POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2004, Vol. 13/54, SI 2, pp. 23–28

FREE RADICALS AND THEIR ANALYSIS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Bronisław K. Głód[1], Cezary Kowalski[2]

[1]Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland; [2]Department of Pharmacology, Faculty of Veterinary Medicine, Agricultural University, Lublin, Poland

Key words: even cells, free radicals, oxygen, HPLC

The hydroperoxyl radical is a much stronger oxidant than superoxide anion-radical and was initially thought to be responsible for the toxic effects of oxygen and cell membrane damage [Liochev & Fridovich, 2001]. $HO_2^{\bullet}$ has a relatively long lifetime and so can diffuse to neighbouring structures. At the physiological pH of 7.4 about 1% of superoxide exists in its protonated form. A quantitative method for hydroperoxide determination has been developed by O'Gara *et al.* [Morisco *et al.*, 2004], based on the oxidation of glutathione (GSH) by hydroperoxide and formation of oxidized glutathione (GSSG). This reaction is catalyzed by glutathione peroxidase. GSH and GSSG were separated on reversed phase column at pH 3 and their concentrations were estimated using electrochemical detection at a glassy carbon electrode. The method was applied for the analysis of picomolar levels of many hydroperoxides in human plasma.

INTRODUCTION

Although oxygen is necessary to live, it is also toxic for living organisms. A big part of oxygen is used as electron acceptor. Some enzymes catalyze oxidation reactions of transform electrons from substrates on oxygen molecule. During its reduction the so-called ROS – reactive oxygen species are created. Among them one can enumerate free radicals and also singled oxygen and hydrogen peroxide. Free radicals are atoms, ions, molecules or their fragments having unpaired electrons, giving them paramagnetic properties. They are present in every living cell (Tables 1 and 2). Their concentration in animal tissue (about 10^{15}/g) changes widely after interactions with some chemical compounds, radiation, sickness, stress (using much more oxygen) and aging. In the living organisms free radicals are generated under physiological as well as pathological conditions mainly on the inner surface of the mitochondrial membrane. Under physiological conditions they do not accumulate in tissue because they are scavenged by the local antioxidative mechanisms. Under pathological conditions they can cause degenaration and even cell deaths.

Free radicals interact with all cell components. The biggest changes they cause in lipids, proteins and DNA. Of the cell organs, the most exposed to the free radicals' attack are mitochondria. Their damage may cause even cell death.

Half spin particles are described by the Fermi-Dirac statistics. According to the probability function, the electron (fermion) appearance increases with a decrease of energy and never exceeds 1. This means that in a given energetic (described by energy, momentum and spin) state there may be only one electron. The electron disposition in the oxygen molecule and its reduction products are presented in Figure 1. In the basic state the oxygen is a triplet biradical, which explains its relatively high reactivity and simultaneously small reactivity as for a radical.

TABLE 1. Generation of free radicals in living organisms.

1. Exposition to high and low oxygen pressure and after ischemic reperfusion.
2. Interaction of chemical compounds (xenobiotics, pesticides (paraquat), tetrachlorocarbon, PCH, antracyclic antibiotics, ozone, nitric oxides, nicotine).
3. Energetic changes (α, β, γ, UV, visible, IR, X radiation, electrical discharges).
4. Metabolic processes in microsomes, mitochondria, during phagocytose, photosyntese, cytochrome reactions, peroxidation of PUFA.
5. Disorders of metabolic processes by avitaminose (A, E and C), aging, sickness (Alzheimer disease, tumor, rheumatism, diabetes, alcoholism, after encephalopathic blood deficiency) vitamin.

TABLE 2. Influence of free radicals on basic components of animals cells.

1. Lipid peroxidation (changing of membrane "liquidity" and permeability, mitochondrium swelling).
2. Amino acids and proteins (modification of amino acids, peptides fragmentation cross linking).
3. Nucleic acids (modification of heterocyclic bases and pentoze rests, tear of glycoside and phosphodiester bonds).

