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Effects of selenium deficiency on the formation and detoxification of endogenous electrophiles in rats

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Selenium deficiency could be expected to lead to enhanced lipid peroxidation through loss of selenium-dependent glutathione peroxidase activity. Such a relation has, however, been difficult to verify. In the present study, the influence of selenium deficiency in rats on in vivo doses of some endogenously occurring low-molecular mass aldehydes and epoxides was determined. In vivo doses were measured by mass-spectrometric analysis according the N-alkyl Edman method of reaction products (adducts) with N-terminal valines in hemoglobin. Despite variations between experiments, the adduct levels of acetaldehyde and malonaldehyde were shown to be significantly higher in rats fed a selenium-deficient diet than in controls fed a selenium-adequate diet. No significant effect was found for the other aldehydes measured. In contrast, the in vivo doses of endogenous ethylene oxide and propylene oxide were lowered in selenium-deficient rats, indicating a 1.7-times faster detoxification rate. This was verified by the lower adduct levels in selenium-deficient rats following intraperitoneal administration of these epoxides at moderate doses. In conclusion, the results seem to reflect the complex changes of induced and reduced enzyme activities in response to selenium deficiency. Measurement of reactive compounds through their adducts to hemoglobin has shown its ability to elucidate the effects of selenium deficiency per se. (J. Nutr. Biochem. 11:425–430, 2000)

Keywords: selenium deficiency; lipid peroxidation; gluthathione peroxidase; hemoglobin adducts; aldehydes; epoxides

Introduction

Selenium (Se) is an integral part of the enzyme glutathione peroxidase (GSH-Px) and as such plays a probable role in the cellular protection against lipid peroxidation by scavenging hydrogen peroxide and organic hydroperoxides.[1] Decreased plasma levels of Se have been shown to be associated with several human diseases,[2] and experimental animal models as well as human studies have long indicated a cancer-protective role of Se.[3] These protective effects of Se seem, however, mainly to be due to other mechanisms than those related to protection from oxidative damages. In any case, much attention has been devoted to shed light on the possible relationship between the nutritional antioxidants Se and vitamin E and in vivo lipid peroxidation. Markers of lipid peroxidation such as ethane exhalation,[4] malon(di)aldehyde (MA) in liver tissues[5] and F_2-isoprostane in plasma and other tissues[6] have been used for this purpose. Such studies have shown these markers to increase in combined deficiency of Se and vitamin E, in single vitamin E deficiency, but not in Se deficiency alone.

Peroxidative degradation of polyunsaturated fatty acids (i.e., lipid peroxidation) in biological membranes leads to the formation of free radicals and several aldehydes. Many aldehydes exhibit cytotoxic and hepatotoxic as well as mutagenic or other genotoxic properties.[7] Another compound formed through peroxidation reactions is ethene,[8,9] which has been shown to be metabolized to ethylene oxide (EO)[10] in animals[11] and in humans.[12]

The (geno)toxicity of electrophilic compounds such as aldehydes and epoxides depends on the reactivity toward the nucleophilic sites of macromolecules such as nucleic acids and proteins. The reaction products (adducts) formed with nucleophilic atoms in hemoglobin (Hb) and plasma

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Received December 2, 1999; accepted April 19, 2000.

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0955-2863/00/$–see front matter
PII S0955-2863(00)00099-1

proteins can be used to estimate both in vivo doses of endogenously formed reactive compounds and environmental exposure to (geno)toxic compounds. The advantages of using Hb adducts are that blood is an easily available tissue and that these adducts are not the object of repair (as are adducts to nucleic acids), which means that chemically stable Hb adducts are accumulated over the life span of erythrocytes.[13] A sensitive modified Edman method[14,15] has been developed for the analysis by gas chromatography–mass spectrometry (GC-MS) of adducts to N-terminal valine in Hb.

This method has revealed the existence of background levels of adducts from epoxides such as EO and aldehydes in individuals without known environmental exposure.[16] The aldehydes analyzed so far include, among others, formaldehyde, acetaldehyde (AA), hexanal, and MA. In some cases, causative factors to the background levels have been identified. Thus, peroxidation of unsaturated fatty acids and metabolism by intestinal bacteria have been shown to contribute to the background levels of Hb adducts from ethene/EO and MA.[17–19]

The primary aim of the present study was to measure in vivo doses of endogenously formed MA and EO assumed to be markers of lipid peroxidation, and also to measure endogenously formed AA and propylene oxide (PO) in rats fed diets differing only with regard to Se content. In addition, the impact of Se deficiency on the detoxification rates of EO and PO was studied in complementary groups of rats dosed with these compounds.

