REVIEW

Application of medical and analytical methods in Lyme borreliosis monitoring

Magdalena Ligor · Paweł Olszowy ·
Bogusław Buszewski

Abstract Lyme borreliosis (LB) is one of the most common tick-borne diseases in the northern hemisphere. It is a chronic inflammatory disease caused by the spirochaete *Borrelia burgdorferi*. In its early stages, pathological skin lesions, namely erythema chronicum migrans, appear. The lesions, usually localised at the site of the bite, may become visible from a few weeks up to 3 months after the infection. Predominant clinical symptoms of the disease also involve joint malfunctions and neurological or cardiac disorders. Lyme disease, in all its stages, may be successfully treated with antibiotics. The best results, however, are obtained in its early stages. In order to diagnose the disease, numerous medical or laboratory techniques have been developed. They are applied to confirm the presence of intact spirochaetes or spirochaete components such as DNA or proteins in tick vectors, reservoir hosts or patients. The methods used for the determination of LB biomarkers have also been reviewed. These biomarkers are formed during the lipid peroxidation process. The formation of peroxidation products generated by human organisms is directly associated with oxidative stress. Apart from aldehydes (malondialdehyde and 4-hydroxy-2-nonenal), many other unsaturated components such as isoprostenes and neuroprostane are obtained. The fast determination of these compounds in encephalic fluid, urine or plasma, especially in early stages of the disease, enables its treatment. Various analytical techniques which allow the determination of the aforementioned biomarkers have been reported. These include spectrophotometry as well as liquid and gas chromatography. The analytical procedure also requires the application of a derivatization step by the use of selected reagents.

Keywords Lyme borreliosis · Biomarkers · Analytical methods · Malondialdehyde · 4-Hydroxy-2-nonenal · Prostanes

Abbreviations

AA	arachidonic acid
ACA	acrodermatitis chronica atrophicans
APCI	atmospheric pressure chemical ionization
BHT	butylated hydroxytoluene
BL	Borrelia lymphocytoma
CNS	central nervous system
DAN	diaminonaphthalene
DNPH	2,4-dinitrophenylhydrazine
ECD	electron capture detector
EIA	enzyme immunoassay
ELFA	enzyme-linked fluorescent assay
ELISA	enzyme-linked immunosorbent assay
EM	erythema migrans
EPA	eicosapentaenoic acid
ESI	electrospray ionization
FLD	fluorescence detector
GC	gas chromatography
GSH	glutathione
GSHPO	glutathione peroxidase
HNE	4-hydroxy-2-nonenal
HPCE	high-performance capillary electrophoresis
HS-SPME	headspace solid-phase microextraction
IFA	immunofluorescent antibody assay
IFS	immunoaffinity separation

M. Ligor · P. Olszowy · B. Buszewski (✉)
Department of Environmental Chemistry and Bioanalytics,
Faculty of Chemistry, Nicolaus Copernicus University,
Gagarina 7 St.,
87-100 Toruń, Poland
e-mail: bbusz@chem.umk.pl

Iso-Ps	isoprostanes
LA	Lyme arthritis
LB	Lyme borreliosis
LC	liquid chromatography
MDA	malondialdehyde
MRM	multiple reaction monitoring mode
MS	mass spectrometry
Neuro-Ps	neuroprostanes
PCR	polymerase chain reaction
PFB	pentafluorobenzyl ester
PH	phenylhydrazine
PUFAs	polyunsaturated fatty acids
ROS	reactive oxygen species
SIM	single-ion monitoring mode
SPE	solid-phase extraction
TBARS	thiobarbituric acid reactive substances
TCPH	2,4,6-trichlorophenylhydrazine
TLC	thin-layer chromatography

Introduction

In Europe and North America, *Ixodes ricinus* ticks are common ectoparasites (external parasites). Many species of viruses, bacteria and spirochaetes are found in tick vectors. They belong to a phylum of distinctive Gram-negative bacteria, which have long, helically coiled (spiral-shaped) cells. Therefore, ticks are vectors of a number of diseases, including Lyme disease, Colorado tick fever, tularaemia, tick-borne relapsing fever, babesiosis, ehrlichiosis and tick-borne meningoencephalitis, as well as anaplasmosis in cattle and canine jaundice [1, 2]. The cause of Lyme borreliosis is the activity of a single spirochaete species, *Borrelia burgdorferi*. Lyme borreliosis or Lyme disease is one of the most often identified diseases on the northern hemisphere. In Europe and North America, approximately 85,000 and 15,000–20,000 cases are identified each year, respectively. Noticeably, numerous cases of Lyme borreliosis are reported in Poland, Sweden, Lithuania, Latvia, Belarus, Austria, the Czech Republic and Slovenia [3].

When a human organism is infected with Lyme borreliosis, the fastest occurring symptoms are inflammation and changes in the immunological system. The disease causes noticeable damage in various organs including the brain, central nervous system (CNS), heart, eyes, skeleton and joints. The effects of the disease depend on the genotype of the spirochaetes, individual immunological system predispositions and other factors [3]. Usually, antibiotics eliminate symptoms of the infection, especially if the illness is treated in its early stage. If the disease is identified long after the infection took place, delayed or inadequate treatment can lead to more serious symptoms,

which can even become irreversible (post-Lyme borreliosis syndromes [4]).

In this paper, methods used for diagnosing Lyme borreliosis (LB) are reviewed. The developments in laboratory and analytical methods used for the diagnosis of the disease are taken into consideration and discussed in detail. Procedures applied for the determination of LB biomarkers are also presented. Apart from aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), many other unsaturated components, e.g. isoprostenes and neuroprostane, are separated and determined in biological samples. The formation of peroxidation products generated by a human organism is directly associated with oxidative stress. An analysis of human encephalic fluid, blood, urine or plasma, provides much important data. The presence of the MDA, HNE and isoprostanes was confirmed in various types of tissues as well as human body fluids. The fast separation of these compounds from encephalic fluid, urine and plasma, especially in early stages of the disease, makes LB treatment possible. Various analytical techniques which allow the determination of the aforementioned biomarkers have been reported. These include spectrophotometry as well as liquid and gas chromatography. The analytical procedure also requires the application of a derivatization step by the use of selected reagents. Moreover, modern analytical techniques including liquid chromatography coupled with tandem mass spectrometry (LC/MS^n) or a fluorescence detector (FLD) have been used for the determination of such compounds.

Spectrum of Lyme borreliosis

Lyme borreliosis is a multisystemic disease caused by the spirochaete *Borrelia burgdorferi* sensu lato (in Poland these are *B. burgdorferi*, *B. garinii*, and *B. afzelii*), transmitted by *Ixodes* ticks. Lesions are associated primarily with the injury of the skin, joints, nervous system, and heart infections [5–12].

Patients infected with *B. burgdorferi* sensu lato may experience one or more clinical syndromes of early or late LB. The basis for the LB diagnosis in humans is a series of its clinical forms [13–15]. One of these is erythema migrans (EM, creeping rash). The diagnosis is based solely on the clinical picture. Typically, immunoserological tests towards anti-*B. burgdorferi* antibodies are unnecessary. Erythema migrans may appear at the site of a tick bite usually after 1–3 weeks (and even after 3 months). A quickly appearing syndrome takes the form of a macula, and quickly expands showing central clearing. Erythema migrans disappears within a few days of the proper antibiotic therapy, but this fact is not tantamount to the elimination of infection.

Untreated EM may last several months, and its spontaneous regression does not eliminate the infection either. Multiple secondary EM, which shows the infection is spreading, occurs rarely. The changes are usually smaller than those at the primary stage and uniformly coloured.

Diagnosing Borrelia lymphocytoma (BL) requires the determination of IgM or IgG presence in serum and its histological confirmation. BL symptoms are noted in less than 1% of patients, usually within a few weeks after a tick bite, as a single, bluish red, painless nodule. BL is most frequently localised in earlobes, nipples, and the scrotum. BL changes may be accompanied by lymph node enlargement.

Another kind of LB clinical form is chronic atrophic dermatitis on limbs (acrodermatitis chronica atrophicans, ACA). ACA diagnosis requires the determination of IgM or IgG presence in serum and its histological confirmation. In ACA, bluish red changes, initially with the features characteristic of inflammatory edema, and later skin atrophy, are frequently observed. They appear even many years after infection. ACA is most frequently localised on certain parts of the limbs, especially legs. Less commonly, it can be the trunk. These changes may be accompanied by leg pain which often appears simultaneously with the main symptom of peripheral neuropathy and changes in degenerative arthritis beneath the affected skin. ACA is usually diagnosed in elderly people, mostly women.

Moreover, cases of arthritis (Lyme arthritis, LA) are very often observed. The morbidity requires laboratory confirmation by the determination of IgM antibodies in serum at an early stage, or IgG antibodies at the late stage of the disease. LA can take various clinical forms, e.g. wandering pain in bones, joints, muscles and tendons, or chronic arthritis (asymmetric, occurring even a few years after infection, usually preceded by recurrent pain or arthritis). Flu-like symptoms, including pain in joints, of varied intensity are present in 40% of cases with erythema migrans. They usually recede spontaneously and have no connection with subsequent LA symptoms observed.

In *B. burgdorferi* spirochaete infection, myocarditis (Lyme carditis) may also occur. A diagnosis requires the confirmation of the presence of IgM antibodies against *B. burgdorferi* in serum and cardiac dysfunction confirmed by an ECG examination.

