Vysoká škola chemicko-technologická v Praze
Fakulta potravinářské a biochemické technologie
Ústav mléka, tuků a kosmetiky

Habilitační práce

Oxidace lipidů a použití antioxidantů

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Obor habilitačního řízení: Technologie potravin

Praha 2021

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V Praze 6. ledna 2021

Poděkování

Chtěla bych poděkovat prof. Ing. Vladimíru Filipovi, CSc. a prof. Ing. Janu Šmidrkalovi, CSc. za cenné rady, podporu a vstřícnost.

Speciální dík patří mým kolegům Ing. Markétě Berčíkové, Ph.D., Ing. Janu Kyselkovi, Ph.D., Ing. Monice Mikoláškové a Ing. Eriku Peškovi za velkou podporu při přípravě práce.

Nejsrdečněji bych chtěla poděkovat mému manželovi, dětem, rodičům a všem, kteří měli vždy pochopení, dodávali mi energii a vytvářeli mi dokonalé zázemí, bez kterého by tato práce nemohla vzniknout.

Seznam zkratek

AA	kyselina askorbová
AČ	anisidinové číslo
AP	askorbylpalmitát
Ant-H	antioxidant
Ant·	radikál antioxidantu
α-TO	alfa-tokoferol
BHA	butylhydroxyanisol
BHT	butylhydroxytoluen
β-TO	beta-tokoferol
γ-TO	gama-tokoferol
δ-TO	delta-tokoferol
FAME	methylestery mastných kyselin (fatty acid methyl esters)
HLB	hydrofilně-lipofilní rovnováha
HO·	hydroxylová radikál
HSS	headspace sampler
MAG	monoacylglycerol
MAG10	monokaprinoylglycerol
MAG14	monomyristoylglycerol
MAG16	monopalmitoylglycerol
MAG18	monostearoylglycerol
MAG18:1	monooleoylglycerol
MAG20	monoarachoylglycerol
MAG22	monobehenoylglycerol
PČ	peroxidové číslo
MUFA	mononenasycené mastné kyseliny (monounsaturated fatty acids)
OSI	Oil stability index
PUFA	polynenasycené mastné kyseliny (polyunsaturated fatty acids)
SFA	nasycené mastné kyseliny (saturated fatty acids)
SPME	solid phase microextraction
R·	radikál mastné kyseliny
RO·	alkoxylový radikál
ROO·	peroxylový radikál
ROOH	hydroperoxid
TAG	triacylglycerol
TOTOX	totální oxidační hodnota

OBSAH

1 OBECNÝ POPIS OXIDACE LIPIDŮ

Oxidace lipidů je vedle Maillardových reakcí nejčastěji se vyskytující reakcí v potravinách, jelikož ve většině potravin se vyskytují ve větší či menší míře lipidy s nenasycenými mastnými kyselinami a zároveň i kyslík jako základní reaktanty této reakce. Je to nežádoucí reakce, která vede k snížení nutriční a senzorické hodnoty potravin a vzniku potenciálně kancerogenních látek.

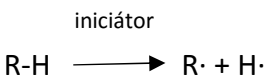
Oxidace lipidů je souhrnný název pro řadu reakcí, z nichž nejrozšířenější je autooxidace. Častým typem oxidace lipidů je také oxidace singletovým kyslíkem, tzv. fotooxidace, méně časté jsou oxidace enzymy, těžkými kovy, peroxidem vodíku či chinony.

1.1 AUTOOXIDACE A REAKCE S NÍ PROBÍHAJÍCÍ

1.1.1 Autooxidace

Autooxidace je radikálová řetězová reakce, která probíhá za podmínek zpracování a skladování potravin. Účastní se jí nenasycené mastné kyseliny a biradikálová forma molekulárního kyslíku, tzv. tripletový kyslík ($^3O_2$). Nasycené mastné kyseliny mohou být tripletovým kyslíkem efektivně oxidovány až za vyšších teplot (pečení, smažení nebo fritování).

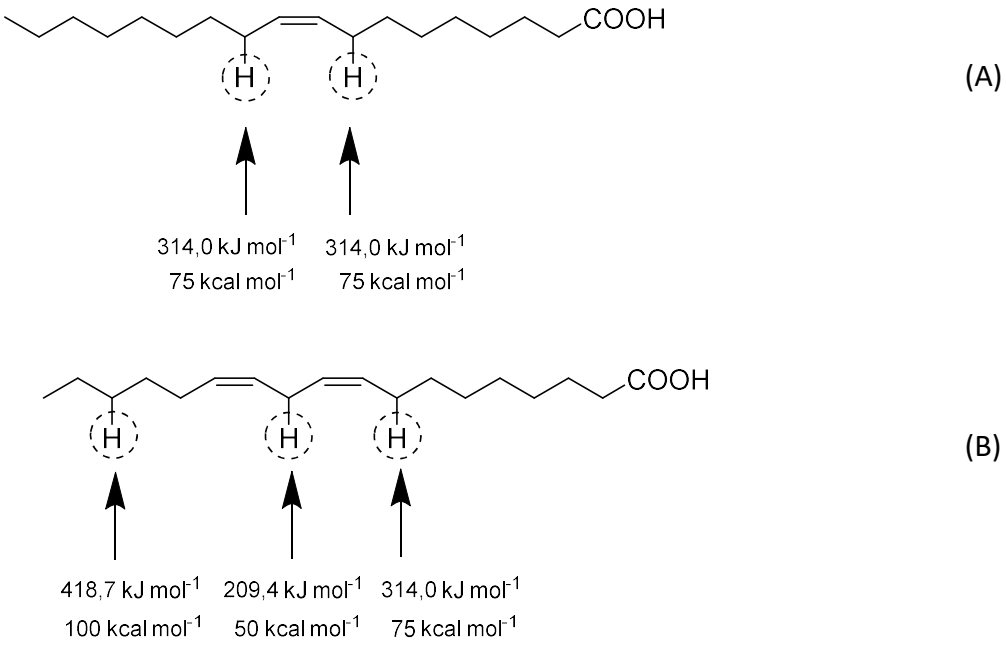
Autooxidace probíhá ve třech krocích – iniciace, propagace a terminace. Během primární iniciace dochází ke vzniku radikálu z mastné kyseliny (obr. 1). Iniciátorem reakce jsou světlo, teplo, peroxidy nebo hydroperoxidy (Schaich K.M., 2020).



Obr. 1: Iniciace autooxidace

V nenasycených mastných kyselinách, v sousedství dvojných vazeb je vodík vázán velmi slabě, tedy se snadno odtrhává. U kyseliny olejové ((9*Z*)-oktadec-9-enová kyselina) jsou přednostními reakčními místy C8 a C11 uhlovodíkového řetězce, u kyseliny linolové ((9*Z*,12*Z*)-

oktadeka-9,12-dienová kyselina) je prioritní reakční místo C11, reaktivita na C8 a C14 uhlovodíkového řetězce se uplatňuje minimálně (obr. 2). S počtem dvojných vazeb v molekule mastné kyseliny stoupá i relativní rychlost autooxidace, pro kyselinu C18:2, C18:3, C20:4 a C22:6 je poměr rychlostí 1 : 2 : 3 : 5 (Frankel E.N.,1998; Cosgrove J.P. a kol.,1987; Min D.B. a Boff J.M., 2002).



