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Antioxidant activity of two edible isothiocyanates: Sulforaphane and erucin is due to their thermal decomposition to sulfenic acids and methylsulfinyl radicals

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ABSTRACT

Sulforaphane (SFN) and erucin (ERN) are isothiocyanates (ITCs) bearing, respectively, methylsulfinyl and methylsulfanyl groups. Their chemopreventive and anticancer activity is attributed to ability to modulate cellular redox status due to induction of *Phase 2* cytoprotective enzymes (indirect antioxidant action) but many attempts to connect the bioactivity of ITCs with their radical trapping activity failed. Both ITCs are evolved from their glucosinolates during food processing of *Cruciferous* vegetables, therefore, we studied antioxidant behaviour of SFN/ERN at elevated temperature in two lipid systems. Neither ERN nor SFN inhibit the oxidation of bulk linolenic acid (below 100 °C) but both ITCs increase oxidative stability of soy lecithin (above 150 °C). On the basis of GC-MS analysis we verified our preliminary hypothesis (*Antioxidants* **2020**, 9, 1090) about participation of sulfenic acids and methylsulfinyl radicals as radical trapping agents responsible for the antioxidant effect of edible ITCs during thermal oxidation of lipids at elevated temperatures (above 140 °C).

1. Introduction

Isothiocyanates (ITCs), the organic compounds with –N═C═S group, are sulfur analogues of isocyanates (R-NCO) and isomers of thiocyanates (R-SCN). Numerous natural ITCs can be found in vegetables and due to their specific odour and taste they formerly were called mustard oils. These compounds are present in plants in the form of glucosinolates, ie., metabolites with S-β-D-glucopyrano unit anomerically connected to an O-sulfated (Z)-thiohydroximate function, see Scheme 1 (Blažević et al., 2020; Fahey, Zalcmann, & Talalay, 2001; Fenwick, Heaney, Mullin, & VanEtten, 1983). During cutting, slicing, or grinding, they are enzymatically transformed giving a gentle, specific flavor and taste. Formation of ITCs from glucosinolates occurs in the presence of myrosinase (EC 3.2.3.1.147), a glycoprotein present in plant tissues, and proceeds via Lossen rearrangement (Hayes, Kelleher, & Eggleston, 2008). Recently, the great attention has been paid to naturally-occurring ITCs with methylsulfinyl and methylsulfanyl moieties, namely sulforaphane (4-methylsulfinylbutyl isothiocyanate, SFN) and erucin (4-methylthiobutyl isothiocyanate, ERN) (Dinkova-Kostova & Kostov, 2012; Fahey et al., 2001; Palliyaguru, Yuan, Kensler, & Fahey, 2018; Posner, Cho, Green, Zhang, & Talalay, 1994; Yuanfeng et al., 2020; Zhang, Talalay, Cho, & Posner, 1992), see Scheme 1B. Both ITCs are the components of our diet when consuming common *Cruciferous* vegetables (Blažević et al., 2020; Fahey et al., 2001; Fenwick et al., 1983): SFN is present in broccoli, some cultivars of cabbage and Brussels sprouts while ERN is predominantly found in rocket (*Eruca vesicaria*) (Azarenko, Jordan, & Wilson, 2014; Fahey et al., 2001; Fahey, Zhang, & Talalay, 1997; Fenwick et al., 1983).

SFN and ERN are promising compounds for the use in medicines, food supplements and cosmetics. Some dietary supplements contain glucoraphanin with active myrosinase or stabilized "free" SFN (Avmacol®, TrueBroc®, and Prostaphane®) and a few cosmetics with SFN are accessible on the market, with the names relevant to broccoli and sulforaphane (BroccoFusion® Sulforaphane Lotion, Broccoli serum and CBD/Sulforaphane serum). SFN is also being tested as a potential drug in about 70 clinical trials (according to clinicaltrials.gov., date: 24.07.2020). Medical applications (eg., treating autism, psoriasis, and bladder cancer) and cosmetic applications of ITCs are subject of

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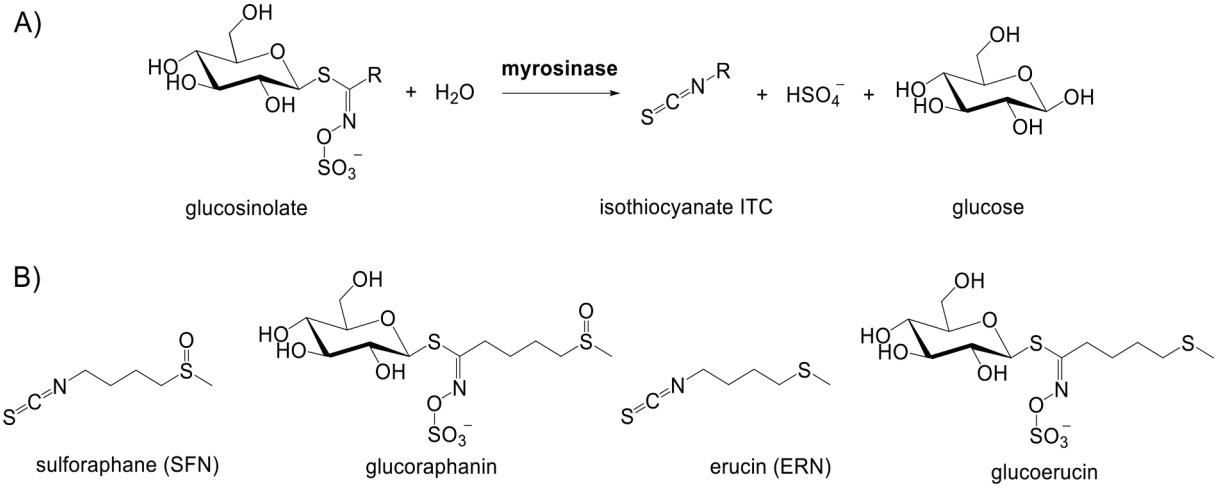
numerous patents. Moreover, ERN is listed in the flavor library of Flavor Extract Manufacturers Association (FEMA) as cabbage flavor (no. 4414).

Talalay and co-workers (Fahey et al., 1997; Posner et al., 1994; Zhang et al., 1992) discovered and documented that among natural ITCs sulforaphane is the most potent inducer of carcinogen detoxification enzymes (eg., quinone reductase, glutathione-S-transferase, heme oxygenase) and SFN is one of the most potent phytochemical with the inducing activity of cytoprotective enzymes (Fahey et al., 1997; Yagishita, Fahey, Dinkova-Kostova, & Kensler, 2019; Zhang et al., 1992). ITCs are considered as chemopreventive agents (Dinkova-Kostova & Kostov, 2012; Hayes et al., 2008; Zhang et al., 1992) due to their role in the induction *Phase 2* enzymes of the xenobiotic metabolism (this process involves Nrf2 signaling) and inhibition of *Phase 1* enzymes. SFN or ERN are also recognized as inducing some apoptotic pathways, anti-inflammatory effects, inhibition of angiogenesis, and their H_2S-donating ability might be important in redox homeostasis (Cho, Lee, & Park, 2013; Citi et al., 2019; Houghton, 2019; Melchini & Traka, 2010). Health benefits of dietary intake of SFN include prevention of different types of cancer (eg., prostate, bladder and lung cancer), asthma, schizophrenia, neurodegenerative diseases (such as Alzheimer and Parkinson diseases), diabetes, cardiovascular diseases (CVDs), chronic obstructive pulmonary disease (COPD) or autistic spectrum disorders (ASDs) (Houghton, 2019; Tarozzi et al., 2013; Yagishita et al., 2019). ERN, a less extensively studied analogue of SFN, also have a beneficial impact on human health, being a potential chemopreventive and anti-cancer agent (Azarenko et al., 2014; Citi et al., 2019; Melchini & Traka, 2010; Posner et al., 1994).