Singled oxygen

Figure 1 shows the products of oxygen reduction. In the basic state oxygen is a triplet biradical and in the excited

Author's address for correspondence: Cezary Kowalski, Department of Pharmacology, Faculty of Veterinary Medicine, Agricultural University, Akademicka 12, 20-033 Lublin, Poland; fax: (48 81) 445 60 06; e-mail: Cezaryk@agros.ar.lublin.pl

state it appears as a singlet which is present in cytoplasm at a concentration of about 10^{-15} mol/L (compared to about 10^{-5} mol/L for the triplet form). Two singlet oxygen states are known: delta ($^1\Delta gO_2$) and sigma ($^1\Sigma g^+O_2$). They are created during the oxidation of superoxide anion radical with iron (Haber-Weissa reaction) and oxyalcohol, during phagocytose, non-enzymatic lipid oxidations, peroxide dismutation *etc*.

Superoxide anion-radical, $O_2^{\bullet-}$

The first products in the oxygen reduction scheme in Figure 1 are the superoxide anion-radical ($O_2^{\bullet-}$) and its protonated form, the hydroperoxyl radical ($HO_2^{\bullet}$, pK_a=4.88). The concentration of these species in the biological cell is about 10^{-11} mol/L and is kept essentially constant by superoxide dismutase (SOD) [Michelson *et al.*, 1977]. In 1954 Gershman, Gilbert and Fridovich suggested that it is responsible for oxygen toxicity. However it turned out that although it is highly reactive, it is non-reactive in aprotic environment and for amino acids and lipids.

Hydroperoxyl radical, $HO_2^{\bullet}$

The hydroperoxyl radical is a much stronger oxidant than superoxide anion-radical and was initially thought to be responsible for the toxic effects of oxygen and cell membrane damage [Liochev & Fridovich, 2001]. $HO_2^{\bullet}$ has a relatively long lifetime and so can diffuse to neighbouring structures. At the physiological pH of 7.4 about 1% of superoxide exists in its protonated form. A quantitative method for hydroperoxide determination has been developed by O'Gara *et al.* [Morisco *et al.*, 2004], based on the oxidation of glutathione (GSH) by hydroperoxide and formation of oxidized glutathione (GSSG). This reaction is catalyzed by glutathione peroxidase. GSH and GSSG were separated on reversed phase column at pH 3 and their concentrations were estimated using electrochemical detection at a glassy carbon electrode. The method was applied for the analysis of picomolar levels of many hydroperoxides in human plasma. This method and its modifications have been recently reviewed [Hensley *et al.*, 1999]. The next reaction product in Figure 1 is hydrogen peroxide, which can be determined by a range of methods, *e.g.* a detection limit of 6 ng can be obtained with fluorescence detection [Wang & Glaze, 1998].

Hydrogen peroxide, H_2O_2

Hydrogen peroxide is generated during dismutation reaction. It is also generated by L-amino acid and glycolan oxidases which carry 2 electrons. In all other cases reaction goes in two steps. In the presence of transition metal ions (iron, copper) hydrogen peroxide dissociated with the production of very reactive hydroxyl radical, according to Fenton's reaction described in 1884:

$$Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH^{\bullet} \quad (1)$$

Already oxidized metal can be reduced, it means that it plays the role of catalyst:

$$Fe^{3+} + O_2^{\bullet-} = Fe^{2+} + O_2 \quad (2)$$

The both reactions give the Haber-Weiss one (year 1934):

$$O_2^{\bullet-} + H_2O_2 = O_2 + OH^- + OH^{\bullet} \quad (3)$$

Hydroxyl radical, $OH^{\bullet}$

Reduction of hydrogen peroxide gives the very reactive hydroxyl radical, $OH^{\bullet}$. This radical interacts with a number of organic compounds by the addition, free radical substitution and electron transfer reactions. It does not penetrate the cell and interacts only with molecules that are in close proximity. Its concentration in biological systems is of the order of 10^{-11} mol/L. Because of their extreme reactivity, hydroxyl radicals are primarily analyzed using radical trapping agents followed by electron spin resonance (ESR) or HPLC determinations. The ESR is highly sensitive but is difficult to be applied directly to biological systems. Free radicals may be investigated using this technique by transforming them into more stable species using the so-called "spin trapping" method. When this method is used for biological analyses the radical trap agent should be selected extremely carefully to avoid toxicity problems. Examples of