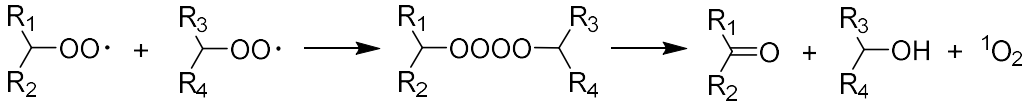
Obr. 2: Reakční místa a energie potřebná k odtrhnutí vodíku z olejové (A) a kyseliny linolové (B) (Min D.B. a Boff J.M., 2002)

V propagačním stupni reaguje radikál s tripletovým kyslíkem za vzniku peroxylového radikálu, který dále odtrhává vodík z další mastné kyseliny a vzniká další radikál mastné kyseliny a primární oxidační produkt, hydroperoxid (obr. 3). Reakce radikálu s kyslíkem je rychlá ($k = 10^{9,0-9,5}$ mol dm^{-3} s^{-1}) a nevyžaduje téměř žádnou aktivační energii ve srovnání s následným krokem, reakce peroxylového radikálu s mastnou kyselinou ($k = 10^{2,0}$ mol dm^{-3} s^{-1}). Je to tedy reakce, která určuje rychlost autooxidace a limitní je ochota mastných kyselin poskytnout vodík peroxylovému radikálu. (Frankel E.N.,1998, Cosgrove J.P. a kol.,1987).

$$R\cdot + \cdot O{=}O\cdot \longrightarrow ROO\cdot + R\text{-}H \longrightarrow ROOH + R\cdot$$

Obr. 3: Propagační fáze autooxidace

V terminační fázi oxidace lipidů se v reakčním systému kumulují radikály, které spolu tvoří stabilní molekuly. Za atmosférických podmínek a bez přítomnosti antioxidantu (reakce není zpomalena) je nejdůležitější reakce dvou peroxylových radikálů Russellovým mechanismem, kdy vzniká tetroxidový meziprodukt, který se následně rozpadá na příslušný alkohol, karbonylovou sloučeninu a singletový kyslík (obr. 4). Za vyšší teploty a nízkého tlaku kyslíku dochází k reakci dvou radikálů a vzniku dimerních nebo polymerních sloučenin (obr. 5) (Miyamoto S. a kol., 2014, Frankel E.N., 2012).



Obr. 4: Terminační fáze autooxidace - reakce dvou peroxylových radikálů Russellovým mechanismem; R_1,R_3 – zbytky uhlovodíkového řetězce s $–CH_3$; R_2,R_4 – zbytky uhlovodíkového řetězce s karboxylovou skupinou

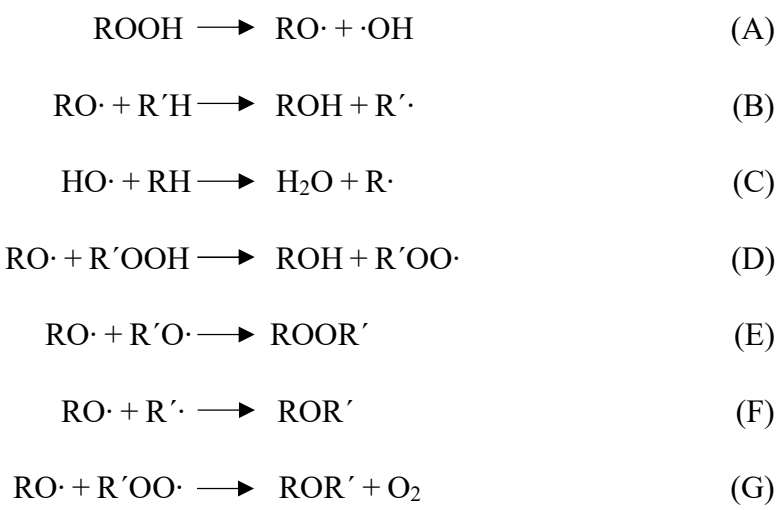
$$R\cdot + R'\cdot \longrightarrow R - R' \qquad (A)$$

$$R\cdot + R'OO\cdot \longrightarrow R\text{-}OOR' \qquad (B)$$

Obr. 5: Terminační fáze autooxidace – A - reakce dvou radikálů mastných kyselin, B – reakce radikálu mastné kyseliny a peroxylového radikálu

Při oxidaci lipidů dochází také k sekundární iniciaci, zde se vzniklé hydroperoxidy přednostně štěpí na alkoxylový radikál a hydroxylový radikál (obr. 6A). Tyto radikály mohou dále vstupovat do propagační (obr. 6B-D) i terminační fáze autooxidace (obr. 6E-F). Iniciační fáze je indikován vyčerpáním antioxidantů a prudkým nárůstem obsahu hydroperoxidů (Frankel E.N., 1998; Roman O. a kol., 2012; Choe E. a Min.D.B., 2007).

Vznik alkoxylového radikálu z hydroperoxidu tzv. β-homolytickým štěpení -O-O- vazby (Obr. 6A) je pravděpodobnější (disociační energie 184 kJ mol^{-1}) než vznik peroxylového radikálu a štěpení -O-H vazby hydroperoxidu (disociační energie 377 kJ mol^{-1}) (Frankel E.N., 2012).



Obr. 6: Vznik (A) a následné reakce alkoxylového a hydroxylového radikálu (B – G)

Rychlost vzniku a poločas rozpadu hydroxylového, alkoxylového a peroxylového radikálu je uvedena v Tab. I a je zřejmé, že hydroxylový radikál rychle vzniká a zároveň zaniká (obr. 6A a 6C) (Namiki M., 1990).

Tab. I: Rychlostní konstanta a poločas rozpadu vybraných radikálů (Namiki M., 1990)

radikál	**název**	**k [s^{-1}]**	**$\tau_{1/2}$[s]**
HO·	hydroxylový	10^8	10^{-10}
RO·	alkoxylový	10^6	10^{-6}
ROO·	peroxylový	10^2	17