Measurement of Hb adducts is used as a new tool in studies of this kind, and a further aim of the present study was to test whether the method is sensitive enough to detect an expected but low enhancement of lipid peroxidation due to Se deficiency alone.

Materials and methods

Chemicals

Pentafluorophenyl isothiocyanate (PFPITC), purum, obtained from Fluka AG (Buchs, Switzerland), was purified on a SEP-PAK silica cartridge (Millipore, Waters Assoc., Milford, MA USA) prior to use.[15] Formamide (analytical grade, Merck, Darmstadt, Germany) was extracted twice with pentane before use. Standard globins with known levels of adducts from EO and PO were prepared and characterized as described earlier.[15] ($^2H_4$)Ethylene oxide-treated globin (d_4EO-globin) and ($^2H_3$)methyl iodide-treated globin (d_3Me-globin), used as internal standards in GC-MS analysis, were prepared and characterized with respect to adduct levels of *N*-[2-hydroxy($^2H_4$)ethyl]valine (d_4HOEtVal) and *N*-[($^2H_3$)methyl]valine (d_3MeVal), respectively, as earlier described.[15,20] All other chemicals and reagents were of analytical grade.

Animals and diets

Male Wistar rats (ALAB, Stockholm, Sweden), 20–22 days old, body weight about 50 g, were divided into two groups and fed a Torula yeast-based diet containing corn oil (40 g/kg), lard (40 g/kg), and providing 50 IU vitamin E per kg as previously described[21] for 11 weeks. Although not specified in some previous reports,[21] this diet is regularly fortified with 1 g/kg of choline chloride. The content of Se in the Se-deficient diet was less than 0.01 mg/kg, whereas the Se-adequate control diet contained 0.2 mg Se/kg as sodium selenite. The animals were housed on a 12-hr light/dark cycle and maintained at 24°C. Diet and drinking water were provided ad libitum throughout the experiment. Tissue preparation and analysis of GSH-Px activity of liver cytosol were performed as described previously.[21] Five replications were carried out in the course of about 1 year in order to determine background levels of Hb adducts. The number of animals varied from 2 to 8 per diet group in different replications.

Detoxification study

For the study of effects of Se deficiency on the detoxification of EO and PO, four Se-adequate and four Se-deficient rats were dosed by intraperitoneal injection of a mixture of 1.14 mmol EO and 0.86 mmol PO per kg body weight (50 mg/kg body weight of each compound). The compounds were dissolved in saline (0.154 mol NaCl/L distilled water). Four controls fed the respective diet were treated with saline solution only. In an additional study, Se-adequate and Se-deficient animals were dosed by intraperitoneal injection of either EO (2.27 mmol/kg body weight) or saline. Rats were sacrificed 24 hr after the treatments.

Blood sample collection and work up

Rats were sacrificed by decapitation and blood was collected from the neck into heparinized tubes. Erythrocytes were isolated by centrifugation, washed twice with saline, and divided into two parts. One half of the erythrocytes was stored for a few days at −20°C prior to precipitation of globin with ethyl acetate from an isopropanol/HCl solution.[22] The other half was worked up on the day they were collected as follows: Two vol. of 0.67 M phosphate buffer, pH 6.5, was added to the erythrocytes, which were then lysed by addition of 1 vol. of toluene. Cell membranes were sedimented at 20,000 *g*, the hemolysates reduced with sodium borohydride ($NaBH_4$), and globin precipitated according to Kautiainen *et al.*[23]

Determination of Hb adducts

The in vivo doses of EO and PO were monitored by determining their reaction products, *N*-(2-hydroxyethyl)valine (HOEtVal) and *N*-(2-hydroxypropyl)valine (2-HOPrVal), with N-terminal valine in Hb. MA reacts with N-terminal valine through formation of enamine, which was reduced to stable *N*-(3-hydroxypropyl)valine (3-HOPrVal) by $NaBH_4$.[23] AA reacts reversibly with valine in Hb, giving rise to an unstable Schiff base that was reduced by $NaBH_4$ to stable *N*-ethylvaline (EtVal). The adducted valines in Hb are specifically detached as pentafluorophenylthiohydantoins (PF-PTHs), according to the N-alkyl Edman method.[15] For this purpose, globin samples (unreduced and reduced) were derivatized with PFPITC, with d_4EO-globin and d_3Me-globin being added as internal standards for the quantification of adduct levels.