Health effects of ITCs have been connected with their ability to modulate cellular redox status due to induction of *Phase 2* cytoprotective enzymes (Dinkova-Kostova and Talalay, 2008). As reviewed by Valgimigli and Iori (Valgimigli & Iori, 2009), there is a general agreement that the enhancement of antiradical defense system is not due to a direct scavenging of radicals and such effect has been named the indirect antioxidant action of ITC. Indeed, the attempts to demonstrate that ITCs are capable to effectively react with reactive oxygen or nitrogen species (ROS, RNS) and prevent the start of peroxidation were only partially successful. Barillari *et al.* showed that glucoerucin (the parent glucosinolate of ERN, see Scheme 1B) efficiently decomposes hydrogen peroxide and *tert*-butylperoxide with the rate constants 3.3×10^{-2} $M^{-1}s^{-1}$ and 2.0×10^{-3} $M^{-1}s^{-1}$ at 25 °C, respectively, proving that glucoerucin is a preventive antioxidant (Barillari et al., 2005), and they suggested that ERN also should be a preventive antioxidant. Experiments with autoxidation of styrene demonstrated that ERN and SFN react too slowly with peroxyl radicals and neither ERN nor SFN are radical-trapping (chain-breaking) antioxidants (Barillari, et al., 2005; Papi et al., 2008). Other reports indicate that SFN and ERN react with hydroxyl radicals ($^{\bullet}OH$) and also can scavenge artificial model radicals, like 2,2′-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) or 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$) (Ligen et al., 2017; Montaut et al., 2017; Yuan, Yao, You, Xiao, & You, 2010). However, the results of $^{\bullet}OH$, $DPPH^{\bullet}$, and $ABTS^{+\bullet}$ assays give the information about overall reducing ability of a tested compound toward particular ROS/RNS (Amorati & Valgimigli, 2015), not about the ability to inhibit peroxidation of organic materials or biomolecules. The conclusion from literature search is that SFN and ERN are not radical trapping antioxidants at ambient temperature, however, there is no information about their chain-breaking activity at temperatures higher than 40 °C. As ITCs are present in food that is subjected to mechanic operations or/and thermal operations like boiling or frying, there is a need to get knowledge about thermal behaviour of this class of compounds at elevated temperature, at conditions similar to those in standard cooking in the presence of lipids as the constituents most sensitive to oxidation (Yuanfeng et al., 2020). In our very recent communication we put the hypothesis that ITCs can act as antioxidant agents during high temperature processing of food (Cedrowski, Dąbrowa, Krogul-Sobczak, & Litwinienko, 2020). In order to confirm the hypothesis, we checked the oxidative stability of two model lipids of different oxidative stability, we also performed chromatographic analysis of decomposition products in order to explain the mechanism of the antioxidant action.

In our former studies we elaborated fast and convenient methodology of measurement of oxidative stability of fats and oil by using differential scanning calorimetry (DSC) in non-isothermal mode, with 5–10 mg samples heated with linear heating rate (β) and recorded heat effect of oxidation (Litwinienko, 2001; Litwinienko, Daniluk, & Kasprzycka-Guttman, 2000; Litwinienko & Kasprzycka-Guttman, 1998a,c, 2000). The methodology of measurements, typical for studies of oxidative stability of polymers and biopolymers (Czochara, Kusio, & Litwinienko, 2017; Ziaja, Jodko-Piorecka, Kuzmicz, & Litwinienko, 2012) can be also applied for food, as alternative to isothermal accelerated tests (isothermal conditions at 100–140 °C, detection of polar volatile products of oxidation and decomposition of the hydroperoxides) as, for example, the oxidative stability index (OSI) or the Rancimat method (RM). Recently, a new methodology of oximetric measurement of the rate of lipid oxidation has been introduced based on the monitoring of the oxygen consumption during high temperature oxidation by means of the fluorescence probe sensing oxygen in head space (Mollica et al., 2020), and this method has great advantages because, unlike OSI and RM, it monitors the oxygen consumption instead of detecting



Scheme 1. A) Reaction scheme of ITC formation during hydrolysis of glucosinolate catalysed by myrosinase. B) Structures of sulforaphane, erucin and their corresponding glucosinolates.

secondary products of oxidation. However, DSC non-isothermal method brings some additional advantages, the samples are small, thermal effect of oxidation is interpreted as due to formation of hydroperoxides (primary products of oxidation) (Litwinienko & Kasprzycka-Guttman, 1998a) and, depending on β, the single run takes 10–30 min comparing to several hours in OSI or RM.

In this report we used DSC to observe the effects of SFN and ERN on the thermal behaviour of linolenic acid (LNA) and soy lecithin (SL) under non-isothermal oxidation conditions. LNA is an example of essential fatty acid and this polyunsaturated component of lipids is one of the most sensitive toward peroxidation. LNA undergoes relatively fast oxidation at elevated temperature, giving a clear and strong thermal effect in DSC. On the other hand, soy lecithin is an important component of a great number of food products and also neutraceutical and food supplement ingredient with many health benefits (List, 2015). Due to lipophilic/hydrophilic character, lecithin is frequently employed as a very good emulsifying agent but there are a number of other applications, and some of them are connected with high temperature operations, lecithins are used as release agents and as wetting agents in industrial bakery and confectionery and also used as pan (also mold and belt) release agents, product separation aids (nonsticking aids in sliced food), and other applications based on heat resistance and surface coating feature of lecithin (List, 2015).

The presented work is an extension of the short communication in which we described that SFN and ERN inhibit oxidation of sunflower oil but do not inhibit oxidation of linolenic acid (Cedrowski, et al., 2020). Here, we compare the kinetics of oxidation of LNA and SL as two lipids of different oxidative stability and dramatically different polarity and both are used for high temperature operations with vegetables containing isothiocyanates, therefore, we decided to use both lipid matrices in our calorimetric studies of the antioxidant effect of SFN and ERN.

2. Materials and methods

2.1. Materials

Detailed description of all chemicals, reagents, synthesis of ERN and SFN, equipment (NMR, GC–MS) and experimental procedures (DSC, preparation of samples) is included in the Supplementary Material. Linolenic acid (98%, *all-cis*-9,12,15-Octadecatrienoic acid, LNA, Sigma-Aldrich) and soy lecithin (95%, SL, MP Biomedicals LLC) were used as lipid matrices. The main constituents of SL as declared by the producer (certified analysis for catalog no. 102147, in %w): phosphatidylcholine (25%); phosphatidylethanolamine (22%); phosphatidylinositol (16%); phosphatidic acid (7%). Linoleic acid (*cis-cis*-9–12-octadecadienoic acid) stands for 70% of fatty acid residues in SL.

2.2. Synthesis of erucin and sulforaphane

The target compounds ERN and racemic SFN were prepared by using method originally proposed by Schmidt and Karrer (Schmid and Karrer, 1948) and modified by us. Full description of procedures, yields, identification by ^{1}H and ^{13}C NMR is provided in Supplementary Material. Here, the symbols **S1**-**S5** denote the compounds in Scheme S1. Potassium phthalimide (**S1**) and 1,4-dibromobutane (**S2**) were reacted in the presence of a catalytic amount of tetrabutylammonium bromide (TBABr) giving 1-phthalimido-4-bromobutane (**S3**, yield 77%). This intermediate was reacted with sodium thiomethoxide (prepared from dimethyl disulfide and sodium) giving 1-phthalimido-4-methylthiobutane (**S4a**) in very good yield (93%). Compound **S4a** was partially oxidized using proper amount of H_2O_2 in glacial acetic acid providing sulfoxide (**S4b**, yield 80%). Next, phtalimide group was deprotected using hydrazine hydrate in refluxing ethanol providing amines **S5a-b** in moderate to good yields (49–81%). The crude amines were reacted without purification with thiophosgene ($CSCl_2$) in a biphasic system (10% $NaHCO_3$/DCM) providing the target products (55–70%). The products were analyzed and the obtained NMR signals were identical with the literature data for natural and synthetic SFN and ERN (Ganin et al., 2013), see Supplementary Material.