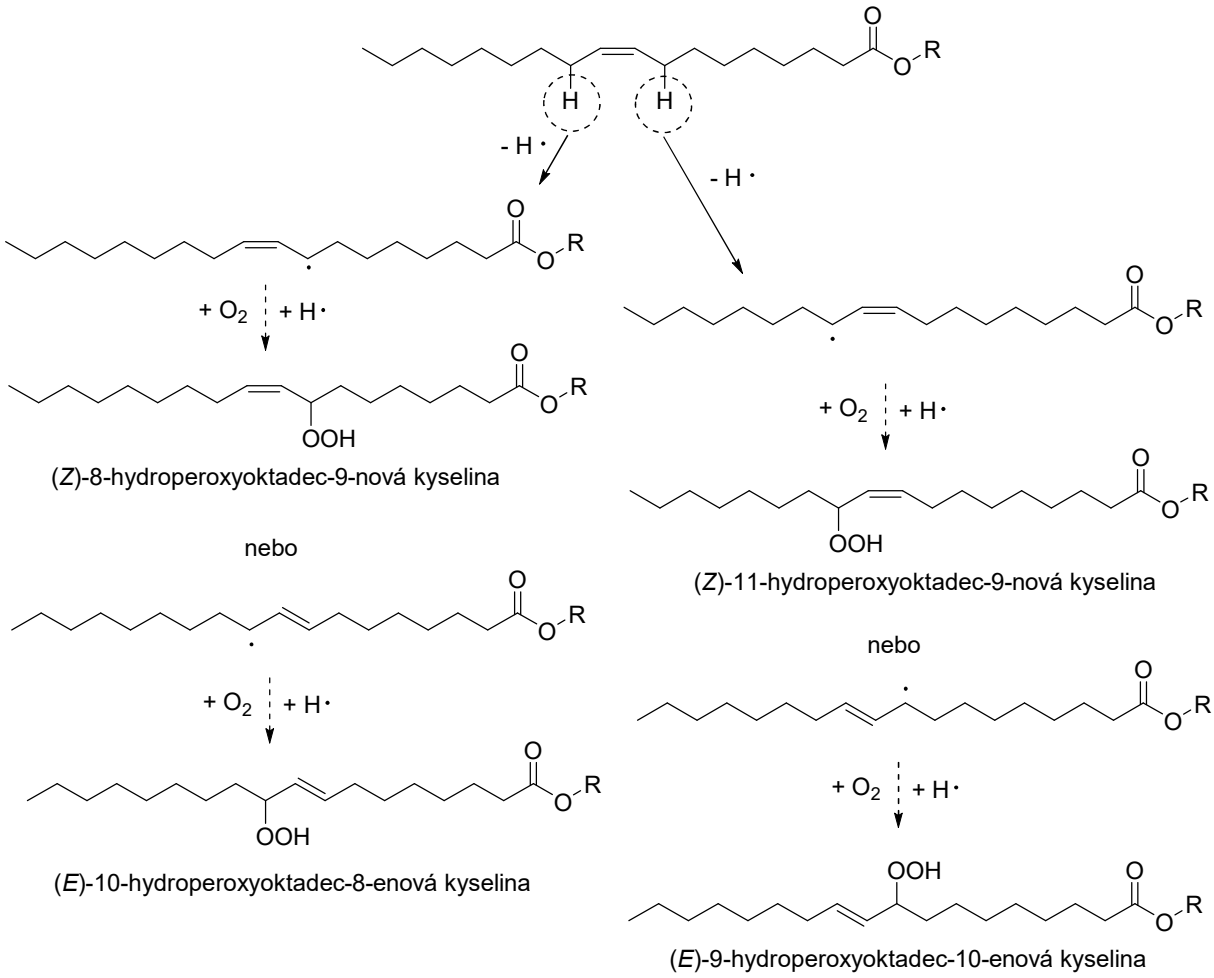
1.1.2 Reakce probíhající s autooxidací

Geometrická, polohová izomerací, polymerace a cyklizace jsou reakce, které probíhají spolu s autooxidací lipidů.

Geometrická a polohová izomerace

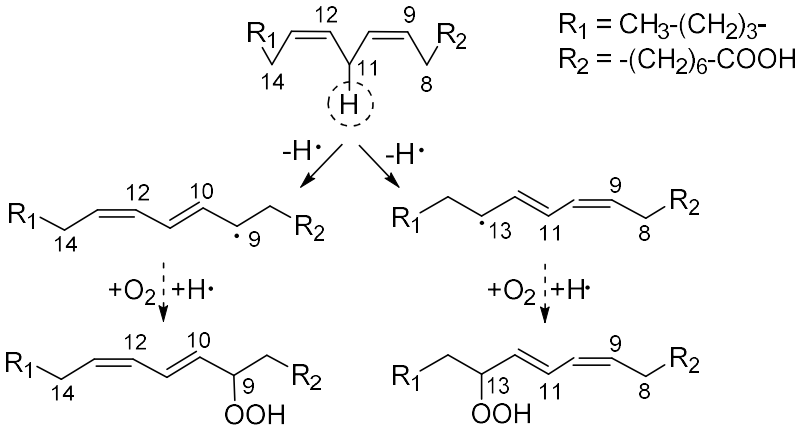
Po vzniku radikálu v mezomerním stavu je možný posun polohy dvojné vazby mastné kyseliny a změna její konfigurace, tedy *cis/trans* (geometrická) izomerace. Obě tyto izomerace přispívají ke snížení energie systému.

Z kyseliny olejové, která obsahuje jednu dvojnou vazbu, mohou vznikat radikály na C8 a C11 uhlovodíkového řetězce. Je tak možná tvorba 4 různých hydroperoxidů - beze změny polohy dvojné vazby se tvoří 8- a 11-hydroperoxid kyseliny olejové, s posunem dvojné vazby (*E*)-10-hydroperoxyoktadec-8-enová a (*E*)-9-hydroperoxyoktadec-10-enová kyselina (obr. 7). Energeticky bohatší *cis*-konfigurace přechází na energeticky chudší *trans*-konfiguraci (Schaich K.M., 2020; Tallman K.A. a kol., 2001).



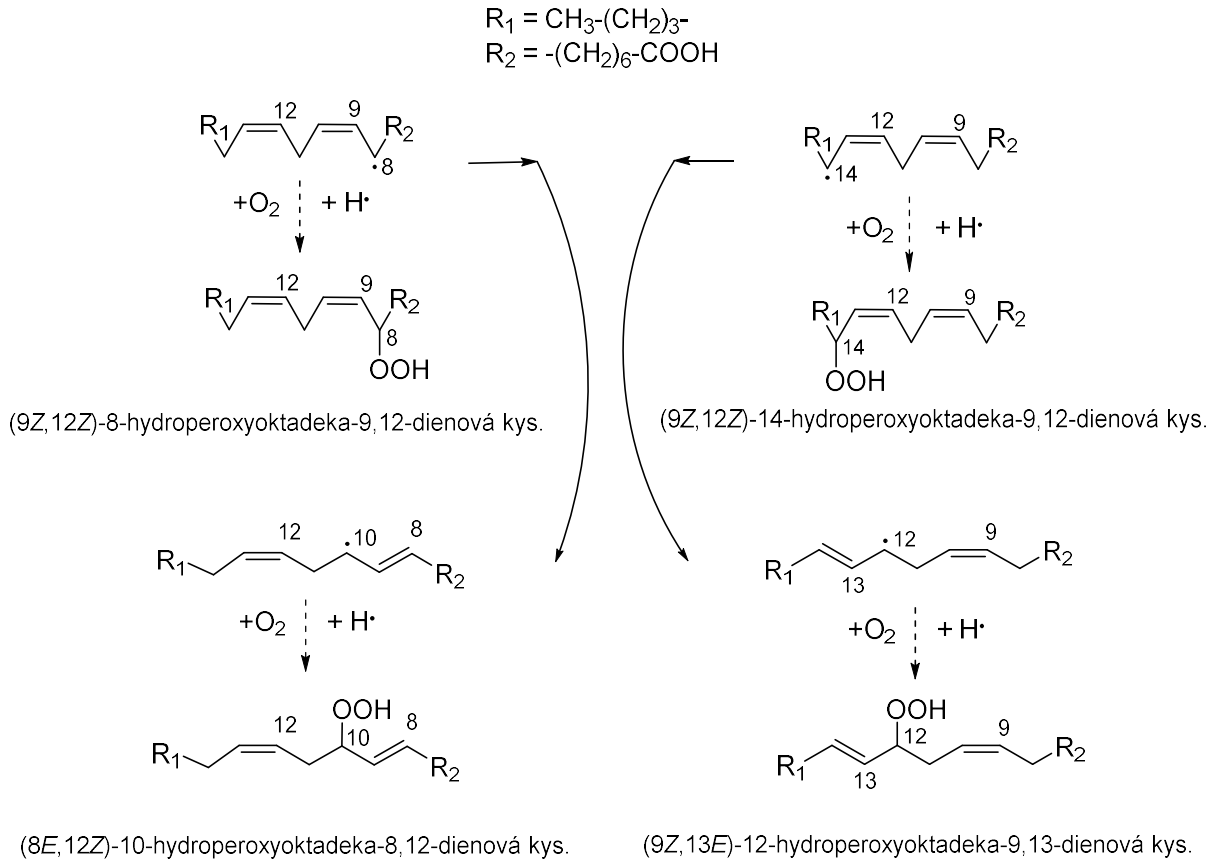
Obr. 7: Vznik hydroperoxidů z kyseliny olejové