GC-MS analyses were carried out using a Varian 3400 GC linked to a Finnigan 4500 (reduced samples) and a Finnigan TSQ 700 mass spectrometer (unreduced samples) (Finnegan, San Jose, CA USA). The GC column used was a 30 m DB-5 (0.33 mm i.d., 1 μm phase thickness) fused silica column (J&W Scientific Inc., Rancho Cordova, CA USA) and helium was used as carrier gas. The MS instruments were operated in the negative ion chemical ionization (NICI) mode with methane as reagent gas. The ions monitored in reduced samples were for 3-HOPrVal-PFPTH: m/z 362, for EtVal-PFPTH: m/z 351, and for the internal standards, d_3MeVal-PFPTH and d_4HOEtVal-PFPTH: m/z 340 and m/z 352, respectively. The unreduced samples were analyzed using tandem mass spectrometry. Product ions monitored, produced by collision-induced dissociation using argon as collision gas (pressure 0.133 Pa), were as follows (precursor ion given in parentheses): m/z 318

Table 1 Adduct levels (mean ± SE) from acetaldehyde (AA), malonaldehyde (MA) and ethene/ethylene oxide (EO) in selenium (Se)-adequate and Se-deficient rats. Adducts from aldehydes measured after reduction of samples

Experiment	n[a]	AA-adduct (nmol/g)		MA-adduct (nmol/g)		EO-adduct (pmol/g)	
		Se-adequate	Se-deficient	Se-adequate	Se-deficient	Se-adequate	Se-deficient
I	6	6.3 ± 0.8	37.7 ± 3.3***	0.06 ± 0.01	0.09 ± 0.01	20.4 ± 1.4	17.0 ± 1.4
II	4	7.0 ± 1.0	10.6 ± 2.4	0.24 ± 0.03	0.65 ± 0.08**	35.0 ± 3.8	18.4 ± 1.1**
III	4	6.8 ± 0.3	7.2 ± 0.6	0.41 ± 0.01	0.54 ± 0.04*	36.4 ± 5.3	24.3 ± 6.0
IV	2	11.0 ± 3.4	34.6 ± 4.0*	0.18 ± 0.04	0.36 ± 0.02	n.a.[b]	n.a.[b]
V	8	1.7 ± 0.2	1.9 ± 0.2	0.53 ± 0.07	0.66 ± 0.06	23.6 ± 2.5	16.2 ± 0.9*
I–V[c]			$P < 0.005$		$P < 0.005$		$P < 0.001$

[a]n: number of animals/diet group.
[b]not analyzed.
[c]P-values for statistically significant effects of Se-deficiency in combined probability analysis of all experiments.
Statistical significance vs. Se-adequate in the separate studies *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

(m/z 348) for HOEtVal-PFPTH, m/z 320 (m/z 352) for d_4HOEtVal-PFPTH, and m/z 318 (m/z 362) for 2-HOPrVal-PFPTH.

The quantitation of levels of HOEtVal and 2-HOPrVal in unreduced samples was carried out using calibration curves.[15] In reduced samples, the measurements of levels of 3-HOPrVal and EtVal were based on ratios of the ions monitored (cf. above) and corresponding internal standards, and should thus be considered as relative adduct levels.

Statistical analysis

Statistical analyses were carried out using Students *t*-test for the significance in specific experiments. Two techniques were used to evaluate the pooled data from the different experiments: a combined probability analysis[24] and a variance component model.[25]

Results

The Se-dependent GSH-Px activity of liver cytosol in samples from Se-deficient groups of rats was less than 0.01 times that of the Se-adequate groups, thus verifying the efficiency of the dietary regimen (data not shown).