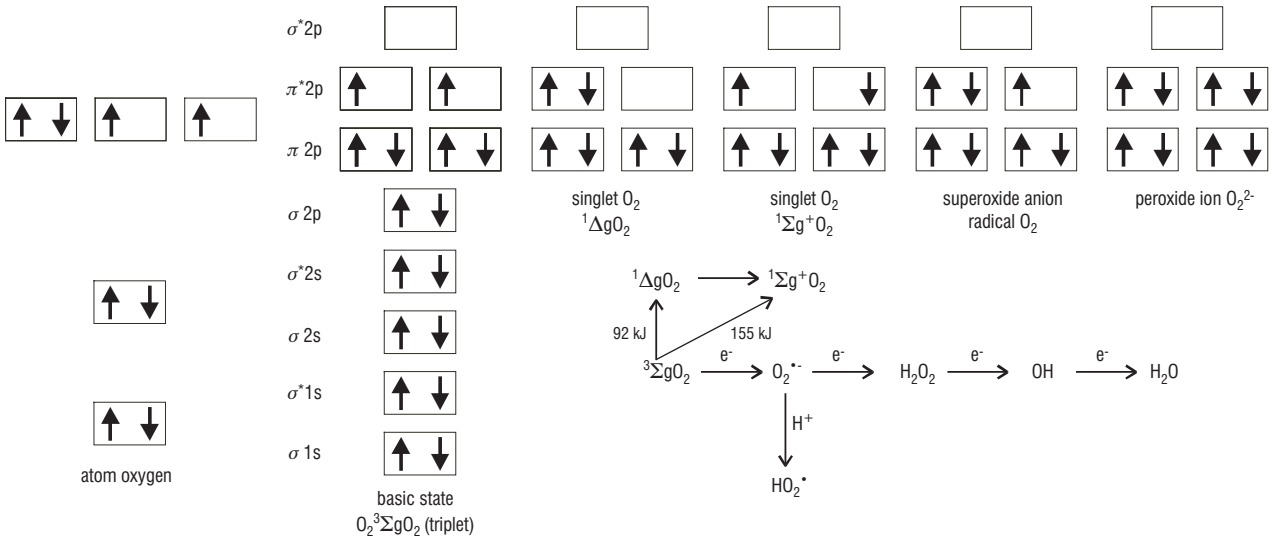

FIGURE 1. Electrons distributions in oxygen and products of its reduction. □ – molecular orbital, * – anti-bond orbital, ↑ ↓ – electron spines.

suitable compounds are phenylalanine (with which hydroxyl free radicals react to produce tyrosines [Halliwell & Kaur, 1991]) or derivatives of aspirin (o-acetylsalicylic acid). In living organisms, aspirin is hydrolyzed rapidly to salicylic acid, which under physiological conditions at pH 7.4 reacts with hydroxyl radicals to give three main products: 2,3-dihydroxybenzoate (49%) and 2,5-dihydroxybenzoate acid (40%) (collectively referred to as DHBA) and o-catechol (11%) (Figure 2). These derivatives can be separated using reversed phase HPLC [Diez *et al.*, 2001] (Figure 3) and detected photometrically or more sensitively by electrochemical detection using a glassy carbon electrode at 0.8 V *vs* Ag/AgCl (detection limits of 1 pg for DHBA and 100 pg for salicylic acid) [Floyd *et al.*, 1986]. DHBA compounds are

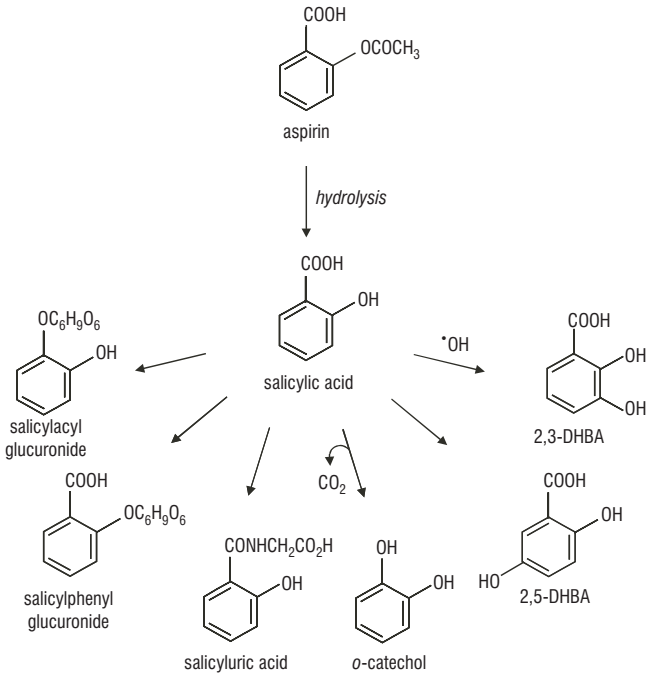

FIGURE 2. Metabolites and products of the reaction of hydroxyl radical with salicylic acid.