U mastných kyselin s dvěma a více dvojnými vazbami vzniká z energeticky náročnějšího pentadienového systému dvojných vazeb energeticky výhodnější systém konjugovaných dvojných vazeb a zároveň dojde také ke změně energeticky bohatší *cis*-konfigurace na energeticky výhodnější *trans*-konfiguraci. Nejjednodušším příkladem je kyselina linolová, která ve své molekule obsahuje 2 dvojné vazby (9, 12-*cis,cis*). Jak již bylo uvedeno v kapitole 1.1.1, obr. 2, během iniciační fáze autooxidace vzniká přednostně radikál na C11 uhlovodíkového řetězce, jelikož disociační energie vazby C-H je pouze 314 kJ mol^{-1} ve srovnání s C8 a C14, které jsou také v sousedství dvojné vazby, ale disociační energie vazby C-H je 368 kJ mol^{-1}. Následně je velmi pravděpodobný přesmyk 9-*cis* vazby na 10-*trans* vazbu a vznik (10*E*,12*Z*)-9-hydroperoxy-oktadeka-10,12-dienové kyseliny nebo 12-*cis* vazby na 11-*trans* vazbu a vznik (9*Z*,11*E*)-13-hydroperoxyoktadeka-9,11-dienové kyseliny (obr. 8). Vznik (9*Z*,12*Z*)-11-hydroperoxy-oktadeka-9,12-dienové kyseliny je nepravděpodobný. Minoritně mohou vznikat radikály i na C8 a C14, beze změny polohy dvojné vazby pak vzniká 8- nebo 14-hydroperoxid kyseliny linolové, se změnou polohy dvojné vazby vznikají (8*E*,12*Z*)-10-hydroperoxyoktadeka-8,12-dienová kyselina nebo (9*Z*,13*E*)-12-hydroperoxyoktadeka-9,13-dienová kyselina (obr. 9). U polynenasycených mastných kyselin je situace s variabilitou vzniku možných hydroperoxidů ještě složitější (Schaich K.M., 2020; Tallman K.A. a kol., 2001).



(10*E*,12*Z*)-9-hydroperoxy-oktadeka-10,12-dienová kys. (9*Z*,11*E*)-13-hydroperoxy-oktadeka-9,11-dienová kys.

Obr. 8: Geometrická a polohová izomerace kyseliny linolové – majoritní produkty

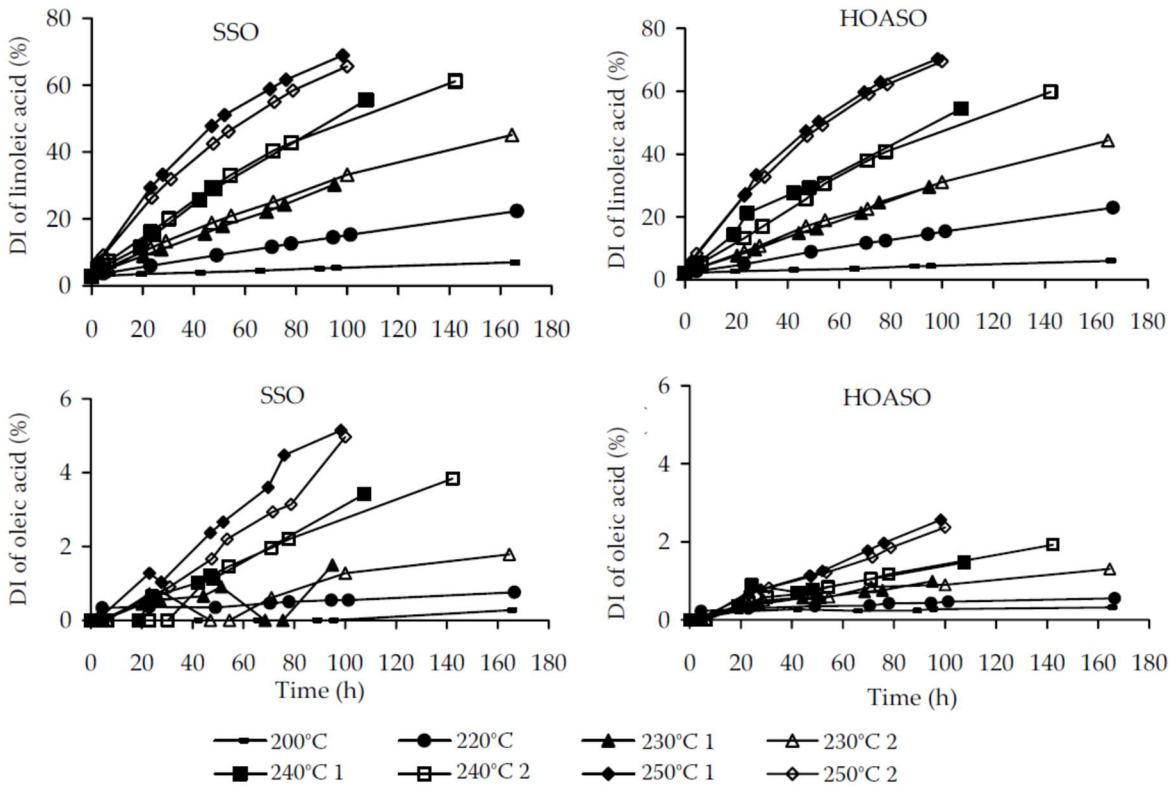


Obr. 9: Vznik minoritních hydroperoxidů kyseliny linolové

Mastné kyseliny v průběhu autooxidace podléhají c*is/trans* a polohové izomeraci jak při teplotě skladování, tak především za zvýšené teploty, během kulinárních úprav (smažení, pečení nebo fritování).

Během technologického zpracování olejů, za vysoké teploty (deodorace, fyzikální rafinace nebo hydrogenace) probíhá *cis/trans* a polohová izomerace při nízkém parciálním tlaku kyslíku. Autooxidace je v těchto případech minoritní reakcí. Při teplotě 200 – 250 °C se z linolové kyseliny tvoří 9-*cis*,12-*trans*-izomer a 9-*trans*,12-*cis*-izomer kyseliny oktadekadienové, dále konjugované izomery oktadekadienové kyseliny, označované CLA (conjugated linoleic acid). Stupeň *cis/trans* izomerace se zvyšuje s rostoucí teplotou, dobou reakce v souladu s Arrheniovou rovnicí a také s peroxidovým číslem vstupní suroviny. Co se týče počátečního obsahu kyseliny linolové a olejové (srovnání standardního slunečnicového oleje s vyšším obsahem kyseliny linolové a slunečnicového oleje s vysokým obsahem kyseliny olejové), bylo zjištěno, že stupeň *cis/trans* izomerace (degree of isomerization) nezávisí na

obsahu kyseliny linolové, ale na obsahu kyseliny olejové (obr. 10) (Cihelková K. a kol., 2009 Cihelková K. a kol., 2013).