2.3. Kinetic measurements

Preparation of lipid samples containing ERN and SFN for DSC analysis is described in the Supplementary Material. The final concentrations of ITCs in samples were 0.17–2.0 mg ITC /g of LNA and 0.2–2.3 mg of ITC/g of SL. The methodology of DSC measurements has been described elsewhere (Czochara et al., 2017; Czochara, Kusio, Symonowicz, & Litwinienko, 2016; Ulkowski, Musialik, & Litwinienko, 2005) and is detailed in the Supplementary Material. The calorimetric measurements were carried out using a DSC apparatus Du Pont 910 differential scanning calorimeter with Du Pont 9900 thermal analyzer and a normal pressure cell, under oxygen flow 6 dm^3/min. 3–5 mg samples were heated from 40 to 200 °C in open pan with linear heating rate β (2.5–20 K/min). The way of determination of kinetic parameters from series of DSC curves is discussed in section 3.1.

2.4. GC–MS measurements

The procedure for thermal decomposition of ITCs and GC–MS measurements of the products is described below. Samples of ITC (1–2 mg) were placed (under air) in ~ 2 ml glass ampoule along with a drop of water and sealed. The ampoule with the sample was heated at 100 °C and 160 °C for 5 h and 30 min respectively. The reaction mixture was then cooled down to −70 °C (dry ice/acetone bath), the head of ampoule was broken and the content was dissolved in 500 μl of methylene chloride (DCM). The solution was moved to vial containing 1 ml of water. The DCM layer phase was subjected to GC–MS analysis directly. GC–MS analysis was performed with HP5 capillary column (0.32 mm diameter, 30 m length, 0.25 μm thickness, Hewlett-Packard) with GC-17A Ver.3 Shimadzu and GCMS-QP5050A Shimadzu mass detector. Mass spectrum was obtained by electron impact ionization mode, scanning from 33 *m/z* to 350 *m/z*. The flow rate of helium gas was 1.5 ml/min, and the split ratio was 5:1. Inlet temperature was 210 °C and oven temperature program was as follows: an initial step starts at 40 °C (isothermal for 5 min); raising with the rate 3 °C/min to 220 °C.

3. Results and discussion

3.1. Kinetic parameters for oxidation of pure linolenic acid (LNA) and soy lecithin (SL)

Fig. 1 presents DSC curves of oxidation obtained for heating rates β ranging from 2.5 to 20 K/min for LNA and 5–20 K/min for SL (for β < 5 K/min thermal effect is extended during long time of experiment and DSC curve is much flatter than the curves obtained for bigger β). Linolenic acid (C18:3) is polyunsaturated fatty acid with two *bis*-allyl C—H bonds and is one of the most sensitive toward oxidation. Thermal effect of LNA oxidation is clear, with start of oxidation at temperature 90–120 °C (depending on β). In contrast, soy lecithin is more stable, with linoleic acid (C18:2) residues as the main fatty acid represented, thus, the oxidation of SL starts at temperatures ca. 150 °C, and, as a consequence of higher temperature, a shape of DSC curve is more sheer (compare Fig. 1A and B). Non inhibited oxidation of LNA and SL was carefully discussed and interpreted in our previous publication (Ulkowski et al., 2005), so, a brief information will be presented here. The observed thermal effect is a consequence of formation of hydroperoxides as primary products of oxidation. Hydroperoxides decompose at temperatures above 200 °C forming second peak on DSC curve (not shown on the plots in Fig. 1, see DSC at temp. range 50–350 °C in our previous work (Ulkowski et al., 2005)).

For low degrees of conversion α (low extent of reaction) and at oxygen pressure bigger than 13 kP the autoxidation can be described as a

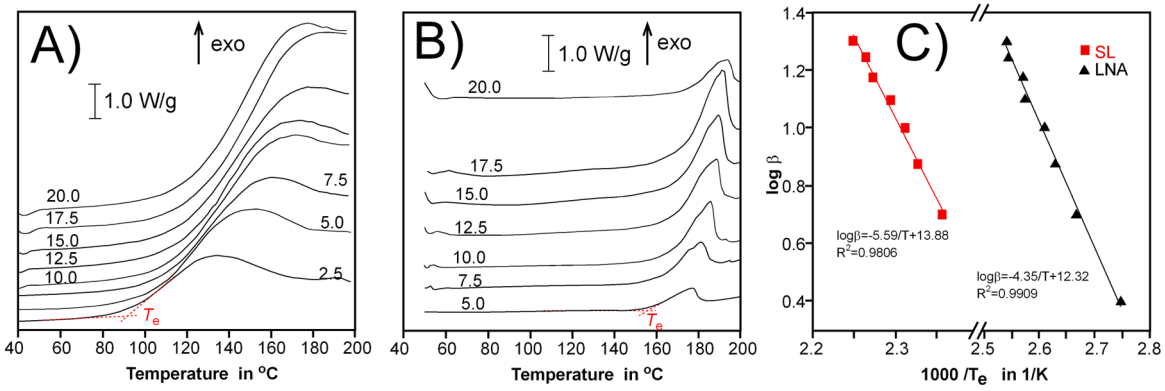


Fig. 1. DSC curves obtained during thermoxidation of linolenic acid (LNA) shown in panel A, and soy lecithin (SL) shown in panel B. The number above each thermal curve is the heating rate (β in K/min). T_e is extrapolated start of oxidation. The curves have been vertically shifted for better visual clarity. Panel C: plots of logβ versus $10^3/T_e$ for LNA and SL constructed on the basis of T_e values determined from DSC curves.

Table 1
Temperatures of extrapolated start of oxidation (T_e) of linolenic acid and soy lecithin obtained for experiments with various heating rates (β), parameters *a* and *b* of equation (4) with standard deviations for the slope (σ and $\sigma_{90\%}$)[a], square regression coefficients (R^2), and calculated kinetic parameters: overall activation energy E_a, pre-exponential factor *Z*, and overall rate constant of oxidation (*k*) calculated for 100 °C and 150 °C.

Linolenic acid (LNA)				**Soy lecithin (SL)**			
β [K/min]	T_e [°C]	kinetic and statistical parameters		β [K/min]	T_e [°C]	kinetic and statistical parameters	
2.5	90.8	*a*=	−4.3468			*a*=	−5.5859
5.0	101.8	*b*=	12.3207	5.0	151.2	*b*=	13.8830
7.5	107.1	R^2=	0.9922	7.5	156.5	R^2=	0.9891
10.0	110.0	σ =	0.16	10.0	159.4	σ =	0.262
12.5	115.2	$\sigma_{90\%}$=	0.29	12.5	162.7	$\sigma_{90\%}$=	0.487
15.0	115.8	E_a=	79 ± 5 kJ/mol	15.0	166.8	E_a=	102 ± 9 kJ/mol
17.5	120.0	*Z*=	4.54×10^{10} min^{-1}	17.5	168.4	*Z*=	1.29×10^{12} min^{-1}
20.0	120.3	$k_{100°C}$ =	0.38 min^{-1}	20.0	171.6	$k_{100°C}$ =	7.49×10^{-3} min^{-1}
		$k_{150°C}$ =	7.7 min^{-1}			$k_{150°C}$ =	0.36 min^{-1}

[a] Standard deviations (σ) and errors calculated for confidence level 90% ($\sigma_{90\%}$).