Influence of Se-deficiency on the production of endogenous electrophiles

The influence of Se deficiency on endogenously formed electrophiles such as some aldehydes and epoxides, was measured by means of their adducts to N-terminal valines in Hb. Levels of Hb adducts from aldehydes were measured in reduced (nmol/g Hb) samples and epoxides in unreduced (pmol/g Hb) samples. The levels of adducts from AA, MA, and ethene/EO in Se-deficient and Se-adequate control animals are shown in *Table 1*. Large variations in the adduct levels (particularly with regard to the aldehydes) between the studies were observed. Despite this, the same tendency for Se-dependent differences were observed in each of the five studies and for each of these Hb adducts. A combined probability analysis based on *P*-values in individual experiments indicated significance for higher levels of AA and MA adducts and lower levels of EO adducts in Se-deficient compared to Se-adequate animals (*Table 1*).

The large variation in the effect of Se deficiency studied by a variance-component analysis (*Table 2*) gave *P*-values for this effect and for its interaction with experiment. For the AA-adduct level, this interaction effect is strongly significant and, evidently, an expression of the fact that a marked effect of Se-deficiency was at hand only in Experiments I and IV, whereas in the other experiments, this effect was weak or practically absent (cf. *Table 1*). The analysis was, in principle, two-sided; therefore, $P = 0.09$ for an independent effect of Se may be taken to indicate borderline significance ($P_{one\text{-}sided}$ 0.045). For the MA adduct, there are weak significances for the interaction as well as an independent effect of Se. The effect on the EO-adduct level is highly significant (as given in *Table 1*), considering the nonsignificant interaction effect.

The levels of a few analyzed aldehyde adducts other than AA and MA, that is, those from formaldehyde (mean adduct level ~ 30 nmol/g Hb), glycolaldehyde (~2 nmol/g Hb), methylglyoxal (~1 nmol/g Hb), and hexanal (~0.001 nmol/g Hb) were not affected by Se deficiency (data not shown). The level of Hb adducts from ethene/EO and propene/PO were calculated as the ratio of Se-deficient/Se-adequate and also found to be significantly lower ($P < 0.001$)—about 0.6 for both compounds—in Se deficiency (*Table 3*).

Detoxification rate of EO and PO

Detoxification rates of epoxides were studied by the treatment of rats with a mixture of 1.14 mmol EO and 0.86 mmol PO per kg body weight (50 mg/kg body weight of each compound; *Table 4*), leading to adduct levels about 10^3 times higher than the background level from EO. The adduct levels following administration of epoxides were

Table 2 Hypothesis tests for effect of selenium level considering selenium level/experiment as a random component

Adduct	Effect of selenium level	Interaction selenium-level/experiment
Acetaldehyde	$P = 0.09$	$P < 0.001$
Malonaldehyde	$P = 0.03$	$P = 0.04$
Ethylene oxide (two outliers excluded)	$P = 0.02$	$P = 0.30$

Table 3 Levels of Hb adducts from ethene/ethylene oxide (EO) and from propene/propylene oxide (PO) in selenium-deficient (Se^-) animals compared to selenium-adequate (Se^+) controls. Mean ± SE of five experiments is given. Hb-adduct levels of EO and PO in Se-adequate animals were about 30 pmol/g and 2 pmol/g, respectively

Experiment	Se^-/Se^+ *n*	EO-adducts Ratio Se^-/Se^+	PO-adducts Ratio Se^-/Se^+
I	6/5	0.83	0.55
II	4/4	0.49	0.46
III	4/4	0.61	0.52
V	8/7	0.69	~0.50[a]
VI[b]	4/4	0.66	0.76
I–VI[b]		0.66 ± 0.06***	0.56 ± 0.05***

Statistical significance between Se^- and Se^+ rats with ***$P < 0.001$.
[a]Several values were below the detection limit for Se^- animals due to low analytical sensitivity at the occasion for GC-MS/MS analysis.
[b]Controls to the EO/PO-injected rats according to *Table 4*.

lower (about 0.6) in the Se-deficient than in the Se-adequate rats. These differences were significant for the EO adduct ($P < 0.01$) but not for the PO adduct (*Table 4*). Saline-treated controls showed adduct levels of EO (data not shown) similar to those found in untreated rats according to *Table 1*.