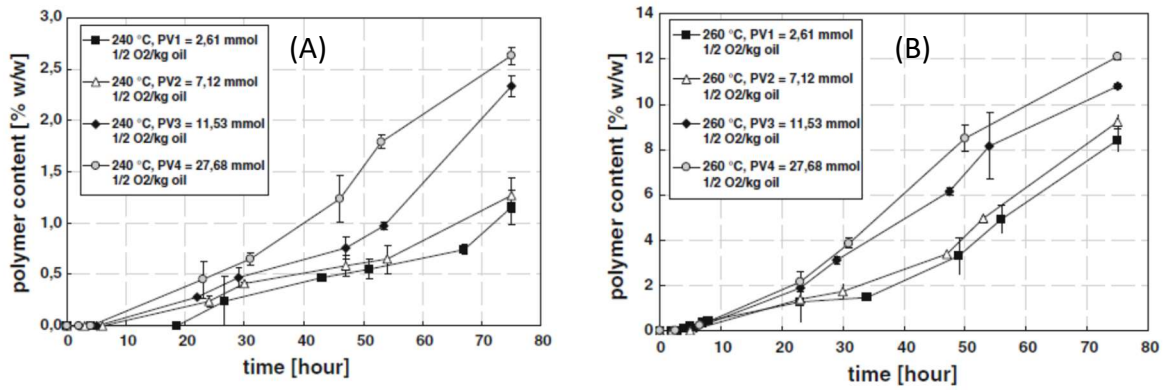


Obr. 10: Časová závislost stupně izomerace kyseliny linolové a olejové ve standardním slunečnicovém oleji (SSO) a slunečnicovém oleji s vysokým obsahem kyseliny olejové (HOASO) v rozmezí teplot 200 – 250 °C (Cihelková K. a kol., 2009)

Pokud jsou v oleji před hydrogenací přítomny hydroperoxidy a karbonyly, v redukčním prostředí z nich vznikají primární a sekundární alkoholy. Při deodoraci (fyzikální rafinaci) dochází ke štěpení hydroperoxidů a vzniklé těkavé sloučeniny odchází do deodoračního kondenzátu (destilátu mastných kyselin). Olej po deodoraci (fyzikální rafinaci) má nulový obsah hydroperoxidů, ale obsah karbonylů není nenulový, jelikož v oleji zůstávají netěkavé karbonylové sloučeniny, triacylglyceroly s *n*-oxokyselinami (tzv. core aldehydes) (Frankel E.N., 2012).

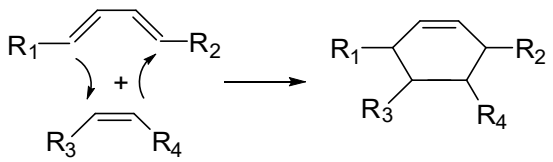
Polymerace

Při vysoké teplotě oleje spolu s *cis/trans* a polohovou izomerací dvojných vazeb dochází také k polymeraci. V terminační fázi autooxidace dochází ke spojení radikálů dvou různých triacylglycerolů ve stabilní vysokomolekulární látky, reakce je pak intermolekulární, nebo spolu reagují dva radikály v rámci jednoho triacylglycerolu, potom se hovoří o intramolekulární reakci. Rozsah polymerace a strukturu vznikajících polymerů ovlivňuje složení mastných kyselin a kvalita výchozí suroviny, teplota a čas záhřevu a přítomnost/absence kyslíku. Se snižující se kvalitou výchozí suroviny (měřená peroxidovým číslem) roste rychlost polymerace (obr. 11) (Cihelková K. a kol, 2009, Cihelková K. a kol., 2013, Fournier V. a kol., 2006).



Obr. 11: Časová závislost vzniku polymerů ve slunečnicovém oleji s různým peroxidovým číslem vzorků při teplotě 240 °C (A) a 260 °C (B) (Cihelková K. a kol., 2013)

Během technologického zpracovaní olejů (deodorace, fyzikální rafinace), při nízkém parciální tlaku kyslíku, kdy převažuje reakce dvou radikálů (obr. 5A), je možný také neradikálový, Diels-Alderův mechanismus vzniku dimerů (obr. 12), jehož se účastní konjugovaná mastná kyselina (nejčastěji CLA) a monoenová mastná kyselina. Obecně vznikají při tomto druhu polymerace sloučeniny nepolární a cyklické (Choe E. a Min D.B., 2007).



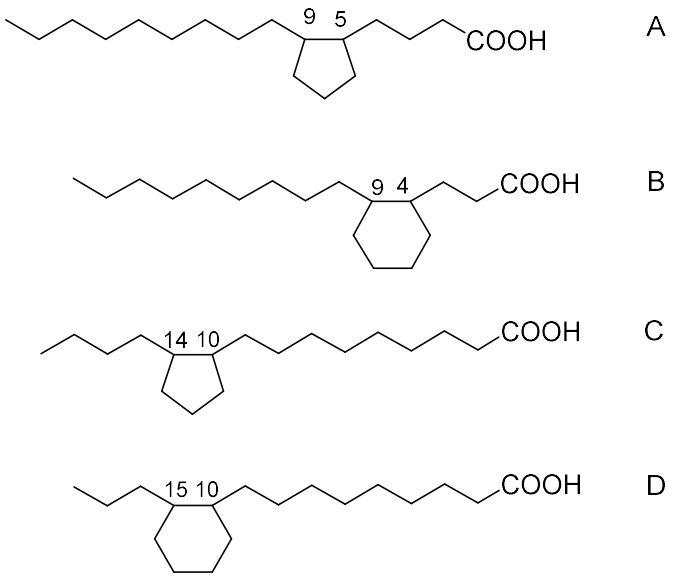
Obr. 12: Diels-Alderův mechanismus vzniku dimerních TAG mezi konjugovanou linolovou kyselinou a monoenovou mastnou kyselinou, R_1,R_3 – zbytky uhlovodíkového řetězce s $–CH_3$; R_2,R_4 – zbytky uhlovodíkového řetězce s karboxylovou skupinou

Cyklizace

Dalším možným typem reakce probíhající za vysoké teploty a při nízkém parciálním tlaku kyslíku je cyklizace mastných kyselin v triacylglycerolech.

Typ nenasycené mastné kyseliny, její koncentrace a *sn*-poloha v rámci TAG mají důležitou roli v množství a struktuře vznikajících cyklických sloučenin. Sébédio J.L. a kol. (1987) zjistili, že v přítomnosti kyseliny linolenové (C18:3) se tvoří až 10x více cyklických sloučenin než v přítomnosti kyseliny linolové (C18:2). Pokud je polynenasycená mastná kyselina v poloze *sn*-2 TAG, probíhá cyklizace snadněji než u polohy *sn*-1 nebo *sn*-3 TAG (Martin J.C. a kol. 1998 a,b,c).

Nejčastěji se vyskytujícími cyklickými sloučeninami vznikajícími z kyseliny olejové jsou nasycené cyklopentylové mastné kyseliny s kruhem mezi C5 a C9 nebo C10 a C14, dále pak cyklohexylové mastné kyseliny s kruhem mezi C4 a C9 nebo C10 a C15 (obr. 13) (Dobson G. a kol., 1996 a). Z dienových mastných kyselin vznikají monoenové monocyklické mastné kyseliny nebo nasycené bicyklické mastné kyseliny (Dobson G. a kol., 1997). U polynenasycených mastných kyselin je variabilita vzniklých cyklických sloučenin vysoká (Berdeaux O. a kol., 2007; Dobson G. a kol. 1996 b).



