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REVIEW ARTICLE

An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples

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Abstract

Lipid peroxidation products like malondialdehyde, 4-hydroxynonenal and F_2-isoprostanes are widely used as markers of oxidative stress *in vitro* and *in vivo*. This study reports the results of a multi-laboratory validation study by COST Action B35 to assess inter-laboratory and intra-laboratory variation in the measurement of lipid peroxidation. Human plasma samples were exposed to UVA irradiation at different doses (0, 15 J, 20 J), encoded and shipped to 15 laboratories, where analyses of malondialdehyde, 4-hydroxynonenal and isoprostanes were conducted. The results demonstrate a low within-day-variation and a good correlation of results observed on two different days. However, high coefficients of variation were observed between the laboratories. Malondialdehyde determined by HPLC was found to be the most sensitive and reproducible lipid peroxidation product in plasma upon UVA treatment. It is concluded that measurement of malondialdehyde by HPLC has good analytical validity for inter-laboratory studies on lipid peroxidation in human EDTA-plasma samples, although it is acknowledged that this may not translate to biological validity.

Keywords: *Oxidative stress, F2-isoprostanes, 4-hydroxynonenal, malondialdehyde*

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Abbreviations: *BHT, Butylated hydroxytoluene; BSA, Bovine serum albumin; BSTFA,* bis-*(trimethylsilyl) trifluoroacetamide; CV_{inter}, Inter-laboratory coefficient of variation; CV_{intra}, Intra-laboratory coefficient of variation; DNP, 2,4-Dinitrophenylhydrazone; DNPH, 2,4-Dinitrophenylhydrazine; ELISA, Enzyme-linked immunosorbent assay; F2-IsoPs, F_2-isoprostanes; GC-NICI-MS, Gas chromatography negative ion chemical ionization mass spectrometry; HNE, 4-Hydroxy-trans-2-nonenal; HPLC, High performance liquid chromatography; LC-MS, Liquid chromatography mass spectrometry; MDA, Malondialdehyde; ODS, Octadecylsilane; PBS, Phosphate buffered saline; PUFA, Polyunsaturated fatty acid; RP, Reverse phase; TBA, Thiobarbituric acid; TBARS, Thiobarbituric acid reactive substances; TMB, 3,3',5,5'-Tetramethylbenzidine; TPP, Triphenylophosphine; UVA, Ultraviolet light A.*

Introduction

Biological membranes and plasma lipoproteins all contain significant proportions of polyunsaturated fatty acids (PUFAs), which make them susceptible to oxidative attack by free radicals and results in peroxidation of the fatty acyl chains. Following the generation of peroxyl radicals and hydroperoxides, rearrangement can lead to formation of relatively more stable long-chain oxidation products such as isoprostanes, which have prostaglandin-like structures but are not synthesized by cyclo-oxygenase [1]; the F2-isoprostane forms (F2-IsoPs) are the most abundant in plasma and urine. Alternatively, peroxyl radicals or endoperoxides may undergo cleavage to release short chain secondary products, often aldehydes, as in the examples of malondialdehyde (MDA), pentanal and 4-hydroxy-2-nonenal (HNE) [2]. Many lipid peroxidation products, both full chain and chain-shortened, have been reported to demonstrate harmful or pro-inflammatory effects [3–5]. There are many studies supporting the idea that oxidative stress and specifically the oxidative degradation of PUFAs is involved in many pathological processes. Overwhelming evidence has accumulated over the last decade, indicating that oxidation of lipids plays an important role in the development of multiple acute and chronic human diseases like cancer, atherosclerosis and others [6–8]. While cell membrane lipid peroxidation may lead to loss of viability, altered signalling and tissue dysfunction, in plasma the oxidation of lipoproteins is probably a major contributor to the formation of lipid peroxidation products and is widely thought to be involved in atherosclerosis. Malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and F2-isoprostanes (F2-IsoPs) are now the most studied products of lipid peroxidation. MDA and HNE are aldehydes that react with a variety of biomolecules, such as proteins, lipids and nucleic acids, and they are thought to contribute to the pathogenesis of human chronic diseases [6]. Furthermore, their relatively long half-life makes them candidates for the propagation of damage to neighbouring cells. F2-IsoPs exert biological actions and may be pathophysiological mediators of diseases [9,10]; their formation correlates with a variety of pathological syndromes associated with oxidative stress [11].

Quantification of lipid peroxidation can be assessed by the detection of products of PUFA peroxidation. Several markers for lipid peroxidation *in vitro* and *in vivo* are available [12] and different methods for detection of lipid peroxidation in biological samples have been described for each marker by numerous authors [6,13,14]. Previously, the differences in several markers of oxidative stress were investigated using a rodent model of oxidative damage (CCl_4 administration) in the biomarker of oxidative stress study (BOSS), a multi-centre study in which samples were generated in one laboratory and distributed to other laboratories for analysis of each different marker [15]. This study identified plasma MDA and plasma and urinary F2-IsoPs as promising candidates for free radical damage induced by CCl4, especially with analysis by GC/NICI-MS. On the other hand oxidation products of plasma proteins (protein carbonyl, methionine sulphoxide and 3-nitrotyrosine) and leukocyte DNA (comet assay) did not appear to be reliable oxidation markers in this model [15]. Other studies have reported, mainly with respect to analysis of DNA oxidation products, that methods of oxidative stress detection often lack sensitivity and specificity and might be disturbed by interference coming from related products or over-estimation resulting from the analysis conditions, i.e. artefacts [16–18]. It is likely that similar problems may affect markers of oxidative damage resulting from lipid peroxidation. In particular, the combination of parameter and method variety, together with unstable analytes, almost certainly contributes to the high variation seen in some published estimates of oxidative damage to biological lipids. Proudfoot et al. [19] have shown that there are wide limits of agreement between the measurement of F2-IsoPs by an enzyme immunoassay and gas chromatography/mass spectrometry, with only 28% of the values within the 95% confidence limits for the difference. The values for F2-IsoPs reported vary considerably depending on whether total (esterified plus free) or free F2-IsoPs and one or several regioisomers are being measured; e.g. levels of 3–25 pg/mL were observed with LC-(APCI)-MS/MS for free 8-iso-PGF2α [20], whereas values in the range 200–300 pg/mL for total F2-IsoPs have been found by GC-NICI-MS [21–24]. Lykkesfeldt [25] compared malondialdehyde values obtained by

HPLC with fluorescence detection and a direct spectrophotometric method and reported an overestimation by spectrophotometry; a similar finding had been reported previously for a comparison of MDA and TBARS analysis [26]. This discrepancy may be explained by the fact that HPLC with fluorescence detection quantifies only the genuine MDA-$(TBA)_2$ adduct, whereas the total absorbance of several thiobarbituric acid reactive substances (TBARS) is measured by the direct spectrophotometric method. In many TBA-dependent methods lipid hydroperoxides are also degraded to release MDA during the assay [1] and the measured levels can also be influenced by whether acidic hydrolysis is used to release protein-bound MDA. Furthermore, it is likely that the inconsistent results often found in the field of oxidative stress intervention studies might be due to a lack of accuracy and comparability of the available methods for lipid peroxidation detection. It is very important to address this point, in order to understand the implications on studies of oxidative stress where lipid peroxidation parameters are measured.