In an additional study, with the aim to study genotoxic end-points in addition to levels of Hb adducts, 2.27 mmol EO/kg body weight was administered intraperitoneally to Se-adequate and Se-deficient rats, with controls receiving saline. In addition to a more than 1,000-fold increase in the level of the EO adduct, the levels of adducts from formaldehyde, AA, and MA were three times higher in these Se-adequate rats treated with EO than in untreated controls (data not shown). This high dose of EO caused the death of all rats in the Se-deficient group. No mortality was noted in the other groups of rats, although Se-adequate rats treated with EO showed obvious signs of toxicity such as inactivity and ruffled fur. Because all EO-treated Se-deficient rats died, no meaningful analysis of genotoxic end-points could be performed.

Discussion

The levels of Hb adducts from aldehydes were found, at least in some of the experiments, to be enhanced in Se-deficient animals, whereas the opposite was valid for Hb adducts from the analyzed epoxides. One characteristic of analysis of Hb adducts is that at a given amount of a compound endogenously formed or absorbed per kg body weight, the dose and the determined adduct level is inversely proportional to the rate of disappearance of the compound. Therefore, changes in the rates of enzymatic detoxification of precursors affect the levels of Hb adducts.

Table 4 Levels (means ± SE for 4 rats) of Hb adducts from ethylene oxide (EO) and propylene oxide (PO) in selenium-adequate (Se^+) and selenium-deficient (Se^-) rats dosed with 50 mg/kg each of EO and PO and sacrificed one day after the treatment

Diet and treatment	EO-adduct (nmol/g Hb)	PO-adduct (nmol/g Hb)
Se^+	46.0 ± 1.9	6.0 ± 3.0
Se^-	28.2 ± 3.0**	3.9 ± 0.6
Ratio Se^-/Se^+	0.61	0.65

Statistical significance between Se^- and Se^+ rats with **$P < 0.01$.

Se deficiency and enzymes associated with the detoxification of xenobiotics

Se deficiency has been shown to change the levels of several enzymes, among which are loss of Se-dependent GSH-Px activity and less dramatic changes in the activities of at least 15 other enzymes known to play a role in the detoxification of xenobiotics.[26,27] Among enzyme changes in Se deficiency are the enhanced activities of glutathione S-transferase (GST) isoenzymes. In line with these enzymatic changes is the rule of thumb that the toxicity of compounds that are metabolized to form free radicals often increases in Se deficiency, whereas the opposite is common for compounds detoxified by GST conjugation.[28]

In addition to the conjugation reactions, all rat GST isoenzymes seem to possess the capacity of catalyzing the destruction of organic peroxides[29] first described as a non-Se-dependent GSH-Px activity[30]. The induction of GST in Se deficiency might therefore compensate for some of the loss of GSH-Px. The effects of Se deficiency on GST is important in the present context because some precursors of the Hb adducts studied here are partly detoxified by GST.[31]

In the study by Olsson et al.,[27] which was comparable to the present study regarding rat strain and diet regimens, the level of hepatic cytosolic GSH-Px decreased to less than 1% of the control values in Se-deficient rats. A similar decrease in GSH-Px activity of Se-deficient rats was found in the present study. For these reasons, it could be assumed that effects of Se deficiency on several other enzyme activities, as reported earlier,[27] were at hand in the present study as well. It was thus reported that the activity of cytosolic GST increased 3-fold due to Se deficiency when measured with 1-chloro-2,4-dinitrobenzene as the substrate. Furthermore, enzyme activities of microsomal and mitochondrial epoxide hydrolases as well as microsomal GST were shown to be significantly increased in Se-deficient rats.[27]

Aldehydes

The levels of AA and MA adducts were significantly increased in Se-deficient rats (*Tables 1 and 2*). Many aldehydes are formed as products of lipid peroxidation, MA being one of the major compounds.[7] Lipid peroxidation has been shown to be induced by treatment with different toxins (e.g., paraquat[32] and choral hydrate or trichloroacetic acid[33]). Such treatments result in highly elevated levels of MA, formaldehyde, and AA;[32,33] that is, the aldehydes discussed in the present study. Aldehydes such as AA and MA are detoxified by liver cytosolic and mitochondrial aldehyde dehydrogenases (ALDH)[34,35] and the cytosolic ALDH increases significantly in Se deficiency.[5] No increases of lipid peroxidation, as such, have been found in Se deficiency when measured as thiobarbituric acid-reactive

substances in liver samples from rats.[5] However, combined Se and vitamin E deficiency or vitamin E deficiency alone has been shown to lead to increased levels of both thiobarbituric acid reactive substances and free MA.[5] By using the more sensitive analysis of Hb adducts, we were able to detect increased lipid peroxidation in single deficiency of Se. For unknown reasons, the level of the AA adduct to Hb varied significantly between the experiments, and therefore lowered the significance for a Se-dependent difference when tested in a variance component model. The differences between experiments with respect to means of recorded adduct levels, particularly of AA and MA, might partly arise from a variation in response factors in the analysis by GC-MS NICI.