One of the most common effects caused by the spirochaete *B. burgdorferi* is neuroborreliosis. When diagnosing, it is necessary to identify other typical LB symptoms, particularly erythema migrans. It is necessary to confirm the presence of IgM or IgG antibodies against *B. burgdorferi* in serum, and in the case of the brain and spinal cord inflammation, additionally their intrathecal production. These confirmations are needed to exclude the passive transfer of antibodies through the blood–brain barrier. Some patients with neuroborreliosis in the early stages of LB (the first weeks after getting infected) show no presence of antibodies in serum. In such cases, the test should be repeated 2 weeks after the symptoms of the disease were observed. The seroconversion occurring confirms the diagnosis. In the course of neuroborreliosis, cerebrospinal fluid (CSF) of patients with meningitis is characterized by lymphocytic pleocytosis, a moderate increase in protein concentration, and normal glucose concentration in serum. In its first period, neuroborreliosis may proceed as follows: cranial nerve palsy, nerve roots or individual peripheral nerves paralysis, lymphocytic meninges inflammation (meningitis), brain inflammation (encephalitis) or brain and spinal cord (encephalomyelitis) inflammation. In the late stage of neuroborreliosis, the following symptoms may occur: inflammation of the brain and spinal cord (encephalomyelitis), peripheral neuropathy, chronic encephalopathy (with dominant memory and/or concentration impairment, irritability, sleepiness and personality changes).

Laboratory diagnosis

Many achievements in the field of LB laboratory diagnosis have been discussed and published in numerous reviews [16–20]. In order to identify each of the Lyme disease clinical forms (except EM), laboratory diagnosis requires a two-stage diagnostic protocol. The first step should confirm the presence of specific IgM or IgG (depending on the clinical form) on the basis of the immunoenzymatic method. In the second stage, for patients with positive or dubious results, Western blotting methods should provide unambiguous results. Both methods complement each other as immunoenzymatic tests are usually characterized by high sensitivity and relatively low specificity. Meanwhile, the Western blot method is characterized by high specificity with lower sensitivity. IgM antibodies can be detected as early as in the second week of illness, but in most patients, their presence can be observed a few weeks later. The late stage of LB is usually characterized by the presence of IgG antibodies.

Herpes virus infections (especially Epstein–Barr virus) or other spirochaetes, and autoimmune diseases can cause false positive serological test results. A seropositive result without clinical symptoms typical of LB does not provide the basis for diagnosing the disease and its treatment.

One of the optimal techniques for a potential diagnosis in the early stage of LB is the polymerase chain reaction (PCR) [16, 21]. The true frequency and clinical correlates of PCR-documented, blood-borne infection in the spread of Lyme disease are widely determined. PCR detection of *B. burgdorferi* is a method at least three times more sensitive than the cultivation of microorganisms for identifying

spirochaetaemia in early LB. The PCR detection may be useful for a rapid diagnosis.

PCR-based methods are useful for the laboratory diagnosis of LB [16]. The first PCR assays for specific detection of a chromosomally encoded *B. burgdorferi* sensu lato gene were reported 20 years ago. Various other PCR assays were subsequently developed for detection of *B. burgdorferi* sensu lato DNA in clinical specimens. Given that the number of spirochaetes in infected tissues or body fluids of patients is very low, appropriate procedures for sample collection and transport and preparation of DNA from clinical samples are critical for yielding reliable and consistent PCR results. A variety of clinical specimens from patients with suspected Lyme disease have been analysed by PCR assays. Skin biopsy samples taken from patients with EM or ACA have been the most frequently tested specimens. Depending on the clinical manifestations of the patients, appropriate body fluid samples can be collected and analysed by PCR. The sensitivity of PCR assays may be reduced by degradation of the *B. burgdorferi* sensu lato DNA during sample transport, storage and processing [16]. The sensitivities of the PCR assays varied from 36 to 88% for patients with EM and from 54 to 100% for patients with ACA. The median sensitivities of the reported PCR assays were in the range from 69 to 76%. The sensitivity of PCR assays for detection of *B. burgdorferi* sensu lato in whole blood (plasma or serum) and CSF specimens is low [16]. By contrast, higher PCR sensitivities were reported with synovial fluid samples from patients with LA. Also PCR is a very sensitive approach when it is employed to detect *B. burgdorferi* sensu lato DNA in skin biopsy and synovial fluid specimens from patients with LB, whereas the diagnostic value of PCR assays for detection of *B. burgdorferi* sensu lato DNA in blood (plasma or serum) and CSF specimens is low. Moreover, PCR assays have not been widely accepted for laboratory diagnosis of LB because of low sensitivity in CSF and blood. PCR as a diagnostic tool may be hampered by potential false-positive results due to accidental contamination of samples with a small quantity of target DNA. Although PCR is highly sensitive for detection of DNA of *B. burgdorferi* sensu lato in skin biopsy samples from patients with EM, such testing is rarely necessary, as a clinical diagnosis can be easily made if the characteristic skin lesion is present [16].

Bloodstream invasion is an important and common mechanism for spreading the Lyme disease spirochaete. As a result of insufficient standardization in diagnosing infections that occur in Poland, PCR is not routinely used. PCR can detect spirochaete DNA without specifying whether it comes from living organisms, so a positive result does not mean an active infection.

Generally, in a laboratory diagnosis of LB, various methods for direct or indirect detection of *B. burgdorferi* can be applied. Particular data and some examples of methods are listed in Table 1.

The decision about diagnosis and further treatment of patients with Lyme disease should be made only by a physician who interprets the symptoms taking into account the results of additional tests. A therapy at least 21 days long is based on putting a patient on an antibiotic. The choice of the antibiotic depends on the clinical form of the disease and an individual's tolerance.

Others methods of diagnosis

Laboratory diagnosis needs CSF (liquor cerebrospinalis) or joint fluids from patients suffering from LB. The detection of *B. burgdorferi* microscopically or by the cultivation of spirochaetes derived from blood or CSF is difficult and burdened with high rates of false results. Currently in Poland, the study of cerebrospinal or joint fluid, in the case of infections caused by *B. burgdorferi* spirochaetes, is nonspecific and does not belong to routine diagnostic procedures. However, it is known that in the early stages of inflammation, the interaction between *B. burgdorferi* and multinucleated granulocytes and endothelial cells is observed, which leads to the generation of reactive oxygen species, lipid peroxidation products and other inflammatory mediators. Therefore, it is extremely important to develop new analytical methodologies aimed at the effective determination of lipid peroxidation products and other mediators of inflammation, as biomarkers of LB and other diseases.

The reaction of an organism during the infection or persistent disease is an inflammation, which is responsible for the damage of organs and systems. An inflammation of various organs is linked to the production of free radicals including reactive oxygen species (ROS). Free radicals play an important role in numerous biological processes, some of which are necessary for life, such as the intracellular killing of bacteria. On the other hand, because of their high reactivity, free radicals also can participate in unwanted side reactions resulting in cell damage [22, 23].

When patients suffer from various diseases including LB, free radical activity rapidly increases. This process is also connected to lipid peroxidation, which refers to the oxidative degradation of lipids, and is especially dangerous for brain cells, resulting in their damage. Metabolism processes in brain cells require more oxygen and glucose concentrations than other cells and tissues. Moreover, brain cells contain large amounts of polyunsaturated fatty acids (PUFAs) and iron ions. In addition, these ions are catalysts of certain reactions in which free radicals take part. All these processes are induced by free radicals and their reaction with lipids in cell membranes, which has an effect

Table 1 Methods recently developed for the direct and indirect determination of *B. burgdorferi*

Diagnosis	Methods	Ref.
Direct detection of *B. burgdorferi* sensu lato	*Microscope-based assays* (have limited clinical utility in laboratory confirmation of LB because of the sparseness of organisms in clinical samples) *Detection of B. burgdorferi-specific proteins or nucleic acids* (detects antigens in CSF of patients with neuroborreliosis and in urine samples from patients with suspected LB; their reliability is poor or at best questionable) *Culture* (the liquid media currently used to grow *B. burgdorferi* sensu lato are derived from the original Kelly medium through various modifications made over time; cultures are incubated at 30–34 °C under microaerophilic conditions for up to 12 weeks; detection of growth is accomplished by periodic examination of an aliquot of culture supernatant for the presence of spirochaetes by dark-field microscopy or by fluorescence microscopy after staining with the fluorochrome dye acridine orange or a specific fluorescence-labelled antibody) *Culture of clinical specimens* (*B. burgdorferi* sensu lato are recovered from various tissues and body fluids of patients with LB, including biopsy, lavage, specimens of EM skin lesions, biopsy specimens of ACA skin lesions, biopsy specimens of borrelial lymphocytoma skin lesions, CSF specimens, blood specimens, other tissue or fluid specimens like synovial fluid, cardiac tissue)	[13–15]
	Molecular methods of detection—PCR analysis of clinical specimens (analysis of biopsy samples from patients with cutaneous manifestations, blood from patients with LB, analysis of CSF specimens from patients with neuroborreliosis, analysis of synovial fluid from patients with LA) *Molecular techniques based on PCR* have been employed for confirmation of the clinical diagnosis of suspected LB, molecular species identification and/or typing of the infecting spirochaetes in clinical specimens or on cultured isolates, and detection of coinfection of *B. burgdorferi* sensu lato and other tick-borne pathogens	[16]
Immunologic diagnosis	*Immunodiagnosis of antigens* *Antibody detection methods*: indirect immunofluorescent antibody assays (IFA) and a variation of this assay using antigens attached to a membrane (FIAX), enzyme-linked immunosorbent assay (ELISA), enzyme-linked fluorescent assay (ELFA), Western immunoblots and immunochromatographic and dot blot assays *Newer enzyme immunoassay (EIA) antibody tests*: enzyme immunoassays using recombinant antigens, peptide-based immunoassays, combination of recombinant or peptide antigens in immunoassays *Other antibody detection methods*: functional antibodies (borreliacidal antibody assays), detection of antibodies bound to circulating immune complexes; detection of antibodies in CSF—ELISA *Cellular immune response in LB*: T lymphocyte and mononuclear cell proliferation assays	[3, 16]

on cell damage. This reaction involves the oxidation of PUFAs, which are biological membrane components. The peroxidation of cell constituents is highly correlated with the formation of reactive aldehydes. Beside aldehydes, many other unsaturated components are formed during lipid peroxidation [24]. Important biomarkers of lipid peroxidation process are MDA and HNE. Particular MDA and HNE formation steps from PUFAs are presented in Figs. 1 and 2, according to refs. [25, 26].