Although the research reported by the BOSS group provided important information on the potential of various assays of lipid peroxidation to detect free radical damage in a rodent model, there is still a lack of information from studies addressing the inter-laboratory variability of particular lipid peroxidation assays. The present study was carried out to address this issue in human plasma and assess the inter-laboratory and intra-laboratory variation in the measurement of lipid peroxidation. Fifteen laboratories within the European COST Action B35 on 'Lipid peroxidation associated disorders' undertook a multi-laboratory validation study using human plasma samples that were generated by a single laboratory and distributed to the participating groups. The plasma was oxidized by exposure *ex vivo* to ultraviolet A irradiation (UVA) at different doses and three separate markers of lipid peroxidation were measured by different methods. The specific aims of the study were to investigate the intra-laboratory variability with two different batches of five identical samples; to compare analysis of the same lipid peroxidation product between different laboratories; to investigate which methods had the sensitivity to detect increased levels of lipid peroxidation expected with the two severities of treatment *ex vivo*.

Materials and methods

Participating groups

A group of 15 laboratories was set up as an initiative within the European COST B35 action on 'Lipid peroxidation associated disorders'. All the laboratories involved were experienced in the determination of lipid peroxidation and were blinded with regard to the identity of the samples.

Plasma samples

Residual fresh human plasma-EDTA samples of another study were pooled and divided into three batches of ~250 mL. Two of the batches were subjected to UVA irradiation at a dose of 15 or 20 Joule using a UV chamber 'Minitec' (Dr Groebel UV Elektronik GmbH, Ettlingen, Germany). The UVA irradiation source was a TL15W/05 lamp that emitted an energy spectrum in the UVA region (320–400 nm). The emitted dose was calculated using a UV-MAT dosimeter system. Each batch was divided into several petri dishes that were irradiated together in the UV chamber and then re-combined after treatment. The third batch was subjected to the same conditions without irradiation ('Control'). The three fractions were then aliquoted in the required number and sample sizes for the 15 laboratories, with all aliquots in duplicate.

For the measurement of each lipid peroxidation analyte (or for each method where a laboratory used two methods) the laboratories received 15 encoded (blinded) samples randomly distributed consisting of 'Control' ($n=5$), '15J' ($n=5$) and '20J' ($n=5$). All samples were frozen and stored at −80°C and shipped on dry ice. Each laboratory received two batches of samples (batch 1 and batch 2) in order to test the day-to-day variation of the laboratories. The samples of batch 2 were only shipped after receiving the results of the first batch in order to enforce separate analytical runs for the two batches. The overall plan of the study is illustrated in Figure 1. The samples were either stored at <70°C or were analysed within 2 weeks (Table I). The analytes are stable at −80°C for at least 3–4 months, based on previous experience of the laboratories.

Lipid peroxidation markers

Lipid peroxidation was determined by the different laboratories using MDA ($n=8$), F2-IsoPs ($n=3$) and HNE ($n=5$) as the studied markers. A variety of analytical techniques were employed, which are summarized in Table I together with important assay characteristics.

HPLC analysis of MDA-TBA adduct. Five laboratories (D, E, G, I and L) determined MDA-TBA adducts according to Khoschsorur et al. [27] (laboratories D, G and L), Wong et al. [28] (laboratory E) and Londero and Greco [29] (laboratory I). Briefly, plasma was mixed with 0.44 M phosphoric acid and 42 mM TBA, incubated in a water bath for 1 h, cooled and diluted 1:1 with alkaline methanol. The supernatant was separated by HPLC on RP C18 columns and fluorimetric detection was performed with excitation at 515 (G), 525–527 (D, E) or 532 nm (I) and emission at 550–553 nm (D, E, G, I), respectively. Laboratory L

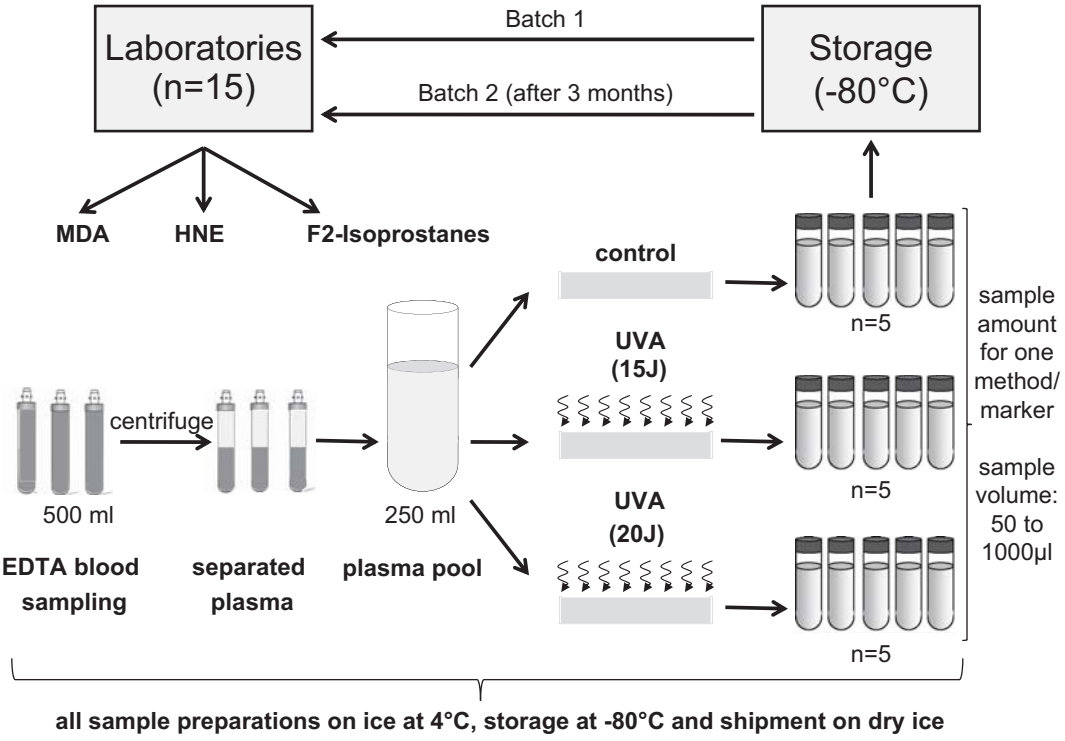

Figure 1. Scheme of sample treatment and distribution in the inter-laboratory validation study.