first order process with the rate law depending on the amount of lipid, LH:

$$v = k_p\left(\frac{R_i}{2k_t}\right)^{1/2}[LH] = k[LH] \quad (1)$$

where the rate of initiation (R_i) together with the rate constant for propagation (k_p) and termination (k_t) can be considered as the overall rate constant $k = k_p(R_i/2k_t)^{1/2}$, thus, the chain process of oxidation can be described by the Arrhenius equation:

$$k(T) = Z\exp\left(\frac{-E_a}{RT}\right) \quad (2)$$

where *Z* is a pre-exponential factor, E_a is activation energy, *R* is the gas constant. During non-isothermal experiment the starting temperature of the sample (T_0) is increased to $T = T_0 + \beta\tau$ with a linear heating rate $\beta = dT/d\tau$ (τ is time). The amount of heat (H) released per time unit (or temperature unit) corresponds to the reaction rate *v*, and can be expressed in term of conversion ($dH/d\tau = v = d\alpha/d\tau = 1/\beta\, d\alpha/dT$), thus, equation (1) defines the rate as a change of conversion:

$$dH/d\tau = d\alpha/d\tau = \beta d\alpha/d\tau = k[LH] = k(1-\alpha) \quad (3)$$

So called isoconversional methods of calculation of kinetic parameters are based on combination of eq. (2) and (3).

Ozawa and, independently, Flynn and Wall proposed a relatively simple way for determination of the kinetic parameters from non-isothermal experiments, nowadays this methodology, named Ozawa-Flynn-Wall method, is one of the most frequently used method in thermal analysis (Flynn and Wall, 1966; Ozawa, 1970). As can be seen in Fig. 1A and 1B, the higher β the higher temperature of start of oxidation (T_e, see Table 1). T_e values correspond to a constant conversion (α = const) and for series of experiments with different β, the straight line dependence of logβ values plotted versus $1/T_e$ (expressed in kelvins) is obtained (see Fig. 1C):

$$\log\beta = \frac{a}{T_e} + b \quad (4)$$

with the slope $a = -0.4567\, E_a/R$ and reciprocal b = -2.315 + log (ZE_a/R), with $R = 8.314$ J mol^{-1} K^{-1} (Litwinienko et al., 2000).

3.2. Kinetic parameters for oxidation of linolenic acid and soy lecithin with ITCs

DSC curves presented in Fig. 1A and 1B indicate that LNA is more sensitive to oxidation (lower T_e) and the kinetic parameters calculated from DSC curves listed in Table 1 confirm this observation. The activation energy for LNA oxidation is in reasonable agreement with determined in our previous works, ranging from 65 to 78 kJ/mol (Czochara et al., 2016; Ulkowski et al., 2005) and E_a for oxidation of soy lecithin is in perfect agreement with 97 ± 8 kJ/mol (Ulkowski et al., 2005). The rate constants clearly demonstrate that below 150 °C oxidation of LNA is faster (at 100 °C is 50 times faster than the oxidation of SL). Therefore, we have two materials with different oxidative stabilities which can be used for assessment of the antioxidant behaviour of ITCs at two different thermal conditions. Choice of more than one lipid model is an important aspect during screening the antioxidant activity of a compound because our previous work with fullerene C_{60} and its derivatives revealed that antioxidant activity depends on the nature of the

oxidized material: the same compound can behave as non-inhibitor in unsaturated hydrocarbons but it turns into an effective antioxidant during oxidation of saturated hydrocarbons (like stearic acid, polymers etc.) at higher temperatures (Czochara, Grajda, Kusio, & Litwinienko, 2018; Czochara, Kusio, & Litwinienko, 2017, 2019; Czochara et al., 2016). There are several reasons for such behaviour, as, for example, different mechanism of high temperature oxidation (oxidation of polymers might be mediated by alkoxyl instead of peroxyl radicals) or not sufficient ratio k_{inh}/k_p, where k_{inh} is the rate constant for reaction of inhibitor with the propagating radicals $Y^{\bullet}$ (reaction (5)).

$$\text{Inhibitor} + Y\cdot \rightarrow \text{non radical or stable radical}; \quad k_{inh} \tag{5}$$

Usually reaction (5) is reduction of radical $Y^{\bullet}$ to Y-H or addition of $Y^{\bullet}$ to the inhibitor. Considering the large stoichiometric excess of oxidized substrate (neat compounds or at molar concentrations) over the amount of antioxidant (micromolar concentrations), k_{inh} should be 10^3 times larger than k_p to effectively suppress the autoxidation. Consequently, in order to keep the proper ratio $k_{inh}/k_p > 1000$, the moderate inhibitor will effectively break the propagation chain during oxidation of a material with relatively high oxidative stability (small k_p), otherwise, the propagation chain will not be sufficiently broken and the inhibiting effect will not be observed.

Addition of an inhibitor should increase the oxidative stability (lag phase, inhibition phase) and during non-isothermal oxidation such lag phase should be manifested as an increasing temperature when the oxidation starts (Litwinienko & Kasprzycka-Guttman, 1998b; Litwinienko, Kasprzycka-Guttman, & Studzinski, 1997; Musialik & Litwinienko, 2007). Table S15 (Supplementary Material) presents the temperatures T_e (for $\beta = 5$ K/min) determined for oxidation of LNA and SL containing various concentrations of ITCs. Unfortunately, the addition of the studied compounds at any concentration within the range 0.17–2.0 mg /g of lipid (corresponding to three concentrations: 1 mM, 5 mM, and 10 mM) did not caused any increase of oxidative stability of LNA and even the opposite effect is observed, oxidation of LNA containing ITCs starts at lower temperatures than T_e for neat LNA (T_e is lower in the range 2.8–4.1 °C for the lowest concentrations of ITCs, and for the highest concentrations of SFN and ERN a decrease of T_e is ca. 11.5 °C, see Table S15). Moreover, addition of ITCs to SL also did not improve the oxidative stability of this lipid. In other accelerated (isothermal) tests like Rancimat and OSI, such results would be concluded as no antioxidant effect (and apparent pro-oxidative behaviour would be observed). However, methodology used in DSC allows to calculate the kinetic parameters that are more informative and can be used for prediction of the oxidative stability of the samples in other temperatures, including temperature before the rapid, spontaneous oxidation starts (corresponding to lag phase in isothermal experiments). Table 2 collects E_a, Z and k parameters determined for thermo-oxidation of LNA and SL containing ITCs. The results for LNA confirm the first observations from Table S15: isothiocyanates do not improve the oxidative stability of polyunsaturated lipid, in contrast to conventional phenolic antioxidants like 2,6-di-*tert*-butyl-4-methylphenol (BHT) or tocopherol (Litwinienko & Kasprzycka-Guttman, 1998b; Litwinienko, Kasprzycka-Guttman, & Jamanek, 1999, 1997; Musialik & Litwinienko, 2007).

Table 2
The values of the apparent activation energy (E_a), pre-exponential factor Z, and overall rate constant of oxidation (k) calculated for 100 and 150 °C for oxidation of linolenic acid (LNA) and soy lecithin (SL) containing two isothiocyanates (ITC) at concentrations 0.17–2.0 mg ITC /g LNA and 0.21–2.3 mg ITC /g SL.