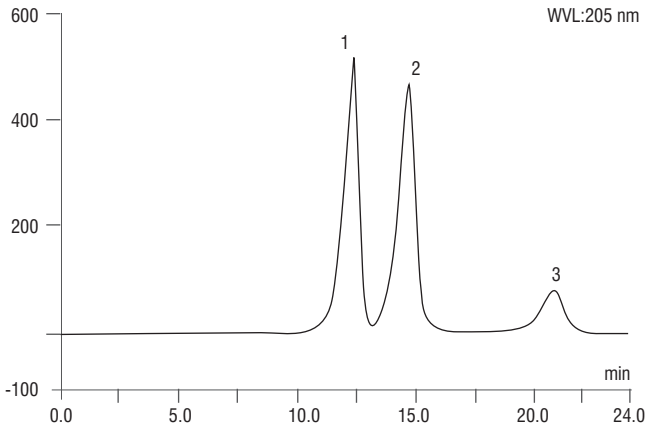

FIGURE 3. HPLC chromatogram of 1 mmol/L standards of acids (1) - 2,5-, (2) – 2,3-dihydroxybenzoicand (3) – salicylic acid. Chromatographic conditions: column – 250×4 mm I.D. Zorbax ODS 5mm (Knauer); mobile phase – acetate-citrate buffer pH 4.3, 1 mmol/L KCl, 0.25 mmol/L EDTA, 5% MeOH, 3 mmol/L TBAP; temp. – 20°C; flow rate – 0.9 mL/min; detector – UV-205 nm [Głód & Grieb, 2002].

eluted near the column dead volume, together with other species such as catecholamines. For this reason, the use of ion-pairing chromatography [Głód *et al.*, 2000] is the preferred separation mechanism. The use of salicylic acid as the radical trap agent has some disadvantages [Ste-Marie *et al.*, 1996], especially the determination of 2,5-dihydroxybenzoic acid due to endogenous production of this species by, for example cytochrome P-450 [Halliwell & Kaur, 1991], and the sensitivity of this system is reduced because there are two products of the reaction.

To avoid these problems Ste-Marie *et al.* [1996] have proposed the use of *p*-hydroxybenzoic acid as the radical trap agent, and this species has recently been applied using ion-exclusion chromatography to measure the product of reaction (3,4-dihydroxybenzoate) [Floyd *et al.*, 1986]. This method has a number of advantages, in that only one isomer is formed by hydroxylation, so the detection signal is not split between two compounds, and the separation is relatively simpler.

Nitric oxide

Recently, there has been intense investigation of the free radical, nitric oxide [Murad, 1996]. This species is a neurotransmitter (in the central as well as peripheral neuronal systems) and a free radical scavenger, but being a free radical it is also neurotoxic and generates other free radicals. The effects of NO are probably dependent on concentration, with NO acting as free radicals at high concentrations, and being protective at low concentrations. In the biological cells, nitric oxide is generated during oxidation of arginine to citrulline (in the presence of NADPH as an electron donor) by nitric oxide synthase (NOS) [Moncada & Higgs, 1993]. In some tissues the only source of citrulline is NOS, thus the determination of citrulline provides the possibility to measure NOS activity and indirectly, the nitric oxide concentration. The standard method HPLC method for this determination is based on derivatisation with *o*-phthalaldehyde, followed by separation on a C18 stationary phase and fluorometric detection at the excitation/emission wavelengths of 338/425 nm [Carlber, 1994]. This method provides a detection limit of 0.1 pmole.