detected MDA-TBA adduct elution by UV/VIS detection at 533 nm. 1,1,3,3-tetra(m)ethoxypropane hydrolysed with 1% sulphuric acid was used as a standard.

HPLC analysis of MDA-DNP adduct. Two laboratories (C and F) determined MDA-DNP adducts according to Mateos et al. [30] (laboratory C) and Punchard and Kelly [31] (laboratory F). Briefly, alkaline or acidic hydrolysis of protein bound MDA was achieved by incubating samples with NaOH (C) or HCl (F) in a 60°C water bath for 30–40 min. The protein was precipitated with perchloric acid (C) or trichloroacetic acid (F) and removed by centrifugation. For derivatization, DNPH (5 mM in 2 M HCl) was added to the supernatant and incubated for 20 min (C) or 60 min (F) at room temperature in the dark. MDA was analysed as its 2,4-dinitrophenylhydrazone derivative at 310 nm following separation by HPLC on RP C18 columns. 1,1,3,3-tetraethoxypropane hydrolysed with 1% sulphuric acid was used as a standard.

Spectrophotometric analysis of MDA with 1-methyl-2-phenylindole. Laboratory A determined MDA according to Gerard-Monnier et al. [32]. Briefly, bound MDA was hydrolysed in the presence of butylated hydroxytoluene in acetonitrile, at pH 1–2 at 60°C for 80 min. A final concentration of 10 mM 1-methyl-2-phenylindole (in 3:1 acetonitrile/methanol) was added to each sample. After centrifugation the reaction was started by adding concentrated HCl (one volume of HCl to five volumes of the reaction mixture). After 60 min incubation at 45°C, the absorbance of the supernatant was measured at 586 nm. 1,1,3,3-tetramethoxypropane in the range 0.25–4 µM was used as a standard.

HNE-His ELISA. The three laboratories H, J and L determined HNE-histidine conjugates based on previously described procedures [33]. Briefly, samples were added to wells containing 25 mM (J, L)/100 mM (H) carbonate buffer pH 9.6 and incubated overnight in the fridge. The wells were washed with ddH_2O (J, L) or 0.05% Tween 20 (H), before incubating at room temperature for 3 h with blocking solution (5% fat-free milk powder in ddH_2O (J, L) or in 5% Tween 20 in PBS (H)). Subsequently they were washed again with water (J, L)/0.05% Tween 20 (H), before addition of anti-HNE-His antibody (H, 1/200; J and L, 1/100 dilution) and incubation for 2 h at room temperature. At the end of this time the wells were washed thoroughly with wash buffer (0.05% Tween-20 in PBS) followed by treatment with 0.1% sodium azide, 1.5% (J, L) or 5% (H) H_2O_2 in PBS for 30 min to inhibit sample peroxidase activity. After further extensive washing, secondary antibody (HRP-labelled Anti-Mouse IgG at 1/100 dilution (J, L) or 1/500 dilution (H) in PBS containing 1% BSA) was added and incubated for 1 h. After a few washing steps TMB was added. After 5–10 min (J) or 30–60 min (H) incubation, the reaction was stopped with H_2SO_4 and the plate was read at 450 nm. In parallel, a second

Table I. Overview of the used methods in the laboratories for determinations of malondialdehyde, F2-isoprostanes and 4-hydroxy-2-nonenal.

Analyte	Laboratory	Method	Reference	Storage	Assay limits
MDA	A	Spectrophotometric with 1-methyl-2-phenylindole (external standard)	[32]	–80°C, <2 wks	LOD and LOQ <0.1 μM Linearity 0.25–4 μM
	C	HPLC with DNPH (external standard)	[30]	–80°C, 6 mth/2 wks	LOD 0.1 μM, LOQ 0.3 μM Linearity 0.3–20 μM
	D	HPLC with TBA with fluorescence detection (external standard)	[27]	–20°C, <2 wks	LOD 0.1 μM, LOQ 0.2 μM Linearity 0.2–6.4 μM
	E	HPLC with TBA with fluorescence detection (external standard)	[28]	–80°C, <2 wks	LOD 2 nM, LOQ 10 nM Linearity 2–1000 nM
	F	HPLC with DNPH (internal standard)	[31]	–80°C, <4 wks	LOD 0.1 μM Linearity 0.1–50 μM
	G	HPLC with TBA with fluorescence detection (external standard)	[27]	–80°C, 3–4 mth/1–2 mth	LOQ 0.125 μM Linearity 0.25–8 μM
	I	HPLC with TBA with fluorescence detection (external standard)	[29]	–80°C, ~1 wk	LOD 5 μM, LOQ 10 μM Linearity 10–1200 μM
	L	HPLC with TBA with UV/VIS detection (external standard)	[27]	–80°C, <1 mth/3 mth	LOD 0.14 μM Linearity 0.14–36 μM
F2-isoprostanes	B	Commercial ELISA method (by OxisResearch)		–80°C, <6 wks/6 mth	LOD 2.7 pg/mL, LOQ 13.0 pg/mL Linearity 2–500 pg/mL
	I	LC-MS	[36]	–80°C, ~1 wk	LOD 0.5 pg/mL, LOQ 1 pg/mL Linearity 1–1000 pg/mL
	K	GC-NICI-MS (internal standard)	[24,46]	–70°C, 3 mth/3 wks	LOQ 2.5 pg Linearity 0.05–2.5 ng
HNE	H (1)	His-ELISA (external standard)	[33]	–40°C, <2 wks	LOQ 0.2 nmol/ml Linearity 0.25–10 nmol/mg BSA
	H (2)	HPLC with UV/VIS detection (external standard)	[34]	–40°C, <2 wks	LOQ 250 pmol/ml (5 pmoles) Linearity 0.2–12.5 nmol/mg BSA
	I	HPLC with fluorescence detection (external standard)	[35]	–80°C, ~1 wk	LOD 5 μM, LOQ 10 μM Linearity 10–5000 μM
	J	His-ELISA (external standard)	[33]	–80°C, ~6 wks/3 wks	LOD 0.1 nmol/mg BSA, Linearity 0.2–12.5 nmol/mg BSA
	L	His-ELISA	[33,47]	–80°C, <1 mth/3 mth	LOD 7 pmol/mg protein Linearity 7–140 pmol/mg protein

set of samples were run with PBS substituted for the primary antibody, to allow correction for non-specific binding. BSA treated with various amounts of HNE (either prepared in-house or purchased from Enzo Life Sciences, Exeter, UK) was used as a standard, in the range 0.1–12.5 nmol/mg protein.