C_{ITC} [mg/g]	E_a [kJ/mol]	Z [min^{-1}]	k (100 °C) [min^{-1}]	k (150 °C) [min^{-1}]
pure LNA	**79 ± 5**	**4.5×10^{10}**	**0.38**	**7.73**
sulforaphane				
0.20	71 ± 5	3.3×10^{9}	0.42	6.2
1.0	79 ± 6	5.0×10^{10}	0.45	9.1
2.0	91 ± 6	4.5×10^{12}	0.97	30
erucin				
0.17	90 ± 4	1.5×10^{12}	0.45	14
0.85	86 ± 7	6.6×10^{11}	0.62	16
1.7	98 ± 5	6.4×10^{13}	1.1	45
pure SL	**102 ± 9**	**1.3×10^{12}**	**7.5×10^{-3}**	**0.36**
sulforaphane				
0.23	114 ± 12	4.9×10^{13}	5.3×10^{-3}	0.41
1.2	129 ± 12	2.4×10^{15}	2.2×10^{-3}	0.30
2.3	92 ± 12	8.0×10^{10}	1.2×10^{-2}	0.39
erucin				
0.21	112 ± 10	2.2×10^{13}	5.2×10^{-3}	0.37
1.1	130 ± 16	4.1×10^{15}	2.9×10^{-3}	0.41
2.1	160 ± 11	2.4×10^{19}	9.1×10^{-4}	0.41

However, the experiments for thermal oxidation of soy lecithin containing ITCs resulted in the kinetic parameters which differ from the ones obtained for the oxidation of SL without additives. The same Table 2 presents the E_a, Z and k values for three different concentrations 0.21–2.3 mg ITC/g SL (corresponding to 1, 5, and 10 milimoles of ITC per mol of lipid, we used here mg/g units instead of molar concentration because SL is solid). For each compound an optimal concentration can be found at which the lipid is protected against the oxidation. Taking into account the activation energies, a small increase is observed for sulforaphane (present at concentration 1.2 mg SFN/g SL) that, combining with the increase of pre-exponential factor, gives significant decrease of k calculated for 100 °C. Other two concentrations of SFN are not effective, the small concentration 0.23 mg/g is not significant, while ten-fold bigger amount, $C_{SFN} = 2.3$ mg/g, generates pro-oxidative effect (at 100 °C the oxidation is twice faster than oxidation of pure SL), see Fig. 2.

ERN at the smallest concentration (0.21 mg per g of SL) is also not effective, but for five-fold and ten-fold bigger concentrations of ERN the increased values of E_a and Z were determined, giving smaller rate constants for temperatures below 150 °C, see Table 2. For the middle concentration (1.1 mg ERN/g) the rate of oxidation at 100 °C is reduced to half of the value obtained for pure SL. This tendency is also kept for ERN present at concentration 2.1 mg/g: the activation energy increased to 160 kJ/mol and the rate constants calculated for 100 °C is almost 10-times smaller. The effect of addition of ERN and SFN on the kinetics of oxidation of soy lecithin confirms our very recent observation for oxidation of sunflower oil (Cedrowski, et al., 2020): E_a for oxidation of the pure oil was 103 ± 4 kJ/mol and increased to 119 ± 8 kJ/mol in the presence of 10 mM ERN or 5 mM SFN, which corresponds to increased resistance towards oxidation (k calculated at 100 °C ie., during the lag phase for oxidation of pure sunflower oil was 5.8×10^{-3} min^{-1} but in the presence of 10 mM ERN was almost two-fold smaller.

3.3. Plausible mechanism(s) of antioxidant action of ITCs at higher temperature

Oxidation of LNA was inhibited neither by SFN nor ERN but the same compounds inhibited oxidation of SL (the lipid undergoing oxidation at higher temperatures). We exclude the possibility that ITCs could be activated by the products of SL decomposition because thermogravimetric and calorimetric studies of soy lecithin made by Ross and coworkers (Ross, Lemay, & Takacs, 1985) demonstrated no significant change of mass during heating up to 200 °C, these results were confirmed by Weete, who reported that soy lecithin did not change the composition even if heated at 150 °C during 1 h in "forced-air oven" (Weete, 1994). From the other side, we also exclude a decomposition of isothiocyanate functional group because alkyl isothiocyanates are stable and –N═C═S group is not eliminated during rather harsh conditions of GC analysis with high temperature of injector and increasing temperature of GC column (up to 220 °C) (Jin, Wang, Rosen, & Ho, 1999). We also exclude a reaction of isothiocyanate group with amine functionality in lecithin (such reaction with formation of alkylated thiourea would be possible for primary amines (Jin et al., 1999), thus, in order to check

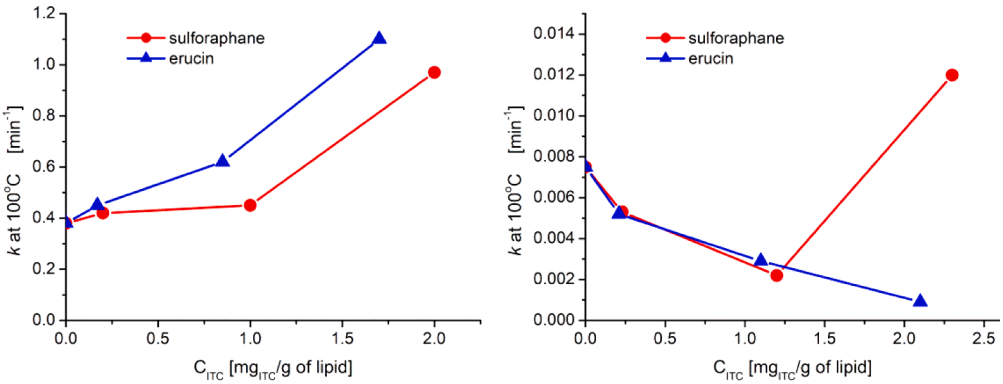


Fig. 2. Comparison of rate constants (*k* at 100 °C) for thermoxidation of LNA (left panel) and SL (right panel) containing ITCs: sulforaphane and erucin at various concentrations (in mg of ITC per g of lipid).