MDA and HNE are generated during all physiological processes. However, their concentrations are sometimes three times higher in inflammation, because free radicals induce their synthesis [27]. These compounds display reactive character toward nucleic acids, proteins and others [28]. Some products of lipid peroxidation react with DNA leading to both genotoxic and mutagenic action. In particular, MDA is the most mutagenic, but HNE the most genotoxic. The aforementioned compounds resulting in DNA damage caused by adducts of lipid peroxidation products with DNA can be removed by the repairing action of glycosylases [28].

The increase of membrane phospholipid peroxidation products in the course of LA has been reported [29]. ROS generated by stimulated phagocytes located near the joints
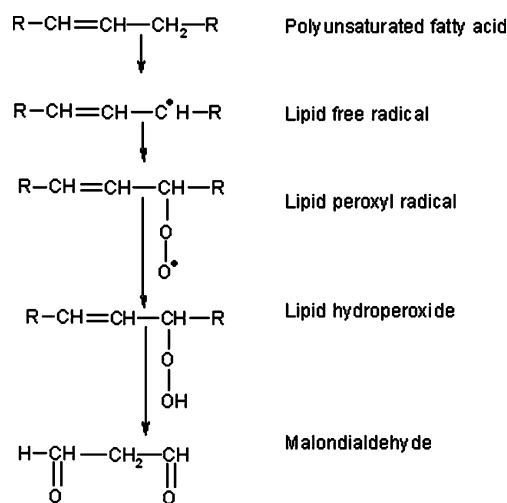
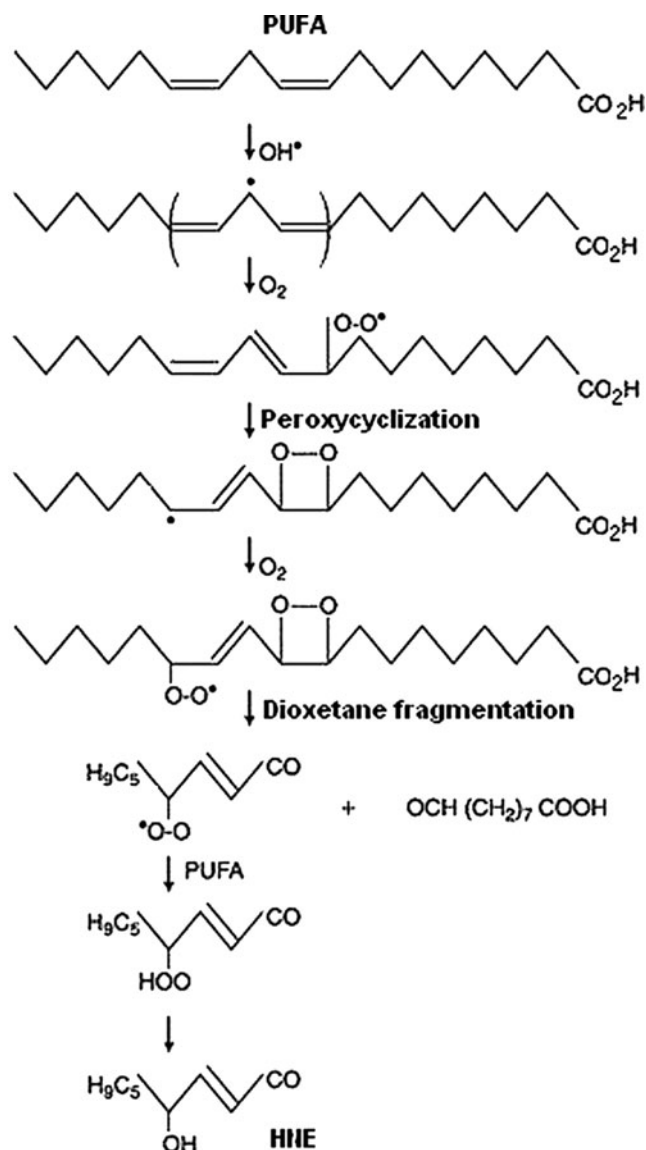**Fig. 1** Formation of MDA during peroxidation of PUFAs [25]

Fig. 2 Pathways of PUFA peroxidation and the formation of HNE [26]

during *B. burgdorferi* infection influence the high concentration of peroxidation products [30]. During inflammation, the lipid peroxidation process is additionally intensified by the decrease in the antioxidants concentration in the lipid phase of the biomembrane [31]. The reaction of ROS with phospholipid PUFAs has an effect on the increased production of superoxides and low molecular weight aldehydes [32]. Results concerning the determination of MDA and HNE in the plasma as well as in the urine of patients with *B. burgdorferi* infection have been published [29]. During LA the level of free form of MDA and HNE in the plasma, as well as in the urine, is about twofold higher in comparison with the control group [29]. The aforementioned compounds have a longer lifetime than ROS and can diffuse through biomembranes and cause greater cell

damage. MDA and HNE are very reactive electrophilic compounds. These compounds react with antioxidants participating in diminishing lipid peroxidation, such as glutathione (GSH) and glutathione peroxidase (GSHPO) [29, 33]. High reactivity of aldehydes generated during lipid peroxidation contributes to the fact that the estimation of their total level in biological samples is difficult. For that reason, other lipid peroxidation products such as prostaglandin F_2-like compounds (including more stable isoprostane 8-isoPGF$_{2\alpha}$) are determined in the plasma and urine samples obtained from patients with LA [29].

Main reactions occurring during peroxidation of PUFAs, which result in the synthesis of isoprostanes, are presented in Fig. 3.

Many classes of F_2-isoprostanes can arise from arachidonic acid (AA). On the contrary, peroxidation of eicosapentaenoic acid (EPA) is predicted to lead to the synthesis of six classes of F_3-isoprostanes, but α-linolenic and γ-linolenic acids to two classes of E_1- and F_1-isoprostanes, and docosahexaenoic acid to eight classes of D_4-isoprostanes and eight classes of E_4-isoprostanes. The formation of F_2-isoprostanes involves the formation of positional peroxyl radical isomers of AA, which undergo endocyclization to PGG$_2$-like compounds, which are subsequently reduced to PGF$_2$-like compounds. F_2-isoprostanes are formed in situ in phospholipids by free radical-catalysed peroxidation of esterified AA and subsequently are released in free form, presumably by phospholipases. The scheme of the formation of various classes of compounds from AA is presented in Fig. 4.

Methodology use for the determination of biomarkers of Lyme borreliosis and other inflammatory diseases

Malondialdehyde

MDA exists predominantly in the enol form, and it is highly reactive. Therefore, in laboratories MDA is obtained by hydrolysis of 1,1,3,3-tetramethoxypropane (commercially available compound). MDA is easily deprotonated to give the sodium salt of the enolate.

Various methods have been developed for the determination of MDA including spectrophotometry, fluorimetric detection, immunotests, high-performance liquid chromatography (HPLC) and gas chromatography (GC) [25, 35, 36].