HPLC analysis of free HNE. Laboratory H determined free HNE as described previously [34]. Briefly, plasma was mixed with BHT and desferrioxamine corresponding to ~ 1 mg protein/ml. The sample was then poured onto an Extrelut column and dichloromethane was applied to collect the eluate in a flask containing 0.1 M acetate buffer. In order to obtain an aqueous phase dichloromethane was removed with a rotary evaporator not exceeding 20°C. The residual aqueous phase was applied to the ODS-disposable pre-conditioned column and the residual sample was rinsed in a flask with methanol/water (15:85) and applied to the column. Free HNE was analysed at 223 nm. BSA treated with various amounts of HNE (prepared in house) was used as a standard, in the range 0.25–10 nmol/mg protein.

HPLC analysis of HNE-1,3-cyclohexandione adduct. Laboratory I assayed HNE as a fluorimetric derivative with 1,3-cyclohexandione separated by HPLC with fluorometric detection with excitation at 380 nm and emission at 445 nm as described previously [35].

Commercial ELISA for F2-isoprostanes. 8-epi-prostaglandin $F_{2\alpha}$ concentration in plasma samples was assayed by BIOXYTECH® 8-Isoprostane Assay Kit *Oxis*Research™ according to the manufacturer's instructions following the extraction procedure. Briefly, 50 μL of plasma was extracted in chloroform:methanol (2:1 v/v) supplemented with 50 mg/L butylated hydroxytoluene (BHT) and 500 mg/L triphenylophosphine (TPP) cooled to 0°C. Following addition of $MgCl_2$ solution the organic

phase was collected and dried under argon. The lipid pellet was dissolved in methanol containing 50 mg/L BHT, alkalized with KOH solution and incubated at 37°C for 30 min before acidifying to pH 3. The sample was cleaned first on a C18 column by washes with 1 mM HCl and heptane before eluting with ethyl acetate:heptane (1:1 v/v). After drying with anhydrous sodium sulphate, the liquid was transferred to a silica column and washed with ethyl acetate; bound material was eluted with ethyl acetate:methanol (1:1 v/v) and dried under argon. The sample was dissolved in 100 µL of the DB buffer included in the assay kit. Competitive ELISA was performed exactly as in the assay manufacturer's protocol.

F2-isoprostane analysis by GC-MS. F_2-IsoPs (esterified and non-esterified) were analysed according to Wiswedel et al. [24] and as described briefly below. Esterified lipids were hydrolysed in the presence of 0.1 mM BHT as an antioxidant using KOH at 45°C. Thereafter, 9α,11α-$PGF_{2\alpha}$-d_4 was added as an internal standard. The samples were neutralized by addition of HCl to give pH ~ 2. The samples were extracted twice with ethyl acetate, the combined upper phases were evaporated under nitrogen and the residues were reconstituted in ethylacetate. The prostanoid extract was applied to NH_2-cartridges equilibrated with n-hexane and then washed successively with n-hexane/ethyl acetate (30/70, v/v), acetonitrile/water (90/10, v/v) and acetonitrile before elution with ethyl acetate/methanol/acetic acid (10/85/5, v/v/v). Dried extracts were derivatized with pentafluorobenzyl-bromide in the presence of *N,N*-diisopropylethylamine, dried again before silylation with *bis*-(trimethylsilyl) trifluoroacetamide (BSTFA). F_2-IsoPs were separated and measured by GC(NICI)-MS (DSQ/Trace GC Ultra, Thermo Fisher Scientific, Dreieich, Germany) using a DB 5-MS column (J&W Scientific, Folsom, CA). Quantitative analysis was performed using selected ion monitoring of the carboxylate anion $[M\text{-}181]^-$ at *m/z* 569 and *m/z* 573 for F_2-IsoPs and 9α,11α-$PGF_{2\alpha}$-d_4 (internal standard; Cayman Chemicals Co., Ann Arbor, MI), respectively. The internal standard was quantified using a 5-point calibration curve. Each sample contained 0.5 ng of 8-iso-$PGF_{2\alpha}$ and either 0.05 ng, 0.250 ng, 0.50 ng, 1.0 ng and 2.5 ng of 9α,11α-$PGF_{2\alpha}$-d_4.

F2-isoprostane analysis by LC-MS. 8-iso-prostaglandin $F_{2\alpha}$ (8-iso$PGF_{2\alpha}$) was assayed by LC-MS essentially using the method of Coolen et al. [36]. The first step of sample preparation was alkaline hydrolysis. To extract the 8-iso-$PGF_{2\alpha}$, the samples were purified by using SEP-PAK C18 column containing octadecylsilyl silica gel (Waters Associates, Milford, MA, USA). 8-iso$PGF_{2\alpha}$ was separated by HPLC and detected using electrospray ionization mass spectrometry. The separation was carried out on RP C18 with a linear gradient from 100% water adjusted to pH 5.7 with acetic acid to 100% acetonitrile. 8-iso$PGF_{2\alpha}$ in the range 1–1000 pg/mL was used as a standard.

Statistics

Data were analysed using Statistica (StatSoft, Tulsa, OK) and are represented as mean values with standard deviations. Using the results of the five single measurements in one laboratory, the intra-laboratory coefficient of variation (CVintra) was calculated as SD/mean×100 for the three different treated sample types. Inter-laboratory coefficient of variation (CVinter) was calculated for between-laboratory data from each measured parameter, independently to which method was used for the determination of the parameter. Significance of differences between Control-, 15J- and 20J-treated samples was tested using the Student's test for dependent samples considering $p < 0.05$ as significantly different. Pearson's correlation coefficients were calculated for relationships between batch 1 and 2 for the different markers.