Obr. 13: Produkty cyklizace kyseliny olejové – A – 4-(2-nonylcyklopentyl)butanová kyselina, B – 3-(2-nonylcyklohexyl)propanová kyselina, C – 9-(2-butylcyklopentyl)nonanová kyselina, D – 9-(2-propylcyklohexyl)nonanová kyselina

Během oxidace lipidů obecně převládají izomerační a polymerační reakce, ale ani cyklizace není zanedbatelná. Rozsah jednotlivých reakcí je závislý především na teplotě, složení mastných kyselin oleje a na počáteční kvalitě oleje (Cihelková K. a kol., 2013, Fournier V. a kol, 2006).

1.2 FOTOOXIDACE

Reakce lipidů se singletových kyslíkem je dalším častým typem oxidace lipidů. Singletový kyslík ($^1O_2$) může vznikat z tripletového kyslíku různými způsoby ($^3O_2$) - chemicky, enzymaticky nebo fotochemicky. V potravinách je to nejčastěji fotosenzibilizací přirozeně se vyskytujících barviv (chlorofyly, hemová barviva apod.), tyto látky jsou nazývány senzibilizátory. Senzibilizátory v singletovém základním stavu (^1S) přechází do excitovaného stavu absorpcí světelné energie ($^1S^*$), který přechází do tripletového excitovaného stavu ($^3S^*$). Senzibilizátor v excitovaném tripletovém stavu reaguje s tripletovým kyslíkem za vzniku singletového kyslíku a singletového senzibilizátor v základním stavu (obr. 14) (Min D.B. a Boff J.M., 2002; Choe E. a Min D.B., 2005; Choe E. a Min D.B., 2009).

$$^1S \xrightarrow{h\nu} {}^1S^* \rightarrow {}^3S^* + {}^3O_2 \rightarrow {}^1S + {}^1O_2$$

Obr. 14: Vznik singletového kyslíku

Singletový kyslík reaguje s nenasycenými mastnými kyselinami řádově rychleji ($k = 1{,}3 \cdot 10^5$ dm^3 mol^{-1} s^{-1}) než tripletový kyslík ($k = 8{,}9 \cdot 10^1$ dm^3 mol^{-1} s^{-1}). Nejedná se v tomto případě o radikálovou reakci, singletový kyslík se aduje přímo na dvojnou vazbu, a tak i vzniklé hydroperoxidy se liší od hydroperoxidů vzniklých během autooxidace, mohou být jak konjugované, tak nekonjugované. Kyselina olejová po reakci se singletovým kyslíkem poskytuje C9- a C10-hydroperoxidy. Z kyseliny linolové vznikají C9- a C13-hydroperoxidy stejně jako C10- a C12-hydroperoxidy (Choe E. a Min D.B., 2009).

Klasické, primární antioxidanty (kap. 6.4.2) jsou z hlediska působení na singletový kyslík neúčinné, jelikož se nejedná o radikálovou reakci, z toho důvodu se používají tzv. zhášeče singletového kyslíku (karotenoidy, tokoferoly, flavonoidy apod.). Zhášeč singletového kyslíku (Z) v základním stavu reaguje se sigletovým kyslíkem nebo tripletovým excitovaným

fotosenzibilizátorem a vzniká tripletový kyslík a zhášeč v tripletovém excitovaném stavu - $^3Z^*$ (obr. 15A). Zhášeč v tripletovém excitovaném stavu je schopen přejít opět do základního singletového stavu s vyzářením tepelné energie (obr. 15B). Zhášeče singletového kyslíku patří do skupiny tzv. sekundárních antioxidantů a jedná se o karotenoidy, tokoferoly nebo kyselinu askorbovou (Frankel E.N., 2012; Bradley D.G. a Min D.B., 1992; Viljanen K. a kol., 2002; Senanayake S.P.J.N. a kol., 2020).

$$^1O_2\ (^3S^*) + {}^1Z \rightarrow {}^3O_2\ (^1S) + {}^3Z^* \qquad \text{(A)}$$

$$^3Z^* \rightarrow {}^1Z + \text{tepelná energie} \qquad \text{(B)}$$

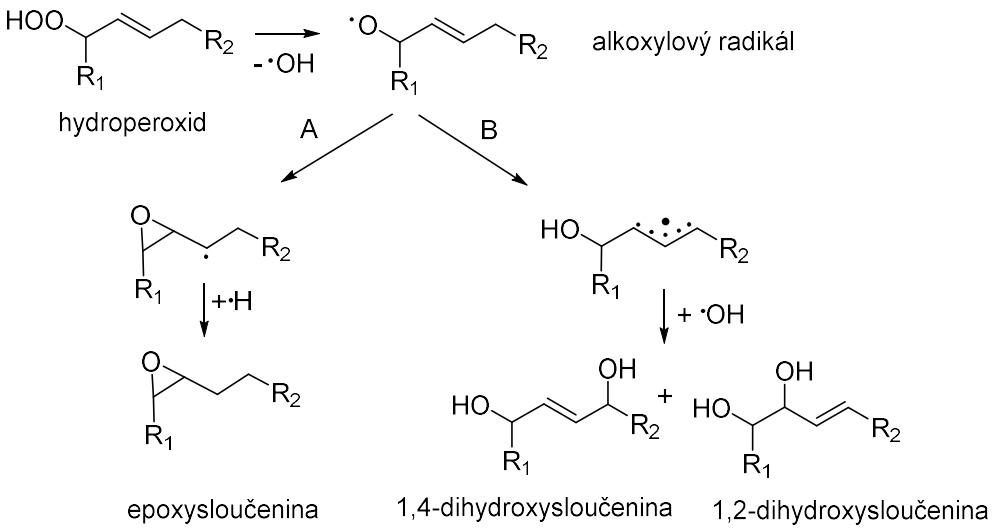
Obr. 15: Reakce zhášeče sigletového kyslíku

1.3 NÁSLEDNÉ REAKCE OXIDACE LIPIDŮ

Primární oxidační produkty, hydroperoxidy, jsou ze senzorického hlediska sice neaktivní, ale zároveň jsou nestabilní a mohou z nich vznikat sekundární oxidační produkty - cyklické látky, látky polární s podobnou molekulovou hmotností jako výchozí surovina, látky těkavé a polární s nižší molekulovou hmotností než výchozí surovina a látky polymerní s vyšší molekulovou hmotností než výchozí surovina.