However, the preparation of biological samples before analysis is the most critical point. In case of plasma analysis, various types of anticoagulant can be used for MDA assay like sodium heparin [37, 38], sodium citrate [37] and tripotassium EDTA [37, 39, 40]. Nevertheless, EDTA is more preferable than citrate and heparin as an anticoagulant, because it provides the lowest limit of
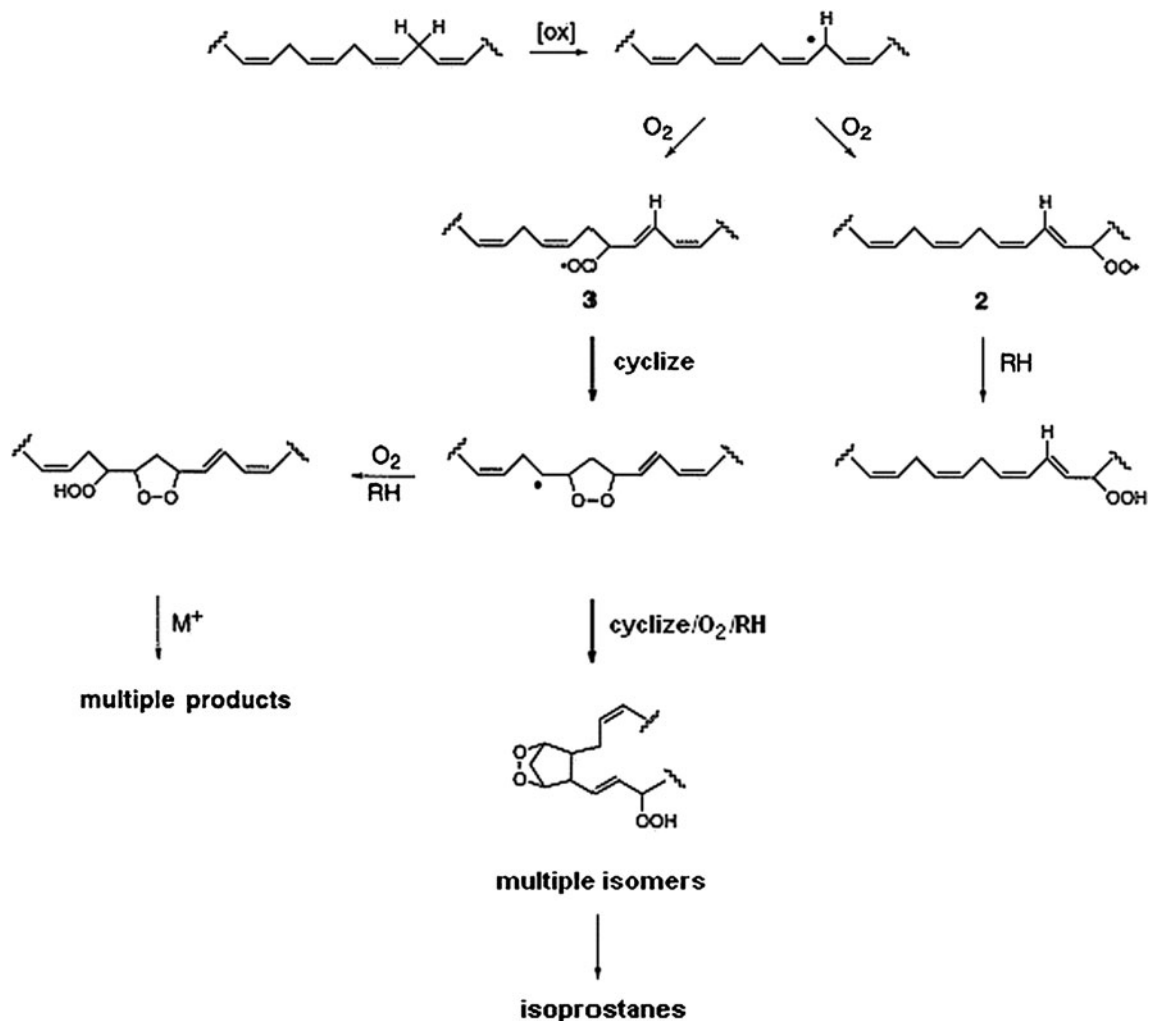
Fig. 3 Proposed pathways of lipid peroxidation which lead to the synthesis of isoprostanes [24]

detection (LOD) of MDA. This is probably related to iron chelation by EDTA and its weak activity e.g. as an antioxidant [41]. Moreover, the Fe–EDTA complex can participate in the Fenton reaction, forming OH· radicals and overestimating the lipid peroxidation [42]. Biological samples are stable at low temperature, which is why a suitable storage temperature is −20 °C for a short period of time, or even −80 °C for storage for more than 1 month [25, 37, 43]. Moreover, the antioxidant butylated hydroxytoluene (BHT) can be used to prevent further formation of MDA during the assay [38, 44–46]. The preparation of biological samples requires the application of an acid to precipitate proteins. Various inorganic acids can be used, e.g. sulphuric acid [39], perchloric acid [47] and (ortho) phosphoric acid [37, 38, 40].

The determination of MDA can be performed on various biological samples. The thiobarbituric acid reactive substances (TBARS) assay has been carried out in plasma [37, 39, 40, 44, 45, 48], serum [47], different tissues [44, 49, 50, 51] and urine [52]. Some investigations report the reaction

of TBA with MDA and linked chromogens to lipoperoxides in biomaterials [53]. Reaction products can be detected by spectrophotometry (λ=532–535 nm) or colorimetry as well as by fluorimetry (excitation at λ=532 nm and emission at λ=553 nm) [54]. The principal reaction between MDA and thiobarbituric acid (TBA) is presented in Fig. 5. Formation of MDA–TBA_2 adduct occurs by a nucleophilic attack involving carbon-5 of TBA and carbon-1 of MDA, followed by dehydration and similar reaction with a second molecule of TBA to produce a red pigment [55]. The intensity of the pink MDA–TBA product formed from this condensation indicates the extent of lipid peroxidation.

Nevertheless, the MDA assay by means of TBARS is limited by the fact that MDA is unstable. Additionally, its oxidation yields organic alcohols and acid [56, 57]. The use of colorimetric or fluorimetric techniques may give some mistakes, especially in the initial stages of human diseases in which lipid peroxidation is lower, when compared with the nonspecific background reaction between TBA and
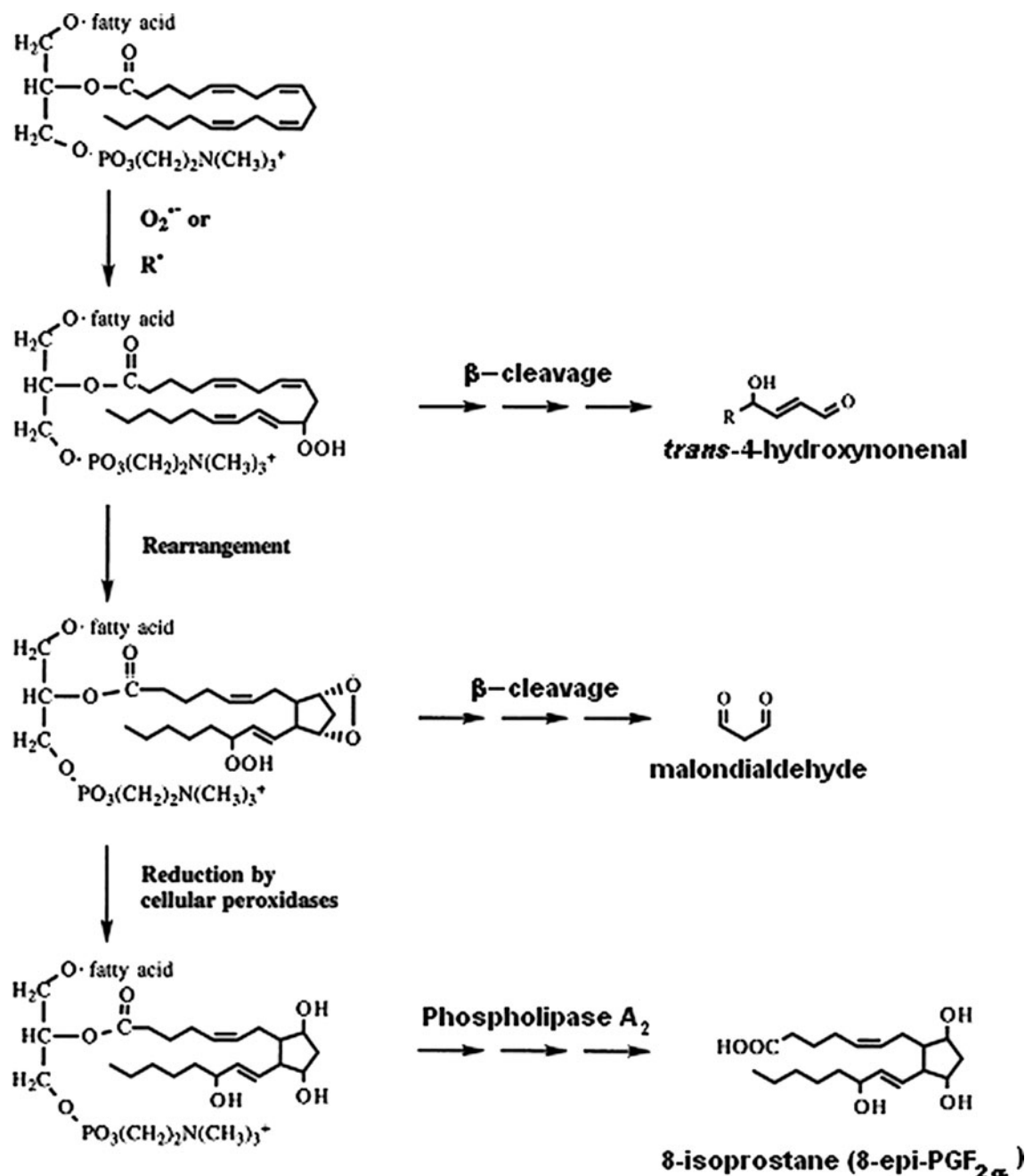
Fig. 4 Formation of various classes of compounds from AA [34]

products not derived from lipid peroxidation [50]. Concentrations MDA or TBARS in plasma obtained with the methods previously developed varied over a very wide range (0–50 mmol/L) [32, 58].

The determination of MDA by use of HPLC utilizes a derivatization as pretreatment. TBA is also used as a reagent for this derivatization. The separation of the MDA–TBA₂ adduct from other compounds by reversed-phase HPLC might be difficult. Several researchers have adapted HPLC methods so that the procedure offers some advantages in terms of specificity, recovery and reproducibility. HPLC assay for MDA is a useful method to measure concentrations of lipid peroxidation products in biological samples [25, 35, 37–39]. Besides, the analytical procedure consisting of TBA addition to the sample followed by extraction with n-butanol has been performed. This step avoids interference formation and extends the lifetime of the column by removing contaminants from the incubation

Fig. 5 Formation of MDA–TBA$_2$ adduct after reaction between MDA and TBA [25]
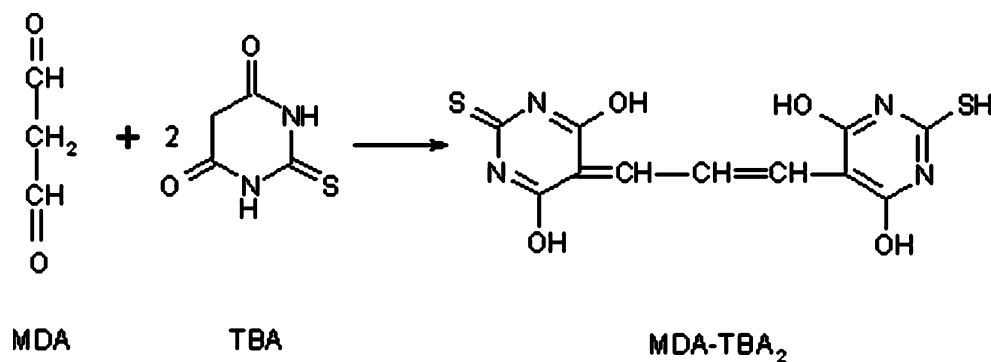
mixture [38, 59]. Moreover, the difference between detection of free and total plasma MDA is discussed [59]. Only low amounts of free MDA are present in biological samples, and an alkaline hydrolysis step by use of sodium hydroxide is necessary to obtain a more complete and uniformed release of MDA from protein–MDA complexes, which results in a higher MDA value, when total MDA is being considered [38, 40].