Results

Of the 15 European laboratories participating in the inter-laboratory validation study, nine aimed to perform measurements on one parameter, four laboratories on two parameters and two on three parameters. Due to methodological problems four laboratories reduced their planned measurements by one parameter and one laboratory returned incomplete results. Finally, for the data analysis of the present work the complete data sets of 16 measurements of 12 laboratories (named A to L) were available and used for data analyses. UVA-irradiation was used as the model system, because it represents a well-documented injury that is oxidative in nature [37].

Intra-laboratory analysis

Intra-laboratory variation (CVintra) for the participating laboratories was calculated for each of the three treatments in both batches (control, 15J and 20J) using the five identical samples in each set. These CVintras represent the within-day-variation of each laboratory. As seen in Table II, CVintra for MDA determination showed a wide range between 1–175%. In batch 1, two of eight laboratories showed intra-laboratory CVs of <10%, four of eight <20% and one of eight <30%. One laboratory was characterized by a CVintra of >110%. Intra-laboratory CVs in batch 2 were in general higher than in batch 1, where again two laboratories showed CVintras <10%. However, calculated intra-laboratory CVs were, except for two laboratories, comparable between the three sample

types. The CVintra for F2-IsoPs and HNE also showed a wide range between 6–112% for F2-IsoPs and 9–136% for HNE (Tables III and IV). In contrast to MDA, CVintra of these parameters were characterized by greater differences between the two batches and less consistent CVs within the individual laboratories.

In order to study the day-to-day variation in the laboratories, correlation coefficients between batch 1 and batch 2 were calculated for all three lipid peroxidation parameters. Correlation analysis indicated a strong statistically significant positive correlation between results of batch 1 and batch 2 for MDA ($r=0.82$) and F2-IsoPs ($r=0.93$), as shown in Figure 2; there was also a weak but significant correlation for HNE ($r=0.62$).

In an additional experiment, one laboratory (laboratory L) performed the determination of MDA and HNE in the same 15 samples. Figure 3 shows the measured MDA and HNE results in ascending order within each treatment set. Interestingly, for MDA the five lowest concentrations were found in the control samples as expected. There was a tendency for the values to increase with increasing irradiation, although the distinction between the 15J and 20J sets is not clear. The values for HNE generally showed a similar pattern within each treatment set, although the values were slightly more variable. In particular, there was no clear trend to higher values with increased severity of treatment, demonstrating a low specificity of this HNE method for detecting oxidative damage to lipids in plasma. It should be noted that the values of the MDA and HNE cannot be directly compared, as the HNE analyses are expressed as nanomols of HNE per mg of protein (i.e. the extent of protein modification within the sample), rather than as an absolute concentration for HNE.

Inter-laboratory analysis

Mean, SD, median and CVinter of MDA, F2-IsoPs and HNE measurements are presented in Table V. The inter-laboratory coefficient of variation for all three markers was calculated to be very high and ranged from 63–190% (Table V), with the inter-laboratory CVs of HNE detection being highest and of MDA detection lowest. A comparison of the data for all three lipid peroxidation analytes and all treatments between batch 1 and batch 2 showed that the means and medians were in almost all cases lower in batch 2, by as much as 5-fold for some sets of data. Due to the high inter-laboratory variation, a statistically significant increase of lipid peroxidation could not be found in the oxidized samples, neither in batch 1 nor in batch 2 (Table V, $p>0.05$).

The mean values of each laboratory for MDA 'control' samples of batch 1 are presented in Figure 4 in ascending order with the median value of 1.11 μmol/L indicated. A factor of 49 separates the highest and lowest values. Only two of the eight laboratories were within ±50% of the median of MDA control samples, whereas four laboratories were within ±50% of the median observed in both UVA-treated samples (data not shown), demonstrating that the variation of the results might be affected by the state of oxidation. Interestingly, the highest values for MDA were determined in laboratories using a method other than TBA-adduct detection by HPLC (Figure 4). The median for MDA detection by HPLC was calculated to be 0.55 μmol/L for MDA control samples. It was also observed that the mean values of ELISA-based HNE-His detection ($n=3$) were up to 20-fold higher than mean values for chromatographic methods ($n=2$; Table VI).

Potential for detection of UVA-induced lipid peroxidation

An important aspect of the study was to test whether the levels of lipid peroxidation products assayed were able to distinguish and predict correctly the induced lipid peroxidation, according to the severity

Table II. Intra-laboratory coefficients (CVintra) of MDA determinations in the single laboratories (A–L) in batch 1 and 2.

	Batch 1			Batch 2		
	C	15J	20J	C	15J	20J
A	10.8%	18.9%	12.5%	45.8%	33.6%	29.3%
C	11.3%	29.6%	23.0%	27.7%	25.4%	12.8%
D	1.2%	1.8%	3.4%	10.7%	6.6%	2.7%
E	11.1%	7.5%	7.7%	22.5%	12.2%	33.8%
F	112.1%	110.2%	135.3%	151.7%	46.0%	49.6%
G	10.9%	13.4%	11.8%	10.1%	8.3%	4.5%
I	6.6%	11.7%	12.3%	17.9%	23.0%	22.6%
L	6.1%	9.9%	3.4%	6.9%	1.7%	2.0%

Table III. Intra-laboratory coefficients (CVintra) of F2-IsoP determinations in the single laboratories (B, I, K) in batch 1 and 2.

	Batch 1			Batch 2		
	C	15J	20J	C	15J	20J
B	27.8%	57.1%	16.9%	18.7%	27.9%	32.8%
I	49.1%	44.4%	29.4%	45.1%	68.6%	56.4%
K	111.7%	39.0%	42.8%	32.2%	22.7%	5.6%

Table IV. Intra-laboratory coefficients (CVintra) of HNE determinations in the single laboratories (H, I, J, L) in batch 1 and 2.