Nitric oxide is quickly degenerated due to interaction with oxygen, nitric dioxide and the superoxide anion-radical. It scavenges the superoxide anion-radical, generating peroxynitrite anion, as follows [Llorens *et al.*, 2002]:

$$NO^{\bullet} + O_2^{\bullet -} = ONOO^- \qquad (4)$$

After protonation peroxynitrite decomposes into two radicals, hydroxide and nitric dioxide, and under physiological conditions it rearranges to nitrate in about 1 s [Groves, 1999]. The neurotoxicity of nitric oxide arises from peroxynitrite and its decomposition products, which interact with lipids, DNA, and the protein tyrosine. In the latter case, the product (nitrotyrosine) may be determined relatively easily by reversed-phase HPLC with photometric (274 nm) [Rehman *et al.*, 1997] or electrochemical [Hensley *et al.*, 1998] detection, or by ion-exclusion chromatography [Głód *et al.*, 2000] together with dihydroxybenzoic acids. The determination of NO_2^- and NO_3^- can also be used as an indirect method for the measurement of nitric oxide. These species are determined using ion-exchange and ion-pair

chromatography [Everett *et al.*, 1997; Ellis *et al.*, 1998], with conductometric, photometric (214 nm), and electrochemical detection (for nitrite) [Everett *et al.*, 1997; Di Matteo & Esposito, 1997]. The high concentration of chloride (10^5 higher then nitrite [Monaghan *et al.*, 1997]) present in biological samples can be removed using a cation-exchange resin in the Ag^+ form [Stratford *et al.*, 1997], or by using chloride as a mobile phase [Monaghan *et al.*, 1997; Raessler *et al.*, 2004]. Good separation was also obtained using capillary electrophoresis [Haddad, 1997].

Lipidic radicals

Free radicals interact with lipids producing other radicals and stable lipid peroxides [Michelson *et al.*, 1977], which usually are more reactive and toxic then the original substrate radicals. Non-metal enzymes (such as lipooxydase) also generate lipid radicals. These species do not contain strong chromophores and are electroinactive in the accessible potential range, but can be determined chromatographically after derivatisation. A sensitive analysis (at the ng level) uses derivatisation to *p*-hydroxyanilides, followed by separation on reversed-phase column and electrochemical detection at 0.7 V [Ikenoya *et al.*, 1980]. Hydroxyperoxides of phospholipids may be determined after derivatisation with 2,2'-azobis(2-amidynopropan)dihydrochloride, followed by separation on a propylamine stationary phase and chemiluminescence detection [Gou *et al.*, 1995].

Degradation of cell membranes is observed during the reaction of free radicals with polyunsaturated fatty acids, PUFA, to yield lipid hydroperoxides, aldehydes and ketones. These compounds are relatively stable and cytotoxic [Głód *et al.*, 2000]. They interact with the cell and membrane components, showing strong chemical affinity. Generated in blood (endothelium, fibroblasts, hearth muscle *etc*.) they are responsible for arteriosclerosis. Additionally they influence the change of electric charge, hydrophobicity and "liquidity" of membranes, thus decreasing their mobility. New electric charges on the membrane surface indicate new carboxylic groups generated during peroxidation. It makes them more hydrophilic, which changes the membrane structure (transport, receptors).

The estimation of oxygen radical damage in a biological system is usually carried out by determination of malondialdehyde (MDA) [Onorato *et al.*, 1998; Debouzy *et al.*, 1992]. The most frequently used method is the thiobarbituric acid (TBA) test. It has been found a reliable assay because of its simple handling and sensitivity [Halliwell & Chirico, 1993]. This is true despite the fact that the TBA assay is intrinsically non-specific and is generally poor when applied to biological samples. A positive response is obtained with sugars, some amino and bile acids, alkenals, alkadienals, *etc*. Certain improvement can be obtained using RP-HPLC separation followed by fluorometric [Volpi & Tarugi, 1988; Bergamo *et al.*, 1998] or photometric detection at 535 nm [Wong *et al.*, 1987; Bagenholm *et al.*, 1997; Suttnar *et al.*, 2001; Khoschsorur *et al.*, 2000; Hong *et al.*, 2000]. In this case thiobarbituric acid influences both the retention (separation) as well as detection conditions. This method still requires a derivatization step. Additionally its results are questionable because identical adducts are yielded from different substances. A similar method is based on the derivatization with 2,4-dinitrophenylhydrazine [Pilz *et al.*, 2000; Bakalova *et al.*, 2000; Fenaille *et al.*, 2001] or diaminonaphthalene [Steghens *et al.*, 2001] followed by photometric detection at 310 nm. Simplification of this method (no derivatization is required and sample preparation is minimal) was shown by the application of reversed phase ion pair chromatography [Waterfall *et al.*, 1995; Bull & Marnett, 1985]. Detection accomplished by monitoring absorbance at 267 nm, enabled analysis of MDA together with two antioxidants, namely ascorbic and uric acids. Unfortunately, the described method produces very broad and small peak of MDA.