Epoxides

The measured background level of adducts from endogenously formed EO and PO (*Tables 1–3*) and adducts formed in response to in vivo treatment with EO and PO (*Table 4*) showed approximately the same influence of Se deficiency; that is, adduct levels about 0.6 times that of Se-adequate rats. The lower background levels of EO and PO in Se deficiency was thus probably due to increased rate of detoxification, meaning that the production of these epoxides is little changed in Se deficiency. These conclusions are strengthened by the fact that EO is detoxified by epoxide hydrolases and by enzymatic conjugation to GSH.[10] The latter detoxification pathway seems to be the major pathway, as GST has been shown to be induced on repeated exposure to EO[36] and, further, that chronic and acute exposure to EO has been found to deplete GSH.[36,37]

Therefore, the activities of GST, epoxide hydrolase, and possibly some other enzymes induced in the Se-deficient rats could be expected to enhance the rate of detoxification and thus lead to reduced doses of EO and PO and of corresponding level of adducts to Hb (*Tables 1–4*).

An unexpected mortality was found in the Se-deficient group after administration of a high dose of EO, in spite of a presumably occurring increase of GST and epoxide hydrolase activities and thus a better capacity to detoxify EO. Se-adequate rats treated with EO showed a highly enhanced adduct level of EO and, in addition, about a 3-fold increase in the adduct levels from formaldehyde, AA, and MA compared to untreated controls, thus indicating that the high dose of EO induced lipid peroxidation. Enhanced lipid peroxidation has previously been reported in the liver membrane fraction of rats treated with EO.[37] The combined effect of the administered epoxide dose and the increases of aldehyde levels, possibly due to peroxidations, was the probable cause of the death of Se-deficient rats.

Overview and conclusions

Se deficiency should be expected to enhance lipid peroxidation due to the lack of the Se-dependent GSH-Px enzyme known to remove hydrogen peroxide and organic peroxides. Such relations have, however, been difficult to verify,[4–6] probably mainly due to the fact that Se deficiency leads to enhanced activities of several enzymes involved in detoxification mechanisms.[26,27] The present findings regarding altered levels of adducts formed from endogenous aldehydes and epoxides in Se-deficient rats are most probably influenced by induction of enzyme activities other than GSH-Px, as discussed above.

From these and earlier[27] results, one may ask whether it might be better to use animals with less severe Se deficiency in studies on the protective role of Se, and thereby avoid the cascade of inductions of enzymes involved in detoxification mechanisms. This is quite possible, as could be deduced from the results of Reiter and Wendel.[26] They found that the Se-GSH-Px activity in the liver of Se-deficient mice (0.01 mg Se/kg diet) fell to 0.01 times that of mice fed a Se-adequate diet (0.5 mg Se/kg diet). In the low-Se group (0.05 mg Se/kg diet) the Se-GSH-Px activity was reduced to just 0.16 times that of Se-adequate mice. Most interestingly, 13 of the other 20 studied enzyme activities were significantly changed in the Se-deficient mice but not in the low-Se group.[26]

In conclusion, the present study demonstrates that Se deficiency, as such, leads to changes in the formation and/or detoxification of some endogenous electrophiles in rats. Furthermore, the method chosen to determine these compounds (i.e., the analysis of their adducts to N-terminal valines in Hb) is sensitive enough to detect the variations in their production due to feeding a Se-deficient diet. Also, the results indicate that ethene/EO (and also propene/PO) is a less useful indicator of peroxidation than malonaldehyde.

Acknowledgements

Ms. Anna-Lena Magnusson, Ms. Eden Tareke, Fil. Lic., and Mr. Vlado Zorzec are gratefully acknowledged for skillful technical assistance. Thanks are due to Professor Lars Ehrenberg for critical reading of the manuscript and for stimulating discussions, and to Fredrik Granath for the statistical variance component analysis of data. This study was supported financially by the Swedish Environmental Protection Agency and the Swedish Cancer Fund.

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