	Batch 1			Batch 2		
	C	15J	20J	C	15J	20J
H	26.7%	38.9%	33.2%	82.2%	31.0%	50.9%
J	91.3%	69.0%	69.0%	14.4%	49.6%	135.8%
L	15.4%	16.9%	10.9%	94.4%	35.8%	22.1%
H	55.5%	26.9%	22.2%	14.7%	33.9%	32.6%
I	58.9%	57.9%	40.4%	9.4%	12.2%	9.9%

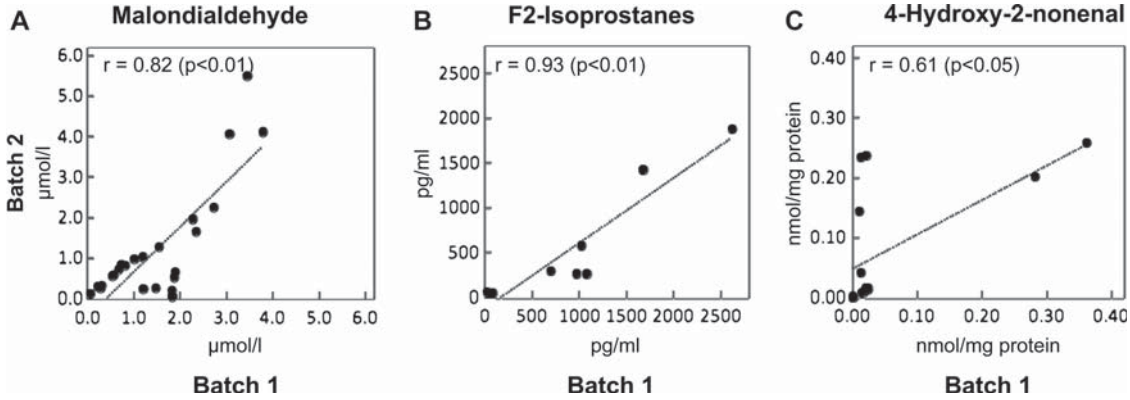

Figure 2. Results of correlation analyses between batch 1 and batch 2 for MDA, F2-isoprostanes and HNE determination. Correlation coefficients and *p*-values are presented.

of the irradiation dose. With regard to MDA determination, five out of eight laboratories showed a significant and progressive increase in MDA in 15J and 20J-treated samples (Figure 5A). These results are in contrast to those of F2-isoprostane and HNE determinations, in which there are only partial significant differences or no significant differences (Figures 5B and C). It was further shown that the simultaneous determination of HNE-His by ELISA (H-1 in Figure 5C) and HPLC (H-2 in Figure 5C) in one laboratory (H) resulted in different HNE concentrations (18-fold for '20J' samples).

Further analyses tested whether differences in lipid peroxidation can also be observed if data of all laboratories were used, and the result is shown in Figure 6. There was no statistically significant rise in MDA, F2-IsoPs and HNE when the data of all laboratories were analysed together. However, the observed changes of HNE were characterized by a very high variation (~3-fold mean value).

Discussion

Besides the quantification of inter-laboratory variations in the determination of lipid peroxidation markers, we were interested to assess the contribution of intra-laboratory variation to inter-laboratory variation. Our data for MDA measurements indicate low within-day variances characterized by intra-laboratory CVs between 1–30% in most of the laboratories. There was one laboratory that had serious difficulties in yielding similar and reproducible results, highlighted by the fact that intra-laboratory CVs for the different sample types ranged between 110–175%; the method used in this case was the DNPH-HPLC analysis of MDA. Within-day variances for HNE and F2-IsoP were characterized by higher within-day variances and less consistency within the single laboratories compared to MDA measurements.

To investigate if the laboratories are able to reproduce their results, identical samples were analysed for second time coded to the laboratories as batch 2 representing the day-to-day variance of the laboratories. We observed a good correlation between the results observed in batch 1 and 2, with correlation coefficients of 0.82 for MDA and 0.93 for F2-IsoP. A statistically significant but only weak correlation was found for HNE. The samples of the second batch were older at the time of measurement than samples of batch 1 (as all the samples were derived from one

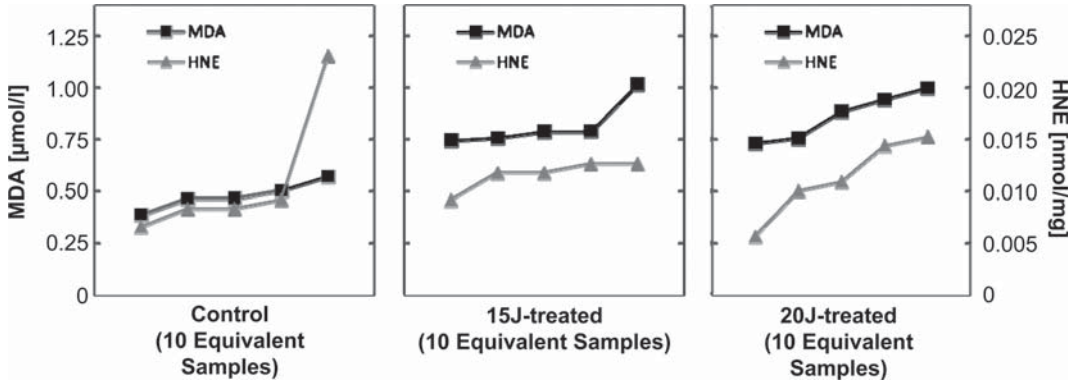

Figure 3. Results of an intra-laboratory comparison of two methods for lipid peroxidation: MDA detection with TBA-HPLC and HNE detection by ELISA. Measurements were carried out on 15 samples in total for each analyte, five for each of the three treatments (control, 15J, 20J). Results of MDA and HNE analysis were both arranged in order of increasing values found within each treatment set; note that the sample sets for the two analytes are separate (10 separate equivalent aliquots per treatment).

Table V. Mean, standard deviation (SD), median and inter-laboratory coefficient of variation in two independent (batch 1 and 2) measurements of Malondialdehyde ($n=8$), F2-isoprostanes ($n=3$) and 4-Hydroxy-2-nonenal ($n=5$).

		Batch 1				Batch 2			
		Mean	SD	Median	CVinter (%)	Mean	SD	Median	CVinter (%)
MDA (μmol/l) $n=8$	C	1.35	1.13	1.11	83.7	1.06	1.41	0.41	133.5
	15J	1.50	1.05	1.52	70.1	1.28	1.76	0.71	138.1
	20J	1.60	1.10	1.28	68.6	1.25	1.26	0.85	100.4
F2-IsoP (pg/ml) $n=3$	C	584.1	498.6	693.8	85.4	303.5	267.9	290.8	88.3
	15J	889.1	829.7	552.8	93.3	580.0	735.6	72.4	126.8
	20J	1257.7	1278.4	1075.0	101.6	727.5	1000.6	260.7	137.5
HNE (nmol/mg) $n=5$	C	0.015	0.009	0.018	63.6	0.081	0.105	0.015	130.2
	15J	0.067	0.121	0.015	180.5	0.091	0.116	0.013	127.4
	20J	0.082	0.156	0.016	189.9	0.065	0.109	0.015	168.1

original plasma pool), which raises the possibility of either an increase in lipid peroxidation due to longer storage and a decrease due to degradation processes. As the absolute values (median) of all markers in samples of the second batch are slightly lower compared to those of batch 1 and there was increased variability, the occurrence of degradation processes seems most likely.