Recently it was shown that ion exclusion chromatography can be applied for the analysis of malondialdehyde [Głód & Kowalski, 2004]. Two methods were compared. The first one was based on its derivatisation with thiobarbituric acid, the second one on its direct separation with ion interaction reagent added to the mobile phase. Better separation was obtained using the second method although lower detection limit offered the first one. Peak broadening was strongly affected by column temperature, which is probably caused by kinetic effects (Figure 4).

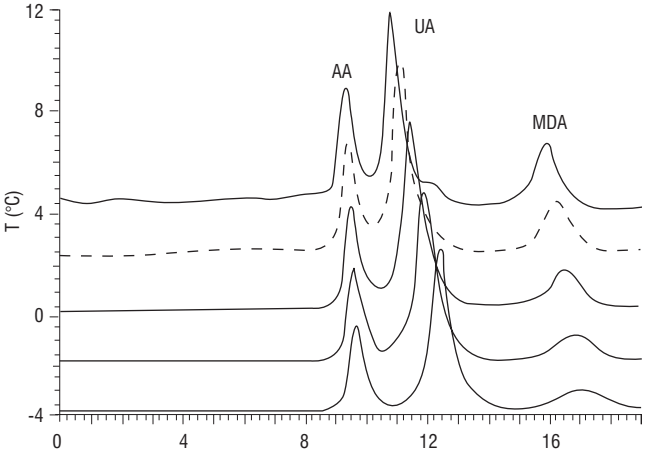

FIGURE 4. IEC chromatograms of ascorbic acid (AA), uric acid (UA) and malonyldialdehyde (MDA) obtained at different column temperatures. Chromatographic conditions: column – 300×7.8 mm I.D. TSK-GEL SCH(H^+) (TosoHaas); mobile phase – 3 mN H_2SO_4, 3 mmol/L TBABr, 5% ACN; detector UV-267 nm; temp. -20°C÷40°C.

CONCLUSIONS

In the paper firstly the role of free radicals in biological systems was briefly presented. Its main idea is to describe HPLC methods of their analysis. It includes three main groups of biologically-active radicals, nitric oxide, oxygen (including hydroxyl) and lipid (including their marker – malonyldialdehyde) radicals. Some free radical scavengers were also shortly mentioned. Reversed phase still remains the main technique widely used for their determination. Ion Exclusion Chromatography finds application in the separation of a wide range of small, neutral or partially-ionized molecules. Relatively simple retention models, based on analytical equations and computer simulation of column performance, enable one to predict the retention and to optimize the separation. The major advantage of IEC lies in the ability to analyze samples having a complex composi-

tion. We would like to show the possibility of its application also for determination of free radicals in biological material. The new idea in this technique, namely applications of weak cation exchangers for the separation of strong acids, like nitrates (they indirectly inform us about the concentration of nitric oxide), is also shortly discussed.