Useful detectors for the determination of MDA are UV-Vis (λ=532 or 535 nm) [37, 38, 45, 51] and FLD [38, 46, 59, 60].

Certain methods consist of plasma sample pretreatment by the acidic hydrolysis of amino acid-bound MDA and consequent protein precipitation [47]. The protein-free plasma is directly injected into the HPLC system and MDA is quickly eluted (monitored at λ= 254 nm) thereby allowing rapid analyses. The LOD of some methods is relatively low (0.012 mmol/L). Another advantage is the low amount of plasma needed for the analysis (50 mL).

GC is also a technique that can be used in the determination of MDA. However, a derivatization step is necessary prior to GC analysis. Some of the main derivatization agents are 2,3-propanediol [61], 2-hydrazinobenzothiazole [62] and pentafluorophenylhydrazine [63]. Depending on the method, either free or bound MDA may be determined and aldehydes other than MDA can be identified. In addition, headspace solid-phase microextraction (HS-SPME) used for the analyses of volatile aldehydes in biological matrices has been recently proposed [64]. The HS-SPME has been applied to prepare samples for MDA analysis, improving the sample preparation steps in lipid peroxidation research. A modified MDA assay involves derivatization and HS-SPME. The product of reaction of MDA and *N*-methylhydrazine is 1-methylpyrazole, which was analysed by GC with a nitrogen–phosphorus detector, and its detection limit was 0.0103 nmol/mL [64].

The most recent and precise methods used to assess MDA in human plasma or serum are briefly reported in Table 2.

4-Hydroxy-2-nonenal

Alkenals, including HNE, are strong electrophiles that react in tissues by alkylating nucleophiles (Michael addition), particularly sulfhydryl groups, amino groups (e.g. lysine), and the imidazole group of histidine [73, 74]. Such reactions can lead to structural alteration and/or cross-linking of proteins, which in turn can cause impairment of protein function. HNE is a relatively stable aldehyde that can diffuse to different subcellular compartments and interact with many different cell proteins [73]. Increased production of HNE has been shown to indicate an increased level of oxidative damage, which may not always be evident using the more common marker MDA [75].

Usually, the conventional analytical method for the quantitative determination of HNE is by derivatization with 2,4-dinitrophenylhydrazine (DNPH) that reacts with HNE to generate 2,4-dinitrophenylhydrazones with characteristic absorbance maxima at λ=360–390 nm. Afterwards, separation by thin-layer chromatography (TLC) or HPLC is possible [76–78]. This two-step method is efficient and applicable to blood plasma samples. Moreover, the quantitative determination of HNE by derivatization with ^{3}H-labelled sodium borohydride (NaB[^{3}H]H$_4$), which converts HNE Michael adducts into ^{3}H-labelled dihydroxy derivatives, has been used as a measure of protein-bound HNE [79, 80]. Michael adducts generated in peptides and proteins, e.g. involving HNE–cysteine, HNE–histidine and HNE–lysine, can be analysed by HPLC with *o*-phthaldehyde derivatization [79, 80]. Reduction of the aldehyde group of primary HNE Michael addition products with NaBH$_4$, which converts them to hydroxy derivatives that are stable to strong acid hydrolysis, forms the basis of methods for the identification and quantification of the HNE adducts by HPLC. By means of these techniques, it has been established that at least 80% of the histidine residues that are lost when low density lipoprotein (LDL) is treated with HNE can be accounted for as the Michael addition products, whereas the Michael addition product accounted for 49% of the lysine residues that disappeared upon HNE treatment. This method has also allowed one to

Table 2 Methods applied in recent years for MDA determination

Methods	Derivatization	Blood anticoagulant	Sample volume (μL)	Concentration of MDA (μmol/L) ± SD	Linearity (μmol/L)	LOD (μmol/L)	Precision	Ref.
HPLC-UV/Vis (λ=532)	TBA	EDTA	250	Healthy (plasma) 0.11±0.03 Renal patients (plasma) End-stage renal failure, diabetic 0.32±0.14 End-stage renal failure, nondiabetic 0.32±0.9 Chronic renal failure 0.14±0.06	0.25–1.0	0.06	Within-run <4.5% Between-run <10.0%	[45]
HPLC-fluometric (λ=515)	TBA	–	50	Normotensive volunteers plasma 0.69±0.13	0–5	0.128	–	
Fluorimetry	TBA	EDTA	200	Healthy (plasma) 0.112±0.034	0.05–0.5	0.015	Between-run 2.0%	[65]
HPLC-UV/Vis (λ=254)	None	None	50	Serum of control 0.50±0.04 Serum of goiter 3.1±0.2	0–50	0.12	–	[66]
HPCE	None	Heparin	500	ND	10–50	12.6	3.86%	[67]
HPLC-UV/Vis (λ=310 nm)	DNPH	EDTA	50	Healthy (plasma) 13.8±1.32	0–100	2.1	Within-run <4% Between-run <7%	[68]
HPLC-UV/Vis (λ=311 nm)	DAN	Heparin	100	Healthy women (plasma) 0.162±0.051 Healthy men (plasma) 0.138±0.028	–	–	Between-run <9.1%	[69]
GC-MS	PH	EDTA	200	Human plasma 1.3±0.07	0. 01–15	–	Within-day <2.0% Between-day <2.1%	[70]
GC-MS and GC-ECD	TCPH	–	–	0.48±0.03 (ECD) 0.50±0.03 (MS)	0.03–3 (ECD) 0.03–20 (MS)	0.03 (ECD)	(MS) <3.3% (EDC) <3.9%	[71]
GC-MS	PH	EDTA	200	Healthy (plasma) 1.41±0.23 Unstable angina (plasma) 2.81±0.52 Stable angina (plasma) 2.40±0.60	0–8	–	Between-day <1.5%	[72]
HPLC-UV/Vis (λ=532)	TBA	EDTA	500	Control (plasma) 1.39±0.17 Patients LA (plasma) 3.13±0.48	0.1–12	0.03	<10.0%	[29]
HPLC-UV/Vis (λ=532)	TBA	EDTA	500	Control (plasma) 0.85±0.25	0.15–12.2	0.03	Within-assay 8.0% Between-assay 10.0%	[60]

ND not determined

quantitate the Michael addition-type HNE–histidine adducts (7–9 mol/mol LDL) and trace amounts of HNE–lysine adducts in Cu^{2+}-oxidized LDL [79].

Currently, LC-ESI-MS/MS methodology is being developed. Chromatography coupled to MS is more sensitive and selective in the quantification of HNE [73, 81]. The determination of the sites of linkage of HNE to protein has been carried out by use of LC/MS^n. The aforementioned method has also allowed for the characterization of HNE-adducted apomyoglobin [82]. The procedure involves an initial analysis by ESI-MS of the adducted protein to determine the stoichiometry of HNE incorporation. The adducted protein is then subjected to proteolytic digestion, followed by direct analyses of the unfractionated digest by ESI-MS for the localization of the sites of HNE adduction. Proteolytic digestion using trypsin produces fragments of suitable length for analysis by tandem MS with low-energy collision-induced dissociation. The components containing HNE-adducted histidine residues are identified by scanning for precursors of m/z 266, which correspond to an immonium ion derived from HNE adducted histidine. Last, product ion scanning of each modified tryptic fragment is performed to provide additional structural detail and to confirm the results obtained by precursor ion scanning.

Application of this approach to the characterization of HNE-modified apomyoglobin indicated that there were between three and ten HNE adducts per protein molecule and that the adduction occurred solely to histidine residues [82].

An interesting aspect in the detection of HNE adducts is the use of antibodies. Some reports concern antibodies to HNE-treated LDL [75, 79]. Others raised the anti-HNE-LDL antibodies interacting with epitopes on Cu^{2+}-oxidized LDL and other modified proteins [74, 83, 84]. It was observed that the antibody–protein interactions can be partly blocked by the HNE-modified forms of several polyamino acids, including polylysine, polytyrosine, polyarginine and polyhistidine [79].

With regard to the fact that HNE is very reactive with proteins and forms stable Michael addition-type adducts, a novel immunochemical procedure has been developed [85]. It is based on the detection of HNE trapped by a protein that has been coated on an immunoplate. The HNE-derived epitopes generated in the coating protein are then detected by an ELISA using a monoclonal antibody (mAbHNEJ2) specific to the haptenic groups of the HNE–protein conjugates [85]. Using this method, it was observed that a considerable amount of HNE was released from human plasma low density lipoproteins treated with copper ions or endothelial cells. Furthermore, a simple and rapid ELISA method for quantitation of HNE-modified proteins has been reported. During this method microtiter plate wells are precoated and blocked simultaneously with epitope-bound bovine caseins as matrix proteins, and aldehyde-modified proteins are quantitated by competition assay with a monoclonal antibody (mAbHNEJ2) specific for the HNE-histidine Michael adduct [86].