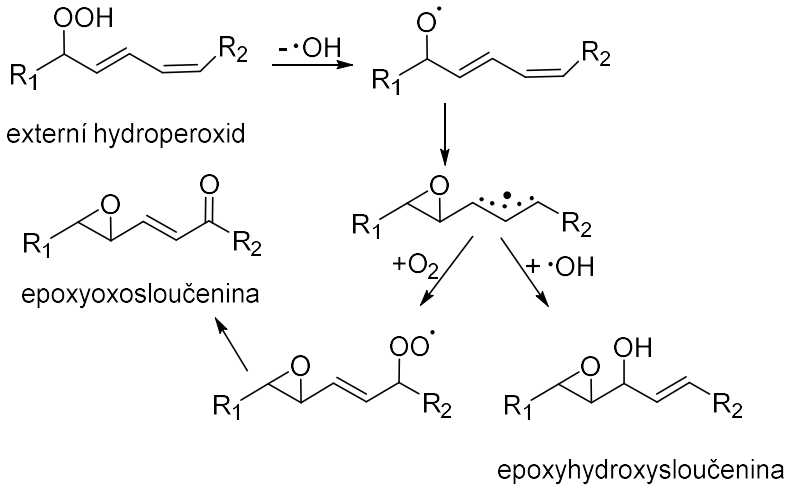
1.3.1 Cyklizace

Oxidací kyseliny olejové, resp. jejího methylesteru, mohou minoritně vznikat epoxysloučeniny. Z hydroperoxidu se vytvoří alkoxylový radikál, který se dále cyklizuje (obr. 16A). Vzniklý cyklický produkt je charakteristický pro daný výchozí hydroperoxid, z 11-hydroperoxidu se tvoří 10,11-epoxyester, z 8-hydroperoxidu 8,9-epoxyester a z 9- a 10-hydroperoxidu 9,10-epoxyester. Vedle cyklizace může docházet i ke vzniku 1,2- a 1,4-dihydroxyesterů (obr. 16B), (Zhang Q. a kol., 2012).



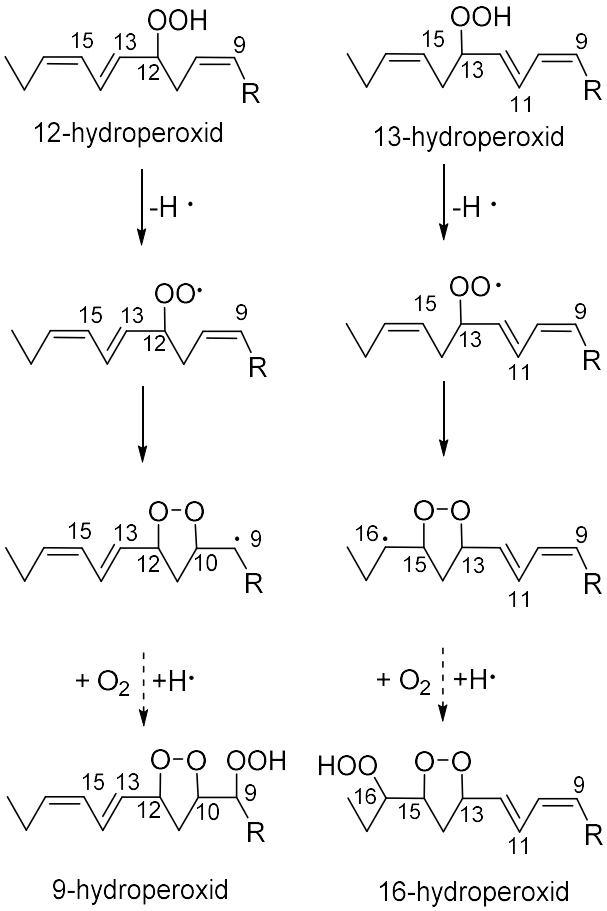
Obr. 16: Cyklizace hydroperoxidu methylesteru kyseliny olejové, R_1 – zbytek uhlovodíkového řetězce s $–CH_3$ (nebo zbytek uhlovodíkového řetězce s karboxylovou skupinou), R_2 - zbytek uhlovodíkového řetězce s karboxylovou skupinou (nebo zbytek uhlovodíkového řetězce s $–CH_3$)

Hydroperoxidy vzniklé oxidací kyseliny linolové, resp. její methylesteru, podléhají také cyklizaci, ale pouze v případě, kdy se -OOH skupina vyskytuje mimo systém dvojných vazeb (tzv. externí hydroperoxidy), jedná se tedy o 9- a 13-hydroperoxid. Z hydroperoxidu vzniká alkoxylový radikál, který následně cyklizuje a po reakci s hydroxylovým radikálem vzniká epoxyhydroxysloučenina nebo po reakci s kyslíkem epoxyoxosloučenina (obr. 17).



Obr. 17: Cyklizace 9- nebo 13-hydroperoxidu methylesteru kyseliny linolové. R_1 – zbytek uhlovodíkového řetězce s $–CH_3$ (nebo zbytek uhlovodíkového řetězce s $–COOCH_3$), R_2 - zbytek uhlovodíkového řetězce s $–COOCH_3$ (nebo zbytek uhlovodíkového řetězce s $–CH_3$)

Oxidací α-linolenové kyseliny, resp. jejího methylesteru, vznikají dominantně 2 externí hydroperoxidy, tedy 9- a 16-hydroperoxid, a asi 3 – 4krát méně 2 interní hydroperoxidy (-OOH skupina je lokalizována mezi *cis,trans*-konjugovaným systémem dvojných vazeb a *cis*-dvojnou vazbou), tedy 12- a 13-hydroperoxid. U interních hydroperoxidů dochází k rychlé cyklizaci v rámci jedné molekuly mastné kyseliny, jedná se o intramolekulovou cyklizaci (obr. 18). Z hydroperoxidu se odštěpí vodík a dojde k 1,3-cyklizaci a tvorbě 5členného cyklu (vznik 1,2-dioxolanu), nový radikál reaguje s kyslíkem, odštěpuje z další molekuly mastné kyseliny vodík a vzniká peroxohydroperoxid (9- nebo 16-hydroperoxid). Je možná i tvorba 9- a 16-hydroperoxid bicyklo endoperoxidů. Oba typy nově vzniklých hydroperoxidů opět nejsou stabilními látkami a podléhají rozkladu za vzniku těkavého malonaldehydu, který je toxický. U interním hydroperoxidů může docházet také ke vzniku 1,2-dioxanových derivátů (6členný cyklus), které jsou opět nestabilní a rozkládají se za vzniku těkavých látek (Frankel E.N., 1984; Frankel E.N.,1998).

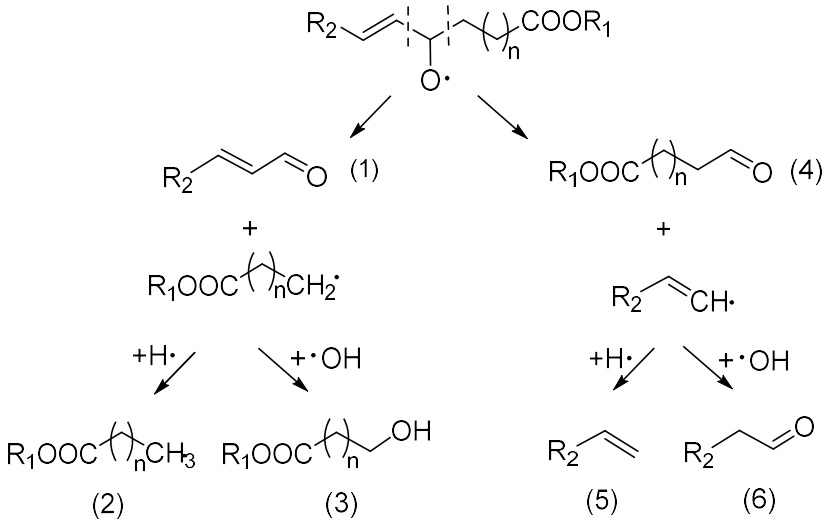


Obr. 18: Tvorba peroxohydroperoxidů z 12- a 13-hydroperoxidu kyseliny α-linolenové, R-zbytek uhlovodíkového řetězce s karboxylovou skupinou

1.3.2 Vznik těkavých látek a látek polárních o nižší molekulové hmotnosti

Těkavé látky vznikající z hydroperoxidů během oxidace lipidů patří ze senzorického hlediska mezi nejvýznamnější sekundární oxidační produkty, často jsou senzoricky aktivní již při koncentraci nižší než 1 mg kg^{-1}. Dříve diskutované oxidační produkty (*cis*/*trans* izomery, cyklické a polymerní látky) neovlivňují oxidovaný vzorek senzoricky, mohou měnit jeho barvu nebo viskozitu (Choe E. a Min D.B., 2007).