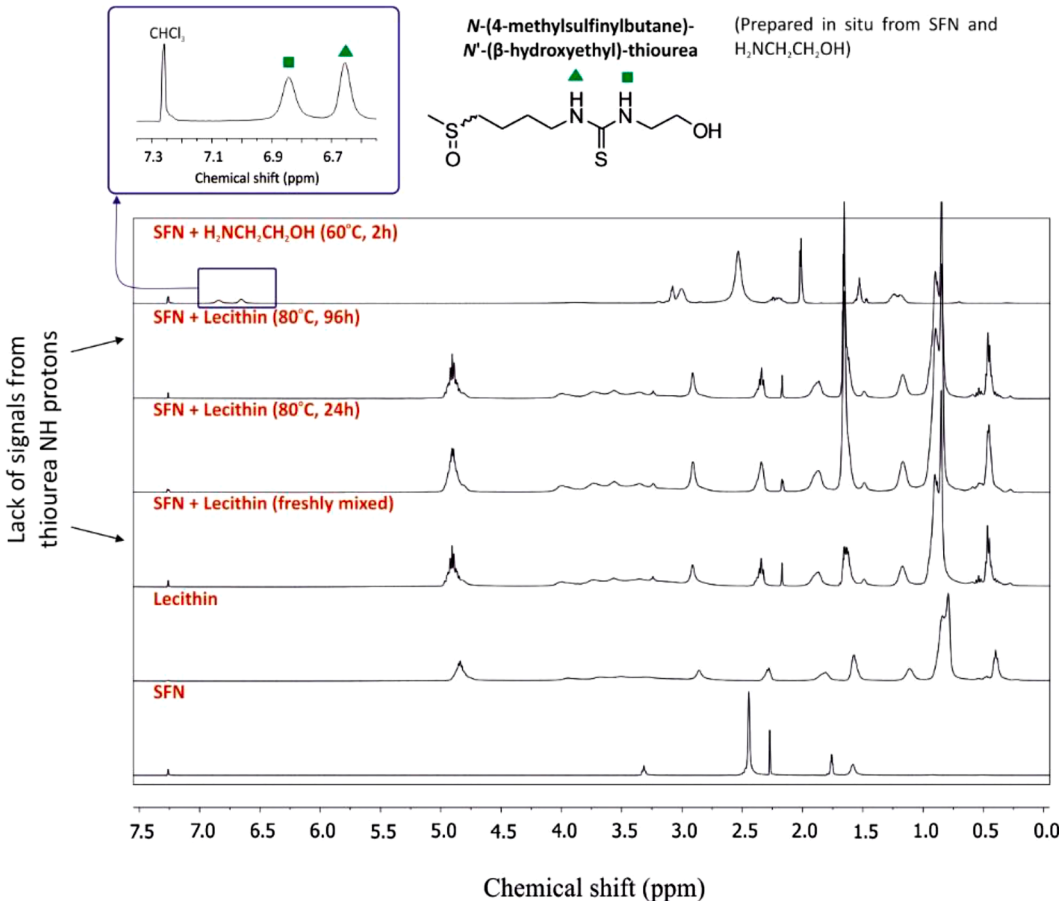
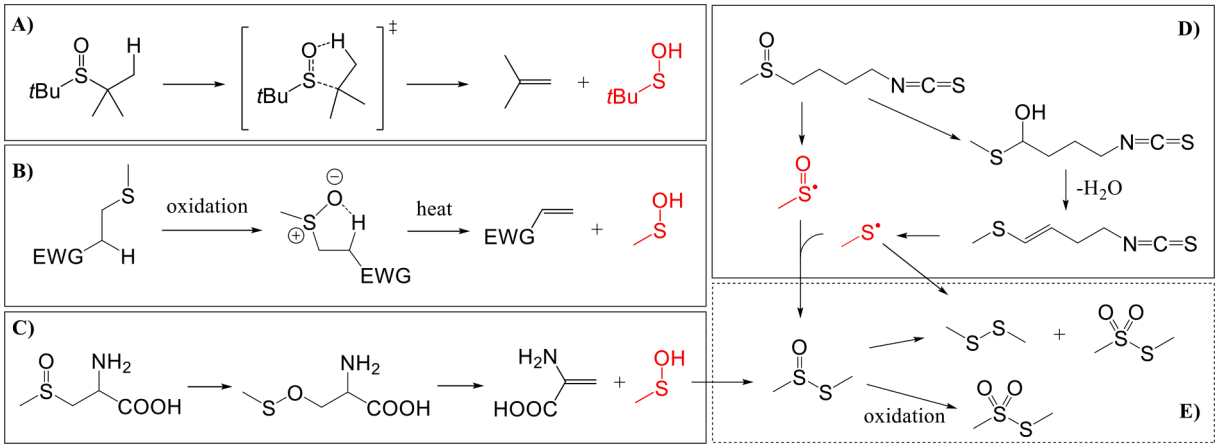


Fig. 3. Stacked ^{1}H NMR plots (400 MHz) in the range of 6–7.5 ppm exemplifying no reaction between SFN (5.6 mg) and lecithin (176 mg) containing phosphatidylethanolamine (~2 equivalents to SFN) leading to thiourea formation, even after prolonged heating in sealed vial in a $CDCl_3$:acetone-d_6 (1:1 v/v) solvent mixture; inset: corresponding thiourea derivative obtained by heating the SFN (20 mg) and ethanolamine (8 mg) in MeCN (2 ml) at 60 °C for 2 h, distilling of the solvent and redissolving the oily residue in $CDCl_3$:acetone-d_6 (1:1 v/v).

whether our assumption is correct, we placed SFN with SL (containing two-fold molar excess of phosphatidylethanolamine over SFN) in glass vial with $CDCl_3$: acetone-d_6 (1:1 v/v), the sample was thermostated at temperature 80 °C during 4 days and we monitored the ^{1}H NMR signals. Fig. 3 demonstrates that no new signals appeared at 6.0–7.5 ppm range, in contrast to control experiment, with ethanolamine instead of soy lecithin, where the NH protons for thiourea were easily recorded (Fig. 3, inset). Additionally, we analysed (^{1}H NMR) the mixture of SFN with soy lecithin heated without solvents at 100 °C during 2 h. The residue was dissolved in $CDCl_3$ containing 10% mol DMSO-d_6 and no signals from the alkylthiourea group were detected (data not shown).

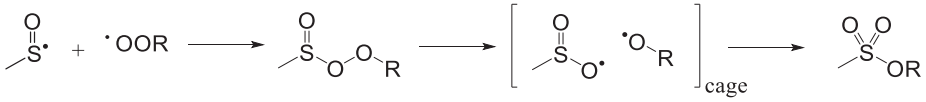
Thus, a functionality other than –N═C═S must be responsible for "antioxidant activation" of SFN and ERN at higher temperatures. The difference between SFN and ERN is the oxidation state of sulfur in sulfides and sulfoxides, however, the literature search indicates that at higher temperatures and in the presence of oxygen sulfides are converted into sulfoxides, and then, sulfoxides undergo decomposition with evolution of the radical trapping agents. Scheme 2 presents examples of such possible conversions at elevated temperatures. First three mechanisms (A-C) include the formation of sulfenic acids R-S-OH, from dialkyl



Scheme 2. Mechanisms of formation of sulfenic acids: A) decomposition of dialkyl sulfoxides in a Cope elimination (during autoxidation of squalene in chlorobenzene at 60 °C (Bateman et al., 1962) or autoxidation of neat tetralin at 60 °C (Koelewijn & Berger, 1972)); B) oxidation of alkylthio-derivatives bearing electron withdrawing group (EWG) at β-carbon with subsequent thermolysis in non-polar solvent (Gupta & Carroll, 2014; Jones, Cottam, & Davies, 1979); C) thermolysis of S-methylcysteine sulfoxide (in water, sealed tube, 80–200 °C (Kubec et al., 1998)); D) and E) thermal decomposition of sulforaphane with generation of methylsulfinyl radical (in water, 50–100 °C (Jin et al., 1999)).

sulfides and sulfoxides (Bateman, Cain, Colclough, & Cunneen, 1962; Gupta & Carroll, 2014; Koelewijn & Berger, 1972; Kubec, Drhová, & Velíšek, 1998; Lynett, Butts, Vaidya, Garrett, & Pratt, 2011; Vaidya, Ingold, & Pratt, 2009).

Sulfenic acids are transient species with very weak O—H bonds (about 70 kcal/mol) which can react with alkylperoxyl radicals with a near diffusion-controlled proton-coupled electron transfer mechanism (Lynett et al., 2011). Formation of sulfenic acids from dialkyl sulfoxides at elevated temperatures were proposed as early as in sixties (Bateman et al., 1962) for sulfoxides with accessible H atoms at β-position, mainly with *tert*-butyl groups (Scheme 2 , panel A). Interestingly, the authors noticed that sulfoxides inhibited autoxidation initiated with hydroperoxides but not with other kinds of initiators, like peroxides or azo-compounds and they interpreted this inhibition effect as a result of inactivation of hydroperoxides due to intermolecular interactions. Ten years later Koelewijn and Berger (Koelewijn & Berger, 1972) ruled out the hypothesis about the role of molecular complexes of hydroperoxides and they proposed that antioxidant action of sulfoxides in tetralin comes from their ability to form transient sulfenic acids during thermal decomposition, and they measured the rate constants for unimolecular decomposition of sulfoxides (k_{decomp} for di-*tert*-butylsulfoxide at 60 °C is 1.3×10^{-5} s^{-1} and for less reactive di-*n*-dodecylsulfoxide $k_{decomp} = 10^{-5}$ s^{-1} at 130 °C). They also measured rate constants for reaction of sulfenic acids with alkylperoxyl radicals:



(8)

$$R\text{-}SOH + RO_2\cdot \rightarrow non-radical\ products \quad (6)$$

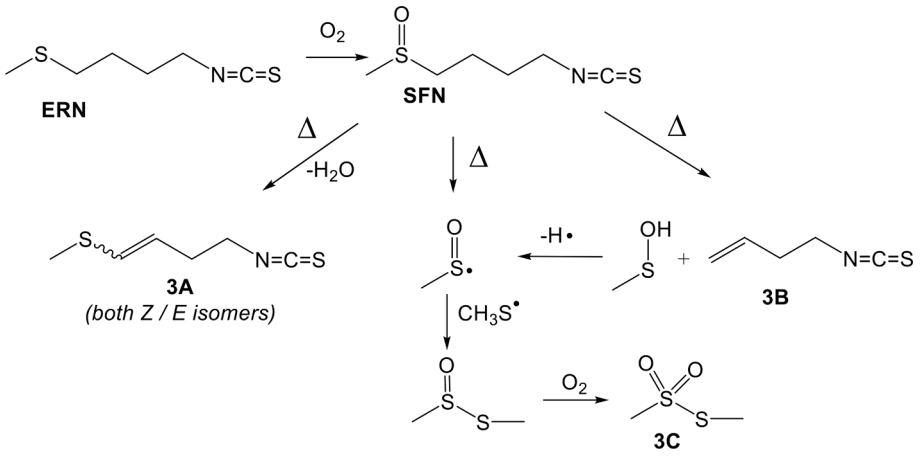
to be higher than 10^7 $M^{-1}s^{-1}$ at 60 °C. If peroxyl radicals are not accessible, alkylsulfenic acids undergo self-condensation giving S-alkyl alkanethiosulfinates (Bateman et al., 1962):

$$2CH_3SOH \rightarrow CH_3(S=O)SCH_3 + H_2O \quad (7)$$

and this thiosulfinate easily decomposes via Cope-type elimination into sulfenic acid and thioformaldehyde. The similar sequence of reaction was proposed for diallyl thiosulfinate (allicin - a component of garlic and onion) (Block, 1992; Lynett et al., 2011; Vaidya et al., 2009).

Another example of formation of sulfenic acid is pictured in panel B of Scheme 2 when initial oxidation of sulfides to sulfoxides make them prone to stepwise β-elimination of acidic hydrogen (Gupta & Carroll, 2014). Electron withdrawing group (EWG) at β-position seems to be helpful but is not necessary (see panel A) and an example of such decomposition not assisted by β-EWG was observed by Kubec et al. (Kubec et al., 1998), see panel C, for S-methylcysteine sulfoxide heated at 120 °C during 1 h (complete conversion in the presence of 10% of water, for dry compound the process was "somewhat slower"). The same authors reported the presence of methyl methanethiosulfinate and dimethyl disulfide (Scheme 2, panel E) as thermal oxidation products, the latter compound responsible for unpleasant smelling during too long frying, baking or roasting (when the food reaches high temperature and the amount of water becomes very low). The formation of unstable sulfenic acid was also observed for cysteine during oxidative stress, meaning that extensive heating is not necessary to initiate this decomposition route (Gupta & Carroll, 2014).

Fourth mechanism we found in the literature is presented in panel D of Scheme 2 (Hanschen, Lamy, Schreiner, & Rohn, 2014) and this is a thermal degradation of SFN with formation of methylsulfinyl radicals. Although the presence of sulfenic acid was not postulated by the authors, the mechanism D also can be used as an explanation of the antioxidant action of sulforaphane, because methylsulfinyl radicals are able to scavenge peroxyl radicals. Moreover, methylsulfinyl radical can be also produced from sulfenic acid (reaction (6) modified to the form: $CH_3SOH + RO_2^{\bullet} \rightarrow CH_3SO^{\bullet} + ROOH$), therefore, mechanism D does not exclude the formation of sulfenic acid (Scheme 2, mechanism A) as an intermediate. Pratt and collaborators in their work on antioxidant action of sulfenic acids (Lynett et al., 2011) paid attention to alkylsulfinyl radicals R-$SO^{\bullet}$ as the products of H atom abstraction from sulfenic acid, and studied thermochemistry of possible subsequent reactions starting from the trapping another alkylperoxyl radical (equation (8)).



Scheme 3. Proposed path(s) of thermal oxidative conversion of erucin (ERN) to sulforaphane (SFN) and subsequent decomposition to the products detected by GC-MS and showed in Table 3. Wavy bond in **3A** means Z/E isomers.

Although formation of peroxysulfinate ester $CH_3(S{=}O)OOR$ is 40 kcal/mol exothermic, it decomposes because of exceptionally weak O—O bond (8–10 kcal/mol). If such decomposition produced additional radicals, no antioxidant action would be observed, but sulfonyl and alkoxyl radicals recombine within the cage to form stable sulfonate ester which is 60 kcal/mol lower in enthalpy than $CH_3(S{=}O)OOR$, and 100 kcal/mol lower than the starting sulfinyl and peroxyl radicals (Lynett et al., 2011). Therefore, the mechanism D with thermal decomposition of sulforaphane and release of sulfinyl radicals that can scavenge peroxyl radicals also can be considered as probable explanation of inhibiting effect of this compound during thermal autoxidation of lipids above 100 °C.

Mechanism D proposes a cleavage of S—C bond proximal to methylsulfinyl group but mechanism B predicts the oxidation of methyl sulfide to methyl sulfoxide as a first step, instead of cleavage of S—C bond. We can justify such different paths because of different stability of C—S bond in sulfides and sulfoxides: bond energy in dialkyl sulfides, depending on the length of alkyl group, is within the range 72 kcal/mol (for CH_3S-C_5H_{11}) to 77 kcal/mol (for CH_3S-CH_3), so, C—S bond is at least 15 kcal/mol stronger than C-(SO) bond in sulfoxides (55 kcal/mol for dimethylsulfoxide) (Luo, 2007). Thus, terminal CH_3S- group is oxidized to $CH_3S(=O)$- group (ERN is easily oxidized to SFN by peroxides even under mild conditions (Barillari et al., 2005)), subsequently decomposes to $CH_3SO^{\bullet}$ or undergoes Cope elimination to sulfenic acid (and then, to $CH_3SO^{\bullet}$), see Scheme 2. We performed experiments with thermal degradation of a few milligrams of SFN and ERN placed in glass sealed vials (aerobic conditions) and kept at temperatures 100–160 °C during 5 h at 100 °C (or 0.5 h at 160 °C) and we analysed the volatile products by GC-MS. The conditions used in our experiments differ from the experimental conditions described in the caption to Scheme 2A-D, however, we succeed in detecting three products of thermal degradation of ITCs that can be important for antioxidant action (see Scheme 3 and Table 3).

All three products **3A**, **3B** and **3C** were detected for both starting compounds, ERN or SFN, proving that oxidation of ERN to SFN is a first step of oxidative thermal conversion. SFN undergoes further processes: dehydratation and formation of **3A** (both Z/E isomers were detected), product of β-elimination **3B** (accompanying sulfenic acid was not detected because of instability) and cleavage of S—C bond with generation of methylsulfinyl radical which, after recombination with methylsulfinyl radical gives S-methyl methanethiosulfinate, further oxidized to **3C** (this process is also depicted in Scheme 2, panelE). Compound **3A** was also detected by Jin (Jin et al., 1999), moreover, Papi et al. (Papi, et al., 2008) described its selective cytotoxic/apoptotic activity and ability to decompose peroxides, however, **3A** was not a good chain-breaking inhibitor of autoxidation of styrene and methyl linoleate.

Comparison of the kinetic parameters collected in Table 2 indicates the highest E_a and Z parameters obtained for oxidation of SL containing 1.2 mg SFN and 2.1 mg ERN per gram of SL. The antioxidant action of both ITCs is dependent on the rate of their decomposition to sulfenic acid (ie., depends on temperature and concentration of ITC) therefore, the observed increase of E_a and Z is an effect of the presence of a new chain-breaking component formed from ITCs. Then, for increasing concentration of SFN from 1.2 to 2.3 mg/g we observe a decrease of effectiveness but such decrease is also observed for other conventional antioxidants when present in too high concentration.