The stability of HNE in whole venous blood stored for different time intervals was studied [87]. The HNE content in venous blood stored in ice was determined (Fig. 6). The

obtained results show a rapid decrease of HNE concentration, especially within the first hour after drawing the blood.

Physiological levels of HNE in human plasma are successfully determined by use of HPLC. The measurement of HNE in human plasma should be done within 30 min after collection. It was established that HNE is a normal constituent of human blood plasma, but other investigations on its occurrence under various conditions may help to clarify the relevance of lipid peroxidation in physiological and pathological processes [76]. The description of some methods applied for the determination of HNE is presented in Table 3.

Iso- and neuroprostanes

Isoprostanes (Iso-Ps) and neuroprostanes (Neuro-Ps) are formed by radical mechanisms and accordingly exist in several forms which in biological matrices undergo additional transformation to other metabolites. This fact creates a necessity to find appropriate methods to enable their analysis. The most often mentioned techniques among researchers involved in trying to develop new methodologies allowing the quantification and qualification of Iso-Ps and Neuro-Ps are GC/MS and LC/MS/MS. However, sample preparation seems to be the most critical point. The most preferable methods are SPE. Also, TLC and derivatization in case of GC analysis are widely used. Choosing LC imposes the use of only the SPE with conventional or specific immunoaffinity sorbent (immunoaffinity separation, IFS). Such a method simplification and the accompanying reduction of cost and time needed for sample preparation have created wide interest in this method. Among useful analytical methods, ELISA [89, 90], which may be also called enzyme immunoassay (EIA) in the literature, should be first discussed because of its utility as a clinical screening test.

ELISA

ELISA is a biochemical technique which is often used in immunology to quantify an antibody or an antigen in samples. A more specific application of this method is in the detection of isoprostanes in clinical analysis [90–95]. Because of its low cost and easy performance, ELISA is used widely in medicine and plant pathology [96–98]. Nevertheless, when we consider the application of ELISA to isoprostanes we see that the estimated level of these compounds is only semiquantitative. The main reason for this fact may be, in compliance with Yan et al. [99], cross-reactivity. Such an interaction may also be observed in future investigations of other prostaglandins. Application of GC allows one to see that ELISA overestimates the isoprostanes level by twofold [99].
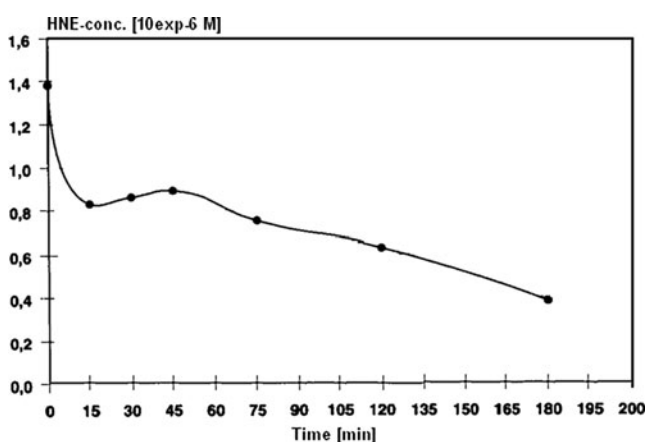

Fig. 6 Content of HNE in venous blood stored in ice vs. time of storage [87]. Methodology: after 3 h of derivatization with DNPH the concentration of HNE was determined by HPLC

Table 3 Examples methodologies used for the determination of HNE

Methods	Derivatization	Sample volume	Concentration of HNE ± SD	Linearity	LOD	Precision	Ref.
SPE (Extrelut®)	DNPH	3 mL	Average human plasma 0.65±0.39 μmol/mL	0.1–0.54 μmol/mL	0.1 μmol/mL	2–6%	[87]
HPLC (λ=370 nm)			Females 0.45±0.23 μmol/mL				
SPE-TLC			Males 0.78±0.43 μmol/mL				
LC-ESI-MS/MS	5,5′-Dimethyl-1, 3-cyclohexanedione	–	Control 0.4±0.1 nmol/mg protein	–	–	–	[73]
			Early Alzheimer's disease hippocampus/parahippocampal gyrus 1.45±0.5 nmol/mg protein				
			Mild cognitive impairment hippocampus/parahippocampal gyrus 1.5±0.3 nmol/mg protein				
			Control superior and middle temporal gyrus 0.8±0.1 nmol/mg protein				
			Mild cognitive impairment superior and middle temporal gyrus 2.4±0.3 nmol/mg protein				
			Early Alzheimer's disease superior and middle temporal gyrus 2.9±0.3 nmol/mg protein				
			Control cerebellum 0.8±0.1 nmol/mg protein				
			Late-stage Alzheimer's disease mild cognitive impairment 1.5±0.2 nmol/mg protein				
ESI-MS	Apomyoglobin	–	–	–	–	–	[82]
HPLC fluorescence detection (λ=380 nm and emission λ=445 nm)	1,3-Cyclohexanedione	1 mL plasma	LA 45.31±16.86 nmol/mL Control 11.24±2.19 nmol/mL	–	–	–	[29]
GC-negative-ion chemical ionization-MS	O-Pentafluorobenzyl oxime	250 μL plasma or CSF	Control 0.66±0.06 nmol/mL HIV 0.81±0.07 nmol/mL AIDS 1.24±0.18 nmol/mL Parkinson's disease CSF 0.20–3.14 nmol/mL Plasma 0.71–6.03 nmol/mL	1–100 ng/mL	–	–	[88]

GC analysis of prostanoids

GC/MS should be sensitive enough to quantify all desirable analytes after appropriate sample preparation. Because of their properties, prostanoids need a derivatization step prior to analysis [91, 100, 101]. SPE has to be performed twice in order to obtain high purity of selected compounds or greater enrichment.

Iso- and neuroprostanes from human plasma and urine are firstly extracted with use of SPE. This step of sample preparation may be performed using a C-18 Sep-Pak cartridge [100, 102, 103]. Other methodologies use two extraction steps. In the first step, reversed-phase C-18 is applied to remove protein and lipids. Subsequently, a Sep-Pak containing NH_2 groups is used [98]. After the prostanoids are eluted from the appropriate cartridge, they are evaporated and then dissolved in a small amount of methanol [100, 104]. Dissolved compounds are applied to a silica TLC plate and developed to the top in a chosen solvent system. According to Morales et al. [100], the separation of isoprostane (15-F_{2t}-IsoP-M) is performed with a mixture of ethyl acetate/acetic acid/methanol (80:0.1:20 v/v/v). The visualization of $PGF_{2\alpha}$ may be done with a 10% solution of phosphomolybdic acid in ethanol followed by heating of the plates. Compounds, which migrated close to the acid, are scraped and extracted from the silica with the use of ethyl acetate/ethanol (50:50 v/v). The obtained samples of 15-F_{2t}-IsoP-M after drying under vacuum or nitrogen are converted to the pentafluorobenzyl ester (PFB) [101]. A second purification with the use of TLC is performed usually in a solvent mixture containing ethyl acetate/methanol (98:2 v/v). Visualization, similarly to the first step, is performed by spraying with phosphomolybdic acid followed by heating. All compounds which migrate near the $PGF_{2\alpha}$ methyl ester are then scraped and eluted with a defined volume of ethyl acetate [100] or other solvent. The eluate is dried (under nitrogen or vacuum) and converted to the corresponding trimethylsilyl (TMS) ether derivative. Such prepared samples are able to be analysed by GC/MS [101, 102, 104, 105].

A shorter and easier method for sample preparation prior to GC/MS analysis was developed and described by Lee et al. in 2004 [106]. Their approach is based on mixed anion exchange solid-phase extraction (MAX-SPE), which may be followed directly by GC/MS analysis by use of various ionization techniques.

Various ionization techniques are frequently used in analysis of both iso- and neuroprostanes by MS. Electron capture negative ionization (GC/ECNI-MS) is preferable due to its sensitivity and because it allows lower LODs [101–103, 107–110].

In the case of very complex matrices a single-ion monitoring (SIM) mode is often used. Additionally, multiple reaction monitoring (MRM) enhances sensitivity and selectivity of tandem instruments.

HPLC analysis of prostanoids

HPLC seems to be a more appropriate method for the analysis of prostanoids. After sample treatment, the obtained extracts may be directly analysed by LC without any derivatization which was necessary in GC. However, the purification of samples is an important step. The sample containing prostanoids needs extraction using e.g. silica-based SPE sorbents [29, 91, 111, 112] or immunoaffinity sorbents [91, 111].

The plasma or other biological samples initially have to be prepared according to an appropriate given protocol. Biological samples with BHT and triphenylphosphine have to be homogenized thoroughly. Then, hydrolysis of esterified prostanoids is performed (heating after adding of KOH solution). Afterwards, samples are diluted with water to prevent precipitation. In the case of urine, only alkaline solution followed by acetic acid has to be added. Such prepared samples may be loaded on the C-18 Sep-Pak columns [112]. Because of the low recovery obtained with conventional C-18 columns (near 20%), it was better to use Oasis HLB cartridges for sample extraction as, depending on the kind of compounds, recoveries were about 80% [111]. The eluate after SPE is evaporated to dryness under vacuum or nitrogen and after reconstitution (usually in mobile phase) may be analysed by HPLC.

Extraction by immunoaffinity bed

In the case of prostanoids, deuterated internal standards and KOH have to be added to the freshly thawed plasma. After short incubation at increased temperature the alkali is neutralized by addition of potassium dihydrophosphate [111]. But urine samples need only dilution in potassium dihydrophosphate and addition of internal deuterated standards. Samples may be analysed by LC/MS after evaporating to dryness and dissolving in mobile phase.