REFERENCES

1. Bågenholm R., Nilsson U.A., Kjellmer I., Formation of free radicals in hypoxic ischemic brain damage in the neonatal rat, assessed by an endogenous spin trap and lipid peroxidation. Brain. Res., 1997, 773, 132–138.
2. Bakalova R., Mileva M., Kotsev C., Bardarov V., Ribarov S., Determination of malondialdehyde in biological samples by solid-phase extraction and high-performance liquid chromatography. Meth. Findings Exp. Clinic. Pharmac., 2000, 22, 267–269.
3. Bergamo P., Fedele E., Balestrieri M., Abrescia P., Ferrara L., Measurement of malondialdehyde levels in food by high-performance liquid chromatography with fluorometric detection. J. Agr. Food Chem., 1998, 46, 2171–2176.
4. Bull A.W., Marnett L.J., Determination of malondialdehyde by ion-pairing high-performance liquid chromatography. Analyt. Biochem., 1985, 149, 284–290.
5. Carlberg M., Assay of neuronal nitric oxide synthase by HPLC determination of citrulline. J. Neurosci. Meth., 1994, 52, 165–167.
6. Debouzy J.C., Fauvelle F., Vezin H., Brasme B., Chancerelle Y., Interaction of the malonyldialdehyde molecule with membranes. A differential scanning calorimetry, 1H-, 31P-NMR and ESR study. Biochem. Pharmacol., 1992, 44, 1787–1793.
7. Di Matteo V., Esposito E., Methods for the determination of nitrite by high-performance liquid chromatography with electrochemical detection. J. Chromatogr. A, 1997, 789, 213–219.
8. Diez L., Livertoux M.H., Stark A.A., Wellman-Rousseau M., Leroy P., High-performance liquid chromatographic assay of hydroxyl free radical using salicylic acid hydroxylation during *in vitro* experiments involving thiols. J. Chromatogr. B., 2001, 736, 185–193.
9. Ellis G., Adatia I., Yazdanpanah M., Makela S.K., Nitrite and nitrate analyses: A clinical biochemistry perspective. Clin. Biochem., 1998, 31, 195–220.
10. Everett S.A., Dennis M.F., Patel K.B., Wardman P., Stratford M.R.L., High-performance ion chromatography applied to free-radical mechanisms in drug design – The problem of ion analysis at high ionic strengths. J. Chromatogr. A, 1997, 770, 273–279.
11. Fenaille F., Mottier P., Turesky R.J., Ali S., Guy P.A., Comparison of analytical techniques to quantify malondialdehyde in milk powders. J. Chromatogr. A, 2001, 921, 237–245.
12. Floyd R.A., Henderson R., Watson J.J., Wong P.K., Use of salicylate with high pressure liquid chromatography and electrochemical detection (LCED) as a sensitive measure of hydroxyl free radicals in adriamycin treated rats. J. Free Rad. Biol. Med., 1986, 2, 13–18.
13. Głód B.K., Czapski G.A., Haddad P.R., Application of high-performance liquid chromatography to the investigation of free radical reactions in biological systems. TRAC, Trends Anal. Chem., 2000, 19, 492–497.
14. Głód B.K. Grieb P., Modified analytical method for hydroxyl radicals using spin trapping and ion exclusion chromatography and its application for antioxidative potential assay. Chem. Anal. (Warsaw), 2002, 47, 399–407.
15. Głód B.K., Kowalski J.C., Analysis of malonyldialdehyde using ion exclusion chromatography. J. Liquid Chromatogr. & Rel. Technol., 2004, 27, 2733–2742.
16. Gou L., Ogamo A., Ou Z., Shinozuka T., Nakagawa Y., Preferential formation of the hydroperoxide of linoleic acid in choline glycerophospholipids in human erythrocytes membrane during peroxidation with an azo initiator. Free Rad. Biol. Med., 1995, 18, 1003–1012.
17. Groves J.T., Peroxynitrite: reactive, invasive and enigmatic. Curr. Opin. Chem. Biol., 1999, 3, 226–235.
18. Haddad P.R., Comparison of ion chromatography and capillary electrophoresis for the determination of inorganic ions. J. Chromatogr. A, 1997, 770, 281–290.
19. Halliwell B., Chirico S., Lipid peroxidation: its mechanism, measurement, and significance. Am. J. Clin. Nutr., 1993, 57, 715S–724S.
20. Halliwell B., Kaur H., Hydroxylation of salicylate and phenylalanine as assays for hydroxyl radicals: a cautionary note visited for the third time. Free Radical Biol. Med.,1991, 27, 239–240.
21. Hensley K., Maidt M.L., Yu Z.Q., Sang H., Markesbery W.R., Floyd R.A., Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. J. Neurosci., 1998, 18, 8126–8132.
22. Hensley K., Williamson K.S., Maidt M.L., Gabbita S.P., Grammas P., Floyd R.A., Determination of biological oxidative stress using high performance liquid chromatography with electrochemical detection (HPLC-ECD). HRC-J. High Res. Chromatogr., 1999, 22, 429–437.
23. Hong Y.L., Yeh S.L., Chang C.Y., Hu M.L., Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method. Clin. Biochem., 2000, 33, 619–625.
24. Ikenoya S., Hiroshima O., Ohmae M., Kawabe K., Electrochemical detector for high performance liquid chromatography. IV. Analysis of fatty acids, bile acids and prostaglandins by derivatization to an electrochemically active form. Chem. Pharm. Bull., 1980, 28, 2941–2947.
25. Khoschsorur G.A., Winklhofer-Roob B.M., Rabl H., Auer T., Peng Z., Schaur R.J., Evaluation of a sensitive HPLC method for the determination of malondialdehyde, and application of the method to different biological materials. Chromatographia, 2000, 52, 181–184.
26. Liochev S.I., Fridovich I., The oxidation of 3-hydroxyanthranilic acid by Cu, Zn superoxide dismutase: Mechanism and possible consequences. Arch. Biochem. Biophys., 2001, 388, 281–284.
27. Llorens S., Jordan J., Nava E., The nitric oxide pathway in the cardiovascular system. J. Physiol. Biochem., 2002, 58, 179–188.
28. Michelson A.M., McCord J.M., Fridovich I., Superoxide and Superoxide Dismutases, 1977, Academic Press, London.