Malondialdehyde, 4-Hydroxy-2-nonenal and F2-Isoprostanes have been extensively used during recent years as markers of lipid peroxidation. However, different analytical methods established for each of these parameters generate the problem of inconsistent results, either due to method specificity or sensitivity. Furthermore, oxidative stress markers are by nature easily prone to over-estimation if stringent precautions are not taken in sample handling and storage. For example, high temperature and low pH during the reaction of MDA with TBA may cause artefactual formation of lipid peroxidation products. Consequently, some of what is measured in the field of lipid peroxidation studies may in fact be artifact. Thus, quantification of lipid peroxidation can be misleading and the range might further be increased by an expected high variance between laboratories and analytical methods. Since there is a lack of knowledge in the literature about the variation in the measurement of lipid peroxidation markers, especially between laboratories, the aim of the present validation study was to quantify intra- and inter-laboratory variation in the determination of lipid peroxidation. Varied protocols in the participating laboratories for analysis of each lipid peroxidation compound were intentionally allowed, in order to mimic the real situation in oxidative stress research.

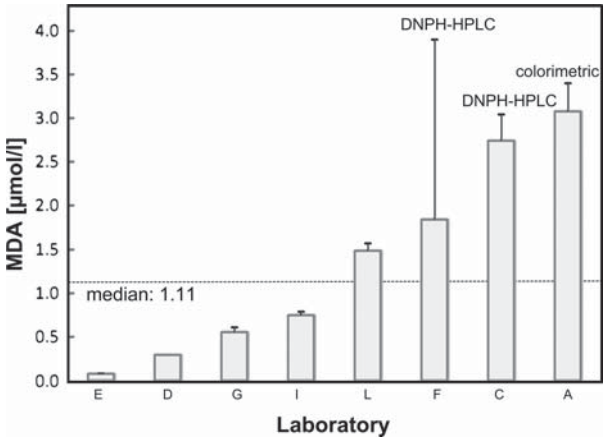

Figure 4. Results of MDA determination of control samples of eight laboratories, arranged in order of increasing values found. HPLC with TBA-adduct detection was employed except where otherwise stated; two laboratories used HPLC with DNP-adduct detection and one laboratory used 1-methyl-2-phenylindole colourimetric detection. $n=5$ for all sets.

As expected, the reported values for the lipid peroxidation markers measured in this group of COST B35 laboratories were characterized by a high variability between laboratories, illustrated by the fact that inter-laboratory CVs were up to 190%. Thus, it appears that there was very limited agreement of the different methods in terms of detection of each parameter. Interestingly, MDA determinations in eight laboratories performed by both different HPLC methods and one colourimetric method showed the lowest inter-laboratory variations. This fits with the observation that, in general, recent measurement of MDA in human plasma of healthy volunteers using TBA falls into a relatively narrow concentration range ~1–5 μmol/L [38–42], although in some cases lower values have been reported [43]. Interestingly, the highest values for MDA were found using 1-methyl-2-phenylindole as the reagent, probably because this method involves an acid hydrolysis step that releases protein-bound MDA, a significant form of MDA in plasma [32]. Likewise, chromatographic separation

Table VI. Comparison of the mean values for determination of 4-Hydroxy-2-nonenal by ELISA ($n=3$) and HPLC ($n=2$).

	Batch 1		Batch 2	
	HPLC (nmol/mg)	ELISA (nmol/mg)	HPLC (nmol/mg)	ELISA (nmol/mg)
C	0.012	0.016	0.008	0.130
15J	0.012	0.103	0.007	0.148
20J	0.010	0.130	0.008	0.103

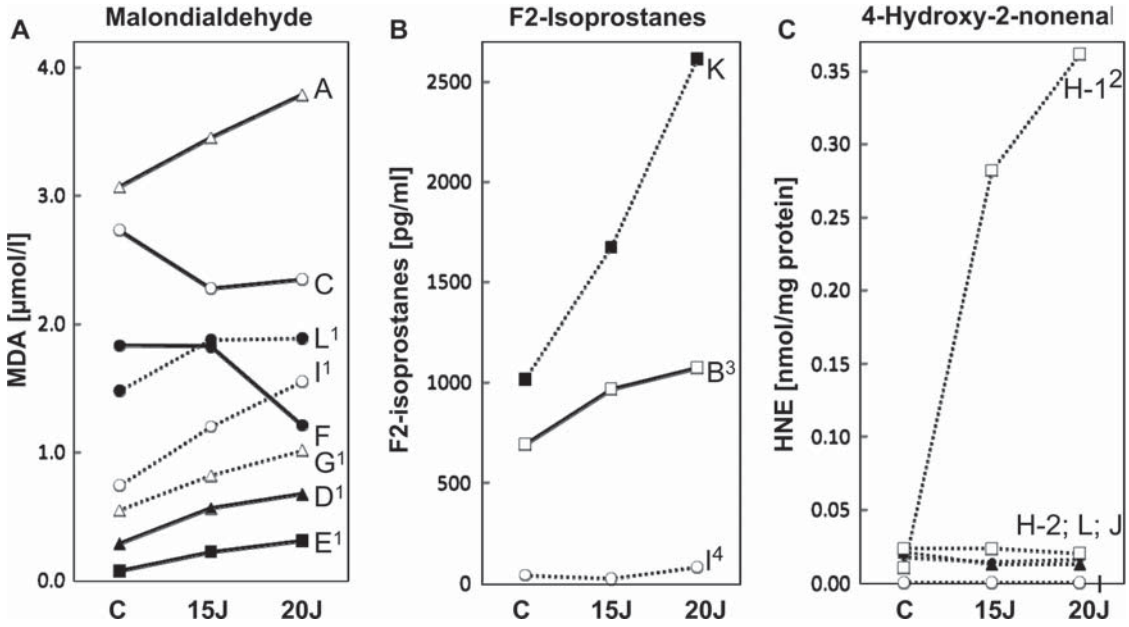

Figure 5. Results of the determination of MDA ($n=8$), F2-isoprostanes ($n=3$) and HNE ($n=5$) in the laboratories participating in the validation study. The laboratories are identified A–L. One laboratory (H) measured HNE both by ELISA (H-1) and HPLC (H-2). Values are means of batch 1. $^1p < 0.05$: C vs 15J+C vs 20J+15J vs 20J; $^2p < 0.05$: C vs 15J+C vs 20J; $^3p < 0.05$: C vs 20J; $^4p < 0.05$: C vs 20J+15J vs 20J.