The literature contains examples of applications using C-18 [29, 91, 111–114] and polar end-capped C-18 [89] columns. Similarly to GC where the most useful tool in prostanoids determination was MS, in LC the same situations are occurring. In LC/MS the most common ionization technique is electrospray (ESI). Also atmospheric pressure chemical ionization (APCI) in prostanoids analysis is recommended [112]. The analysers applied to this aim may be different but in almost all cases a triple quadrupole and quadrupole ion trap (QTRAP) are preferable [89, 91, 111–113]. Beside MS, applications of other detectors are also presented. An interesting example is the application of electrochemical and spectrophotometric detectors [29].

Such detection methods may be applied as an alternative to MS only in limited cases.

The recent methods used to assess isoprostanes and neuroprostanes are presented in Table 4.

Conclusions

Several Central European countries are characterised by the highest incidence of tick-borne diseases in the northern hemisphere. Molecular methods including PCR give possibilities for the detection of *B. burgdorferi* sensu lato and these are the most valuable detection methods. For patients with LB involving systems other than skin, PCR sensitivity is in general low, with the exception of patients with LA. Beside this, PCR is not routinely used in Poland. Therefore, an interesting alternative could be methods for the determination of lipid peroxidation products. The lipid peroxidation process is enhanced during LB and other human body infections. The determination of these products is essential in pathologies including LB and in toxicology associated with oxidative stress. MDA, HNE, isoprostanes and neuroprostanes are suitable biomarkers of various diseases, which refer to viruses and bacteria attack

on an organism and to the increased formation of free radicals and oxidative stress as a consequence. If LB is detected in its early stage, it may be successfully treated. One of the possibilities is the development of methods used for the determination of biomarkers mentioned above. These analytical methods are characterised by high sensitivity, precision, accuracy and are not time consuming.

The concentration of 8-isoPGF$_{2\alpha}$ in plasma and urine of LA patients could be over threefold and eightfold higher, respectively, than that in healthy people. The ratio of free to esterified form of 8-isoPGF$_{2\alpha}$ is significantly smaller if compared with the levels in healthy people. This indicates that the ratio of free to esterified form of 8-isoPGF$_{2\alpha}$ may be useful as an indicator of LA. Moreover, the complementary determination of three lipid peroxidation products (MDA, HNE and 8-isoPGF$_{2\alpha}$) may be very helpful in the diagnosis of LB.

From an analytical point of view, chromatography techniques are the preferred methods to determine the true amount of biomarkers in biological materials, once they are reliable, specific and, moreover, without the presence of artefacts or methodological mistakes. Comparing CG and HPLC methodologies, both are reliable and specific, but CG requires small sample volumes. HPLC UV-VIS is

Table 4 Methods applied in recent years for determination of iso- and neuroprostanes

Method	Compound	Concentration ± SD	LOD (mmol/L)	Precision/accuracy	Ref.
GC/MS	8-epi-PGF$_{2\alpha}$	Human plasma 1.57×10^{-7} to 4.50×10^{-7} mmol/L	2.71×10^{-8}	Within-day CV 4.3–11.1% Day to day CV 9.4–15.1%	[109]
	8-iso-PGF$_{2\alpha}$	Urine $1.06\times10^{-6}\pm4.88\times10^{-7}$ mmol/g creatinine	5.43×10^{-8}	Precision 4% Accuracy 97%	[101]
	15-F$_{2t}$-IsoP-M	Urine (mmol/g creatinine) Normal humans $1.25\times10^{-5}\pm2.44\times10^{-7}$	2.17×10^{-8}	Precision 7% Accuracy 96%	[100]
		Hypercholesterolaemic humans $2.31\times10^{-6}\pm5.70\times10^{-7}$ Hypercholesterolaemic humans administered antioxidants $1.22\times10^{-6}\pm2.71\times10^{-7}$			
	F$_2$-IsoP	Urine (mmol/g creatinine) Healthy volunteers $2.20\times10^{-6}\pm9.77\times10^{-7}$	–	–	[115]
		Polytraumatized patients $1.28\times10^{-5}\pm7.33\times10^{-6}$			
	F$_4$-NeuroP	Brain tissue from rodents $2.30\times10^{-8}\pm5.28\times10^{-9}$ mmol/g wet weight Brain tissue from normal humans $1.29\times10^{-8}\pm1.59\times10^{-9}$ mmol/g wet weight	2.64×10^{-8}	Accuracy 97%	[116]
HPLC/MS	8-iso-PGF$_{2\alpha}$	Urine (mmol/mol creatinine) Healthy volunteers $3.34\times10^{-5}\pm7.60\times10^{-6}$	–	–	[89]
		Patients with obstructive sleep apnoea $5.48\times10^{-5}\pm1.98\times10^{-5}$			
	8-iso-PGF$_{2\alpha}$	Human plasma 2.88×10^{-7} mmol/L	8.14×10^{-8}	Intraday precision 4.68% for plasma and 3.83% for urine	[91]
		Urine 9.77×10^{-7} mmol/g creatinine		Interday precision 3.9% for plasma and 2.98% for urine	

widely used in clinical laboratories and there are methodologies utilizing small sample volume or mass with total reliability and specificity.

References

1. Burgdorfer W (1984) Yale J Biol Med 57:515
2. Rahn DW, Malawista SE (1991) West J Med 154:706
3. Pancewicz SA, Hermanowska-Szpakowicz T, Makarewicz-Płońska M, Witek A, Farbiszewski R, Zajkowska J, Michalska B (2002) Neurol Neurochir Pol 36:767
4. Cairns V, Godwin J (2005) Int J Epidemiol 34:1340
5. Nadelman RB, Wormser GP (1998) Lancet 352:557
6. Stanek G, Strle F (2003) Lancet 362:1639
7. Steere AC (2001) N Engl J Med 345:115
8. Steere AC, Bartenhagen NH, Craft JE (1983) Ann Intern Med 99:76
9. Steere AC, Batsford WP, Weinberg M, Alexander J, Berger H, Wolfson SXMSE (1980) Ann Intern Med 93:8
10. Wormser GP, Nadelman RB, Nowakowski J, Schwartz I (2001) Med Hypotheses 57:435
11. Weber K, Pfister HW (1993) In: Weber K, aBurgdorfer W (eds) Aspects of Lyme borreliosis. Springer-Verlag, Berlin, pp 1–20
12. Wilske B (2003) Vector Borne Zoonotic Dis 3:215
13. Pfister HW, Wilske B, Weber K (1994) Lancet 343:1013
14. Stanek G, O'Connell S, Cimmino M, Aberer E, Kristoferitsch W, Granstrom M, Guy E, Gray J (1996) Wien Klin Wochenschr 108:741
15. Steere AC (1989) N Engl J Med 263:201
16. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP (2005) Clin Microbiol Rev 18:3484
17. Barbour AG (1988) Clin Microbiol Rev 1:399
18. Bunikis J, Barbour AG (2002) Med Clin North Am 86:311
19. Schmidt BL (1997) Clin Microbiol Rev 10:185
20. Wilske B, Preac-Mursic V (1993) In: Weber K, Burgdorfer W (eds) Aspects of Lyme borreliosis. Springer-Verlag, Berlin, Germany, pp 267–300
21. Goodman JL, Bradley JF, Ross AE, Goellner P, Lagus A, Vitale B, Berger BW, Luger S, Johnson RC (1995) Am J Med 99:6
22. Pacher P, Beckman JS, Liaudet L (2007) Physiol Rev 87:315
23. Karthikeyan R, Manivasagam T, Anantharaman P, Balasubramanian T, Somasundaram ST (2011) J Appl Phycol 23:257
24. Marnett LJ (1999) Mutat Res Fundam Mol Mech Mutagen 424:83
25. Grotto D, Santa Maria L, Valentini J, Paniz C, Schmitt G, Garcia SC, Pomblum VJ, Rocha JBT, Farina M (2009) Quím Nova 32:169
26. Awasthi S, Singhal SS, Awasthi YC, Martin B, Woo JH, Cunningham CC, Frankel AE (2008) Clin Cancer Res 14:4372
27. Griffiths HR, Moller L, Bartosz G, Bast A, Bertoni-Freddari C, Collins A, Cooke M, Coolen S, Haenen G, Hoberg AM, Loft S, Lunec J, Olinski R, Parry J, Pompella A, Poulsen H, Verhagen H, Astley SB (2002) Mol Aspects Med 23:101
28. Łuczaj W, Skrzydlewska E (2003) Cell Mol Biol Lett 8:391
29. Łuczaj W, Moniuszko A, Rusak M, Pancewicz S, Zajkowska J, Skrzydlewska E (2011) Eur J Clin Microbiol Infect Dis 30:415
30. Pancewicz SA, Skrzydlewska E, Hermanowska-Szpakowicz T, Zajkowska JM, Kondrusik M (2001) Med Sci Monit 7:1230
31. Bradford A, Atkinson J, Fuller N, Rand RP (2003) J Lipid Res 44:1940
32. Comporti M (1998) Free Radic Res 28:623
33. Kinter M, Roberts RJ (1996) Free Radic Biol Med 21:457–462
34. de Zwart LL, Meerman JH, Commandeur JN, Vermeuln NH (1999) Free Radical Biol Med 26:202
35. Bird RP, Hung SSO, Hadley M, Draper HH (1983) Anal Biochem 128:240
36. Ichinose T, Miller MG, Shibamoto T (1989) Lipids 24:895
37. Nielsen F, Mikkelsen BB, Nielsen JB, Andersen HR, Grandjea P (1997) Clin Chem 43:1209
38. Hong Y, Yeh S, Chang C, Hu M (2000) Clin Biochem 33:619
39. Del Rio D, Pellegrini N, Colombi B, Bianchi M, Serafini M, Torta F, Tegoni M, Musci M, Brighenti F (2003) Clin Chem 49:690
40. Grotto D, Santa Maria LD, Boeira S, Valentini J, Charão MF, Moro AM, Pomblum VJ, Nascimento PC, Garcia SC (2007) J Pharm Biomed Anal 43:619
41. Wong SHY, Knight JA, Hopfer SM, Zaharia O, Leach CN, Sunderman FW (1987) Clin Chem 33:214
42. Tamura H, Kitta K, Shibamoto T (1991) J Agric Food Chem 39:439
43. Gonçalves TL, Erthal F, Corte CLD, Muller LG, Piovezan CM, Nogueira CW, Rocha JBT (2005) Clin Biochem 38:1071
44. Lepage G, Munoz G, Champagne J, Roy CC (1991) Anal Biochem 197:277
45. Templar J, Kon SP, Milligan TP, Newman DJ, Raftery MJ (1999) Transplantation 14:946
46. Lykkesfeldt J (2001) Clin Chem 47:1725
47. Carbonneau MA, Peuchant E, Sess D, Canioni P, Clerc M (1991) Clinical Chem 37:1423
48. Scott B, Deman A, Peeters P, Van den Branden C, Stolear JC, Van Camp G, Verbrrlen D (2003) Nephrol Dial Transplant 18:737
49. Liu J, Yeo HC, Doniger SJ, Ames BN (1997) Anal Biochem 245:161
50. Folmer V, Santos FW, Savegnago L, Brito VB, Nogueira CV, Rocha JB (2004) Toxicol Lett 153:333
51. Cirak B, Inci S, Palaoglu S, Bertan V (2003) Clin Chim Acta 327:103
52. Draper HH, Csallany AS, Hadley M (2000) Free Radical Biol Med 29:1071
53. Yagi K, Nishigaki I, Ohama H (1968) Vitamins 37:105
54. Knight JA, Pieper RK, Mc Clellan L (1988) Clin Chem 34:2433
55. Janero DR (1990) Free Radical Biol Med 9:515
56. Almandos ME, Giannini DH, Ciarlo AS, Boery RL (1986) J Sci Food Agric 37:54
57. Seo CW (1976) J Food Sci 41:594
58. Del Rio D, Stewart AJ, Pellegrini N (2005) Nutr Metab Cardiovasc Dis 15:316
59. Fukunaga K, Takama K, Suzuki T (1995) Anal Biochem 230:20
60. Londero D, Lo Greco P (1996) J Chromatogr A 729:207
61. Lakshminarayana G, Cornwell DG (1986) Lipids 21:175
62. Beljean-Leymarie M, Bruna E (1988) Anal Biochem 173:174
63. Tomita M, Okuyama T, Hatta Y, Kawai SJ (1990) Chromatogr 526:174
64. Fujioka K, Shibamoto T (2005) J Agric Food Chem 53:4708
65. Agarwal R, Chase SD (2002) J Chromatogr B Analyt Technol Biomed Life Sci 775:121
66. Del Rio D, Pellegrini N, Colombi B, Bianchi M, Serafini M, Torta F (2003) Clin Chem 49:690