29. Monaghan J.M., Cook K., Gara D., Crowther D., Determination of nitrite and nitrate in human serum. J. Chromatogr. A, 1997, 770, 143–149.
30. Moncada S., Higgs A., The L-arginine-nitric oxide pathway. N. Engl. J. Med., 1993, 329, 2002–2012.
31. Morisco F., Verde V., Fogliano V., Ritieni A., Marmo R., De Luise G., Tuccillo C., Caporaso N., Oxidative status in chronic hepatitis C: The influence of antiviral therapy and prognostic value of serum hydroperoxide assay. Free Radical Res., 2004, 38, 573–580.
32. Murad F., Signal transduction using nitric oxide and cyclic guanosine monophosphate. JAMA-J. Am. Med. Assoc., 1996, 276, 1189–1192.
33. Onorato J.M., Thorpe S.R., Baynes J.W., Immunohistochemical and ELISA assays for biomarkers of oxidative stress in aging and disease. Ann. NY Acad. Sci., 1998, 854, 277–290.
34. Pilz J., Meineke I., Gleiter C.H., Measurement of free and bound malondialdehyde in plasma by high-performance liquid chromatography as the 2,4-dinitrophenylhydrazine derivative. J. Chromatogr. B, 2000, 742, 315–325.
35. Raessler M., Hilke I., Middelmann S., Determination of trace amounts of nitrate in potassium chloride-extracts by HPLC with ultra-violet-detection. Commun. Soil Sci. Plant Anal., 2004, 35, 69–83.
36. Rehman A., Whiteman M., Halliwell B., Scavenging of hydroxyl radicals but not of peroxynitrite by inhibitors and substrates of nitric oxide synthases. Brit. J. Pharmacol., 1997, 122, 1702–1706.
37. Steghens J.P., van Kappel A.L., Denis I., Collombel C., Diaminonaphtalene, a new highly specific reagent for HPLC-UV measurement of total and free malondialdehyde in human plasma or serum. Free Radic. Biol. Med., 2001, 31, 242–249.
38. Ste-Marie L., Boismenu D., Vachon L., Montgomery J., Evaluation of sodium 4-hydroxybenzoate as an hydroxyl radical trap using gas chromatography mass spectrometry and high-performance liquid chromatography with electrochemical detection. Anal. Biochem., 1996, 241, 67–74.
39. Stratford M.R.L., Dennis M.F., Cochrane R., Parkins C.S., Everett S.A., The role of nitric oxide in cancer – Improved methods for measurement of nitrite and nitrate by high-performance ion chromatography. J. Chromatogr. A, 1997, 770, 151–155.
40. Suttnar J., Masova L., Dyr. J.E., Influence of citrate and EDTA anticoagulants on plasma malondialdehyde concentrations estimated by high-performance liquid chromatography. J. Chromatogr. B, 2001, 751, 193–197.
41. Volpi N., Tarugi P., Improvement in the high-performance liquid chromatography malondialdehyde level determination in normal human plasma. J. Chromatogr. B, 1988, 713, 433–437.
42. Wang K.X., Glaze W.H., High-performance liquid chromatography with postcolumn derivatization for simultaneous determination of organic peroxides and hydrogen peroxide. J. Chromatogr. A, 1998, 822, 207–213.
43. Waterfall A.H., Singh G., Fry J.R., Marsden C.A., Detection of the lipid peroxidation product malonaldehyde in rat brain *in vivo*. Neurosci. Lett., 1995, 200, 69–72.
44. Wong S.H.Y., Knight J.A., Hopfer S.M., Zaharia O., Leach Jr. C.N., Sunderman F.W., Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. Clin. Chem., 1987, 33, 214–220.