with UV detection of the MDA-DNP adduct, which also measures both free and protein-bound MDA, resulted in absolute concentrations of MDA 2–4.5-fold higher than the median of the chromatographic detection with TBA. In fact, the four laboratories (D, E, G, I) that carried out MDA analysis by TBA-HPLC gave the most closely matching profiles, although the absolute values still showed considerable variation. These findings support the theory that the use of different methods contributes to the broad range reported here.

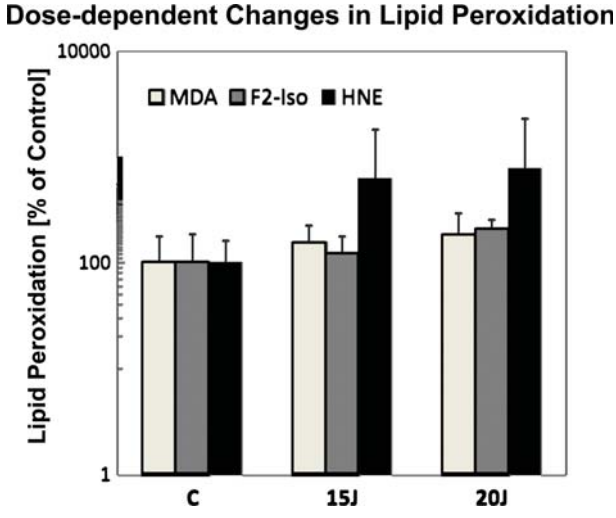

Figure 6. Results of relative changes of MDA, F2-isoprostanes and HNE in control (C) samples and samples treated with 15J and 20J UVA. Bars demonstrate the results of eight (MDA), three (F2-isoprostanes) and five (HNE) laboratories, respectively. The control was set to 100%. Note the log scale.

In our group of laboratories, HNE and F2-IsoP determinations were conducted by two and three methods, respectively, and particularly the high variations in HNE values between the five involved laboratories suggests serious problems, either in the analytical methods or in sample handling. However, sample handling and method variation probably contributes to the variability for MDA also, although this method seems to be more robust. Since it was reported by the laboratories performing HNE-ELISA that the concentrations analysed bordered on the detection threshold of the method and were in the range of the two lowest standards, it appears that the present method is not yet optimal for the analysis of HNE-protein adducts in EDTA-plasma exposed to UVA, possibly because the extent of peroxidation obtained after UVA treatment was low. The HNE-His ELISA used was developed for analysis of cultured cells and cell lysates and has not yet been adapted specifically for HNE-His determinations in the human plasma samples. This study represents the first time that the HNE-His ELISA has been tested in oxidized plasma; previously only HNE spiking of EDTA-plasma was tested and found to give 90% recovery. The methods used for F2-isoprostanes all involved an alkaline hydrolysis step and therefore measured both free and phospholipid-esterified isoprostanes in the plasma. However, in GC-NICI-MS the analysis is based on one gas chromatographic peak that contains 4 regioisomers, whereas in LC-MS the regioisomers can be separated and 8-iso-PGF2α quantified; the ELISA method similarly detects only 8-iso-PGF2α. This in part explains the higher values from the

GC-NICI-MS analysis (laboratory K), although it was noted that the values measured for the control samples were substantially higher than the range of 200–300 pg/mL normally measured by that laboratory [24] and reported in other studies [21–23,44]. Sircar and Subbaiah [45] also reported that F2-isoPs measured by LC-MS were lower than those measured by GC-MS(MS). A previous study using an immunoassay for 8-iso-PGF2α (15-F2t-isoP) reported values in human plasma in the region of 250–350 pg/mL, i.e. lower than those measured in the present study. Thus, again it can be seen that the methodology can change considerably the values of lipid peroxidation products measured.

An important goal of the present study was to test the ability of both the laboratories and analytical methods to discriminate samples with different MDA concentrations induced by an irradiation with UVA at different doses. We observed good correlations between predicted lipid peroxidation and measured MDA concentrations for most of the laboratories. Although F2-IsoP and HNE are also well established parameters of oxidative stress, most of the participating laboratories did not reveal abundant lipid peroxidation in moderately UVA-irradiated samples using MS for F2-IsoPs and HNE-His ELISA or HPLC for HNE. It might be possible that the number of participating laboratories measuring these analytes was too small and that the levels of F2-IsoP and HNE generated by UVA in EDTA-plasma samples used were too low. The fact that the GC-MS method for F2-IsoPs measures several F2-IsoPs both from esterified and free F2-IsoPs, whereas the ELISA method detects only 8-iso-PGF2α probably contributed to the variability observed.

In the past the European Standards Committee on Oxidative DNA Damage [16] was set up to explore the reasons for discrepancies in the measurement of oxidative DNA damage and they were able to make several practical recommendations to reduce oxidation artefacts during DNA isolation. Thus, it appears advisable that a comparable board should be set up in order to resolve the problems associated with high inter-laboratory variability in lipid peroxidation assays and resulting from the use of different analytical methods for each analyte.

An important conclusion from this validation study is that malondialdehyde, F2-isoprostanes and, to a lesser extent, 4-Hydroxy-2-nonenal serve as reliable indicators of lipid peroxidation in human EDTA-plasma samples treated by UVA. Although the inter-laboratory variations were found to be high, the intra-laboratory variations showed an acceptable range. The measurement of MDA by HPLC has emerged as one of the most reliable approaches to assess lipid peroxidation status, because MDA detection was characterized by both the lowest inter-method and inter-laboratory variations and, importantly, the potential to reflect the lipid peroxidation status in human EDTA-plasma samples subjected to different doses of UVA-irradiation. However, it is also necessary to note that the conclusions from this study *in vitro* cannot necessarily be extrapolated to the analysis of free radical-induced damage *in vivo*.

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