67. Wilson DW, Metz HN, Graver LM, Rao PS (1997) Clin Chem 43:1982
68. Sim AS, Salonikas C, Naidoo D, Wilcken DE (2003) J Chromatogr B Analyt Technol Biomed Life Sci 785:337
69. Steghens JP, van Kappel AL, Denis I, Collombel C (2001) Free Radic Biol Med 31:242
70. Cighetti G, Debiasi S, Paroni R, Allevi P (1999) Anal Biochem 266:222
71. Stalikas CD, Konidari CN (2001) Anal Biochem 290:108
72. Cighetti G, Allevi P, Anastasia L, Bortone L, Paroni R (2002) Clin Chem 48:2266
73. Williams TI, Lynn BC, Markesbery WR, Lovell MA (2006) Neurobiol Aging 27:1094
74. Subramaniam R, Roediger F, Jordan B, Mattson MP, Keller JN, Waeg G (1997) J Neurochem 69:1161
75. McGrath LT, McGleenon BM, Brennan S, McColl D, Mc IS, Passmore AP (2001) Oxford J Med QJM 94:485
76. Poli G, Schaur RJ, Siems WG, Leonarduzzi G (2008) Med Res Rev 28:569
77. Esterbauer H, Cheeseman K, Dianzani MU (1982) Biochem J 208:129
78. Winkler P, Lindner W, Esterbauer H, Schaunstein E, Schaur RJ (1984) Biochim Biophys Acta 796:232
79. Uchida K (2003) Progr Lipid Res 26:318
80. Uchida K, Stadtman ER (1993) J Biol Chem 268:6388
81. Williams IT, Lovell MA, Lynn BC (2005) Anal Chem 77:3383
82. Bolgar MS, Gaskell SJ (1996) Anal Chem 68:2325
83. Palinski W, Rosenfeld ME, Yla-Herttuala S, Gurtner GC, Socher SS, Butler SW (1989) Proc Natl Acad Sci U S A 86:1372
84. Chen Q, Esterbauer H, Jürgens G (1992) Biochem J 288:249
85. Uchida K, Osawa T, Hiai H, Toyokuni S (1995) Biochem Biophys Res Commun 212:1068
86. Satoh K, Yamada S, Koike Y, Toyokuni S, Kumano T, Takahata T (1999) Anal Biochem 270:323
87. Strohmaier H, Hinghofer-Szalkay H, Schaur RJ (1995) J Lipid Mediat Cell Signal 11:51
88. Selley ML (1997) J Chromatogr B 691:263
89. Monneret D, Pepin JL, Godin-Ribuot D, Ducros V, Baguet JP, Levy P, Faure P (2010) Free Radical Bio Med 48:619
90. Faure P, Tamisier R, Baguet JP, Favier A, Halimi S, Levy P, Pepin JL (2008) Eur Respir J 31:1046
91. Sircar D, Subbaiah PV (2007) Clin Chem 53:251
92. Liu HM, Liu KX, Cheng MH, Liu Y, Lei S, Irwin MG, Xia Z (2011) J Surg Res 168:18
93. Lucidi V, Ciabattoni G, Bella S, Barnes PJ, Montuschi P (2008) Free Radical Bio Med 45:913
94. Samitas K, Chorianopoulos D, Vittorakis S, Zervas E, Economidou E, Papatheodorou G, Loukides S, Gaga M (2009) Resp Med 103:750
95. Nishibe A, Kijima Y, Fukunaga M, Nishiwaki N, Sakai T, Nakagawa Y, Hata T (2008) Prostag Leukotr Ess 78:257
96. Gong Y, Yi M, Fediuk J, Lizotte PP, Dakshinamurti S (2010) Free Radical Bio Med 48:882
97. Dalaveris E, Kerenidi T, Katsabeki-Katsafli A, Kiropoulos T, Tanou K, Gourgoulianis KI, Kostikas K (2009) Lung Cancer 64:219
98. Kitano S, Hisatomi H, Hibi N, Kawano K, Harada S (2006) World J Gastroenterol 12:5846
99. Yan W, Byrd GD, Ogden MW (2007) J Lipid Res 48:1607
100. Morales CR, Terry ES, Zackert WE, Montine TJ, Morrow JD (2001) Clin Chem Acta 314:93
101. Morrow JD, Zackert WE, Yang JP, Kurhts EH, Callewaert D, Dworski R, Kanai K, Taber D, Moore K, Oates JA, Roberts LJ (1999) Anal Biochem 269:326
102. Roberts LJ, Montine TJ, Markesbery WR, Tapper AR, Hardyi P, Chemtobi S, Dettbarn WD, Morrow JD (1998) J Biol Chem 273:13605
103. Reich EE, Zackert WE, Brame CJ, Chen Y, Roberts LJ, Hachey DL, Montine TJ, Morrow JD (2000) Biochem 39:2376
104. Obata T, Sakurai Y, Kase Y, Tanifuji Y, Horiguchi T (2003) J Chromatogr B 792:131
105. Thomas MJ, Chen Q, Sorci-Thomas MG, Rudel LL (2001) Free Radical Bio Med 30:1337
106. Lee CY, Jenner AM, Halliwell B (2004) Biochem Biophys Res Commun 320:696
107. Mori TA, Croft KD, Puddey IB, Beilin LJ (1999) Anal Biochem 268:117
108. Lawson JA, Rokach J, FitzGerald GA (1999) J Biol Chem 274:24441
109. Nourooz-Zadeh J, Gopaul NK, Barrow S, Mallet AI, Anggard EE (1995) J Chromatogr B 667:199
110. Montine TJ, Montine KS, Reich EE, Terry ES, Porter NA, Morrow JD (2003) Biochem Pharmacol 65:611
111. Liu X, Whitefield PD, Ma Y (2010) Talanta 81:1599
112. Yin H, Porter NA, Morrow JD (2005) J Chromatogr B 827:157
113. Waugh RJ, Morrow JD, Roberts LJ, Murphy RC (1997) Free Radical Bio Med 23:943
114. Łuczaj W, Welerowicz T, Skrzydlewska E, Buszewski B (2008) Toxicol Mech Method 18:483
115. Durand T, Bultel-Poncé V, Guy A, El Fangour S, Rossi JC, Galano JM (2011) Biochimie 93:52
116. Musiek ES, Cha JK, Yin H, Zackert WE, Terry ES, Porter NA, Montine TJ, Morrow JD (2004) J Chromatogr B 799:95