luminolu, tvorba nitritů z chloridu hydroxylamónia
 - biologické – geneticky modifikované kmeny *Salmonella sp.*, nebo *Escherichia sp.*

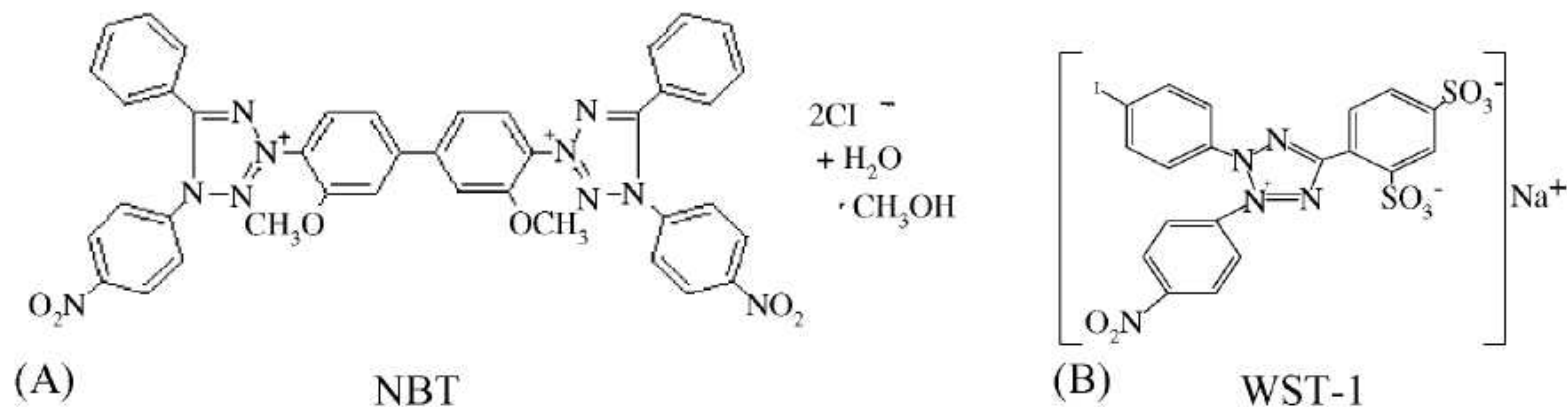


Fig. 1. Chemical structure of NBT (A) and WST-1 (B).