3.4. The problem of peculiarity of start of oxidation at 150 °C

One more problem that needs to be additionally explained is why temperatures of start of oxidation (T_e) of lecithin and lecithin containing ITCs are almost the same, regardless the different kinetic parameters

Table 3
Names, retention times and mass spectral data for three products of thermal decomposition of neat SFN/ERN at temperatures 100 °C and 160 °C.

Compound, symbol, R_T[a]	Experiment[e]	MS spectral data *m/z* (relative intensity)
4-methylthio-3-butenyl isothiocyanate[b] **3A**, R_T = 34.2 min	SFN: 100 °C, 160 °C ERN: 160 °C	161 [(M + 2)$^+$, 9], 87 (22), 85 (24), 72 (42), 61 (100), 45 (34)
but-3-enyl isothiocyanate[c] **3B**, R_T = 11.6 min	SFN: 100 °C, 160 °C ERN: 160 °C	113 (M$^+$, 50), 85 (10), 72 (100), 55 (30), 39 (71)
S-methyl methanethiosulfonate[d] **3C**, R_T = 10.5 min	SFN: 160 °C ERN: 160 °C	128 [(M + 2)$^+$, 8], 126 (M$^+$, 73), 111 (11), 81 (5), 79 (50), 64 (40), 47 (10), 45 (20)

[a] R_T = retention time. [b] Identified basing on NIST Mass Spectrometry Data Center, SRD1A, NIST MS numbers 5649 and 414,629 (accessed on 14.08.2020). [c] Identified basing on NIST Mass Spectrometry Data Center, SRD 69, NIST MS number 414,530 (accessed on 14.08.2020), [d] Identified basing on NIST Mass Spectrometry Data Center, SRD 69, NIST MS number 236,079 (accessed on 14.08.2020). [e] This column indicates in which experiment the compounds were detected for thermal decomposition of SFN /ERN.

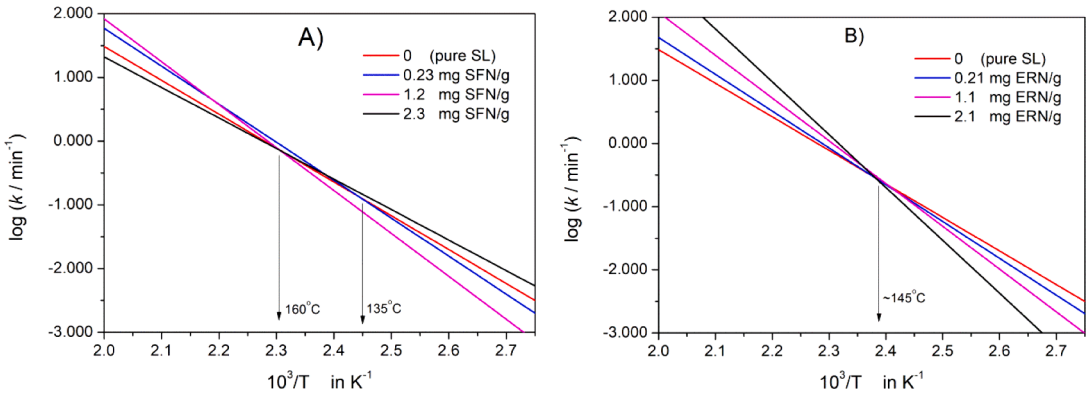


Fig. 4. Comparison of the plots of log *k(T)* versus reciprocal of absolute temperature 363 K-513 K (90–240 °C) for oxidation of pure soy lecithin (SL) and SL containing SFN and ERN. Rate constants were calculated from equation (2), using E_a and Z values listed in Table 2. The vertical arrows indicate the temperatures of intersection of the straight lines (see text).

characterizing the process with and without SFN and ERN, indicating no inhibition effect at temperatures 150 ± 15 °C. Indeed, the rate constants calculated for 150 °C (start of spontaneous oxidation) are very close to each other, within the range 0.30–0.41 min^{-1}, see Table 2. However, such levelling of oxidative stability of inhibited and non-inhibited systems at particular temperature is not surprising and corresponds to commonly found inversion of the relative rates of the processes monitored by thermal analysis techniques. The rate constants of two processes described with different sets of pairs of E_a and Z might cross each other at isokinetic temperature T_{iso} (Litwinienko & Jodko-Piorecka, 2015; Grzegorz Litwinienko, 2005; Ulkowski et al., 2005). Below T_{iso} the process with smaller activation barrier is faster but at temperature > T_{iso} the process described by higher E_a proceeds faster. On the basis of the E_a and Z from Table 2, we plotted the log*k* as a function of reciprocal absolute temperature for oxidation of pure lecithin (red line) and for oxidation of lecithin containing SFN and ERN, see Fig. 4.

The intersections of the red line with other lines are at temperatures 135° and 160 °C for SFN and 145 ± 2 °C for ERN, indicating that during DSC experiment the temperatures of the start of oxidation (T_e from 150 to 170 °C) overlap with T_{iso} 135–160 °C giving completely erroneous impression that both isothiocyanates are kinetically insignificant during autoxidation. However, inspection of the rate constants calculated for a bit lower temperatures, during the induction period (lag phase) for 100 °C, (see Table 2) clearly shows the inhibiting effect at temperatures below T_{iso}. Moreover, some important information can be gained from the above considerations that isothiocyanates at high temperature (ca. 100 °C and higher) are activated via thermal decomposition into reactive transient products which can effectively inhibit oxidation but only at temperatures below 150 °C. Practically, it is possible to decompose ITCs at temperatures above 150–160 °C but after such decomposition the system should be immediately cooled to temperature below T_{iso}, to reach the optimal antioxidant activity of decomposed isothiocyanates.

4. Conclusions

Sulforaphane (SFN) and erucin (ERN) do not inhibit autoxidation of linoleic acid (LNA) at temperatures lower than 100 °C, but both ITCs manifest significant inhibiting effect during autoxidation of soy lecithin (SL) at temperatures above 100 °C. Our results, taken together with the results described in preliminary communication (Cedrowski et al., 2020), suggest that thermal decomposition of ITCs can produce secondary compounds able to trap peroxyl radicals. Literature search indicates that the isothiocyanate (-N=C=S) group is thermally stable and does not exhibit antioxidant activity, thus the other functional groups might contribute to the observed inhibition effect. At higher temperatures methylsulfanyl- (in ERN) and methylsulfinyl- (in SFN) groups can be converted into sulfenic acids and such transient species are responsible for breaking the autoxidation chain. Additional possibility is a thermal decomposition of methylsulfinyl end group with subsequent release of sulfinyl radicals that can react with alkylperoxyl radicals. Interestingly, reaction of sulfenic acids with peroxyl radicals also produces sulfinyl radicals. Possible mechanisms of recombination of the formed sulfinyl radicals with sulfenyl radicals gives also S-methyl methanethiosulfinate, therefore, several organosulfur species and the transient products presented in Schemes 2 and 3 can be responsible for the antioxidant action of SFN and ERN at higher temperatures.

CRediT authorship contribution statement

Jakub Cedrowski: Conceptualization, Funding acquisition, Investigation, GC-MS and DSC measurements, NMR and MS analysis, Visualization, Writing - review & editing. **Kajetan Dąbrowa:** Investigation, Synthesis, GC-MS measurements, NMR and MS analysis, Visualization, Writing - review & editing. **Paweł Przybylski:** Investigation, DSC measurements, Visualization, Writing - review & editing. **Agnieszka Krogul-Sobczak:** Investigation, Writing - review & editing. **Grzegorz Litwinienko:** Conceptualization, Funding acquisition, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129213.

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