Nepřímé metody specifického hodnocení antioxidační aktivity

Superoxid, metody stanovení SOD-aktivity

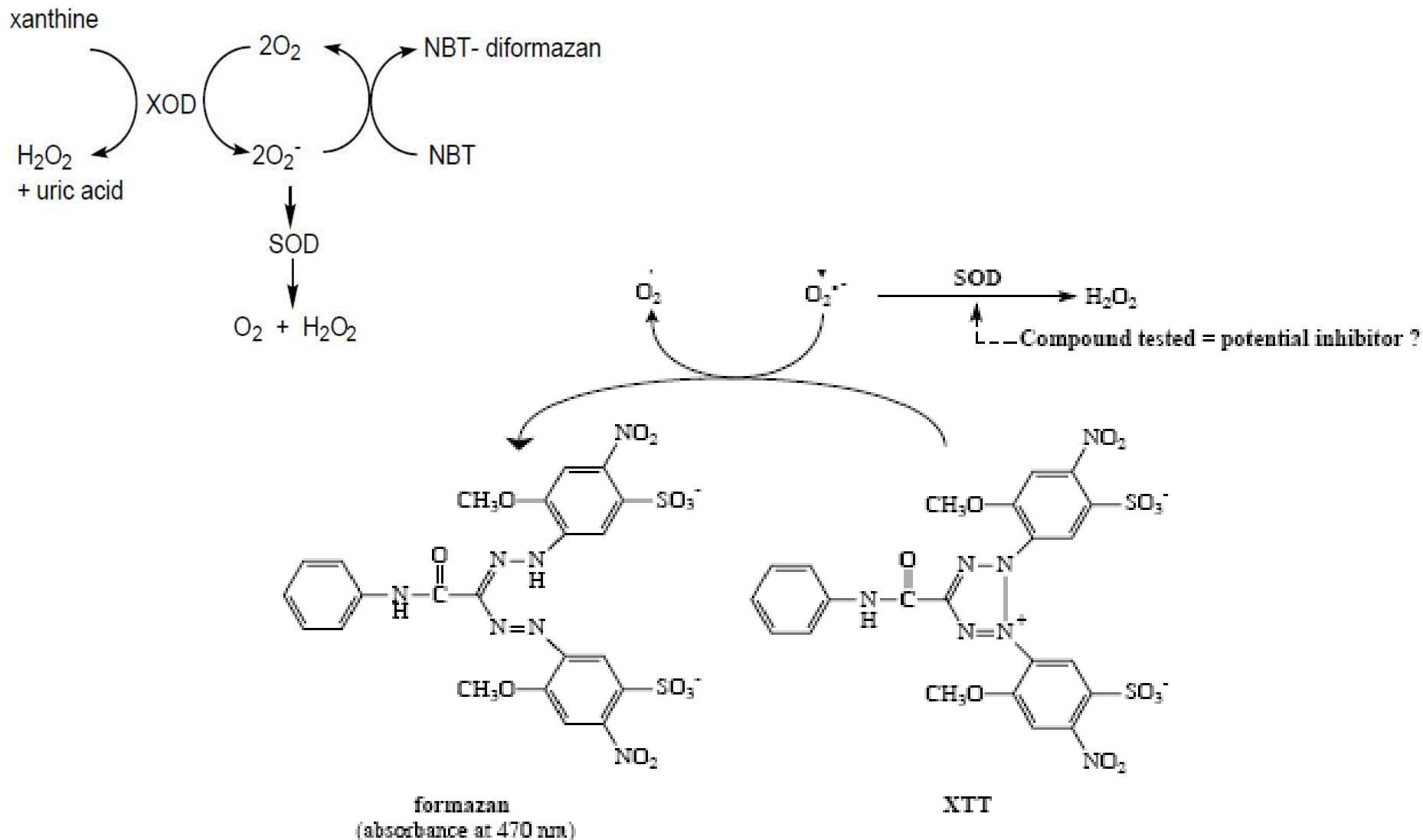
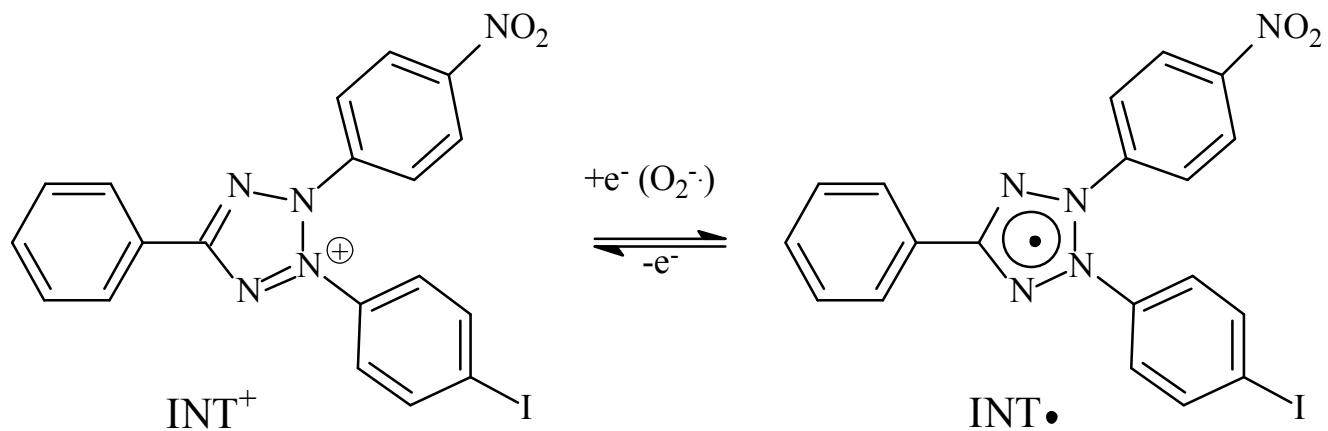


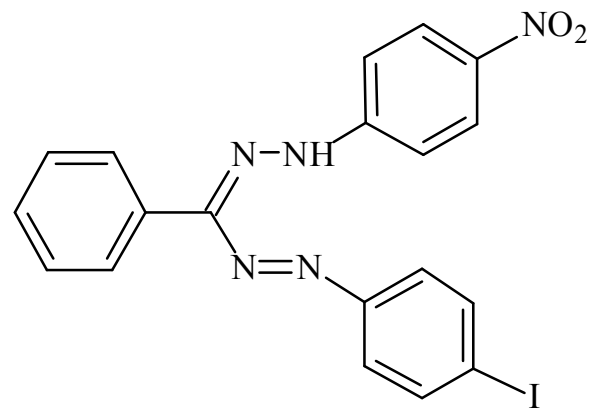
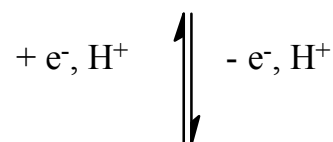
Fig. 3. In vitro SOD assay.

Nepřímé metody specifického hodnocení antioxidační aktivity

Superoxid, metody stanovení SOD-aktivity



2-(4-jódfenyl)-3-(4-nitrofenyl)-5-fenyltetrazól-2-ium



formazán

Nepřímé metody specifického hodnocení antioxidační aktivity

Peroxynitrit

- zdroje: chemické – systém okyselený peroxid vodíku+NaNO₂+ rychlá úprava pH
přídavkem NaOH a odstranění nadbytku H₂O₂ pomocí MnO₂, Angeliho sůl



- detekční systémy
 - nepřímé – nitrace/hydroxylace cílových molekul, např. fenylalanin, tyrosin, proteiny, aminokyseliny
 - biologické – specifické protilátky vůči nitrovaným proteinům