V pokročilém stádiu oxidace, při vyšší koncentraci hydroperoxidů, dochází k sekundární iniciaci a vzniku alkoxylového radikálu. Tento radikál se může štěpit 2 způsoby (obr. 19) (Frankel E.N., 1998; Cao J. a kol., 2020). První cestou dochází ke vzniku těkavého 2-alkenalu (1) a mastné kyseliny (MK), příp. triacylglycerolu (TAG) (2). Vzniklá mastná kyselina, příp. nově vzniklý triacylglycerol po rozpadu alkoxylového radikálu jsou odlišné od původní MK, příp. TAG. Vzniklé oxidační produkty mají nižší molekulovou hmotnost. Je možný také vznik *n*-hydroxykyseliny, příp. TAG s *n*-hydroxykyselinou (3). Ve druhém případě vzniká *n*-oxokyselina, příp. TAG s *n*-oxokyselinou (4), uhlovodík s vinylovou skupinou, což je ale méně pravděpodobný produkt (5) a aldehyd (6).

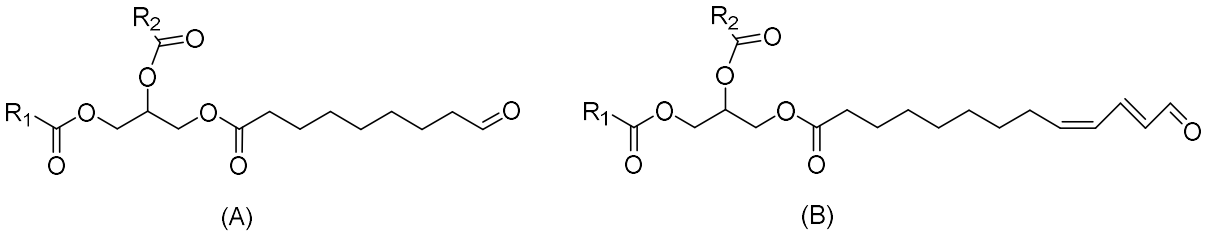


Obr. 19: Štěpení hydroperoxidů na nízkomolekulární látky. R_2 – uhlovodíkový řetězec, R_{1A} = H – kyselina nebo R_{1B} = esterově vázaný diacylglycerol. (1) 2-alkenal; (2) R_{1A}= mastná kyselina, R_{1B}= triacylglycerol; (3) R_{1A}= *n*-hydroxykyselina, R_{1B}= triacylglycerol s *n*-hydroxykyselinou; (4) R_{1A}= *n*-oxokyselina, R_{1B}= triacylglycerol s *n*-oxokyselinou; (5) uhlovodík; (6) aldehyd

Dle profilu těkavých látek oxidované směsi lze stanovit, jaká mastná kyselina se oxidovala a jaký hydroperoxid z ní vznikl. Nejjednodušším příkladem je kyselina olejová. Vznikají z ní 4 hydroperoxidy. Z 8-hydroperoxidu vzniká dekanal nebo 2-undecenal; z 9-hydroperoxidu nonanal nebo 2-decenal, z 10-hydroperoxidu vzniká nonanal; z 11-hydroperoxidu oktanal. Koncentrace těkavých oxidačních produktů se může měnit v závislosti na typu skladování a zpracování vzorků (Cao a kol., 2020).

Netěkavé oxidační produkty (obr. 19), tedy vzniklé mastné kyseliny (2), *n*-hydroxykyseliny (3) a *n*-oxokyseliny (4) nebo triacylglyceroly, které je obsahují, v oxidovaných potravinách zůstávají.

Dominantním netěkavým oxidačním produktem jsou *n*-oxokyseliny, resp. triacylglyceroly obsahující tyto *n*-oxokyseliny. V literatuře se často označují jako tzv. „core aldehydes“ a jedná se reaktivní karbonylové sloučeniny (RCS) vznikající neenzymovými reakcemi. Tyto sloučeniny reagují mechanismem Maillardovy reakce s proteiny za vzniku „Advanced Lipoxidation End Products“ (ALEs), které spolu s AGEs (Advanced Glycation End Products) hrají patogenetickou roli v rozvoji různých nemocí (diabetes, kardiovaskulární onemocnění, chronické selhání ledvin apod.). Zároveň *n*-oxokyseliny mohou podléhat následným radikálovým oxidačním reakcím za vzniku glyoxalu v případě nasycené *n*-oxokyseliny a malonaldehydu v případě nenasycené *n*-oxokyseliny s dvojnou vazbou v konjugaci s *n*-oxoskupinou) (obr. 20). (Velasco J. a kol., 2004; Minamoto S. a kol.,1988; Pamplona R., 2011; Kyselka J. a kol., 2020; Vistoli G. a kol., 2013).



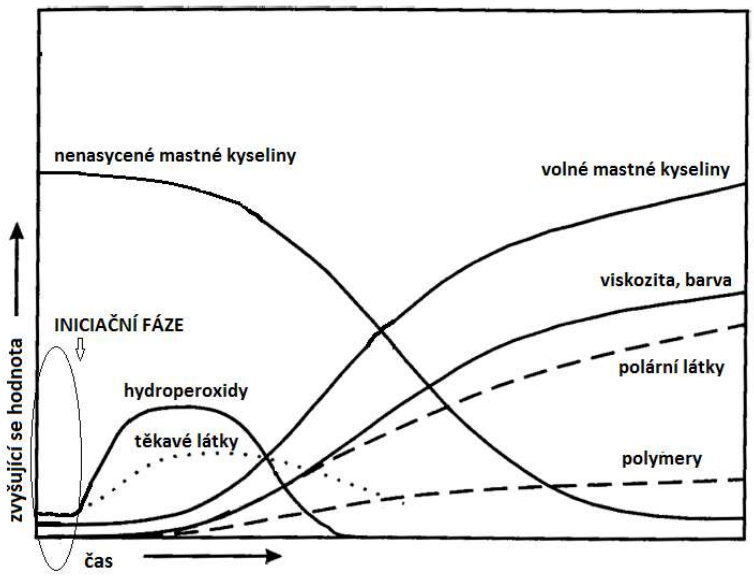
Obr. 20: Triacylglycerol obsahující nasycenou 9-oxononanovou kyselinu (A) a nenasycenou (9*Z*,11*E*)-13-oxotrika-9,11-dienovou kyselinu (B)

1.3.3 Oxypolymerace

Hydroperoxidy se mohou štěpit na alkoxylový nebo peroxylový radikál a dále se účastnit terminační fáze oxidace. Jak je uvedeno na obr. 5B a 6E, vznikají tak polymery obsahující peroxidové vazby, nebo jak je uvedeno na obr. 6F-G, vznikají polymery s etherovými vazbami. Pokud dochází k oxypolymeraci methylesteru kyseliny linolové, vznikají dimery obsahující i hydroperoxy-, hydroxy- nebo oxoskupinu již při zvýšené teplotě. Dimery s peroxidovou vazbou jsou nestabilní a mohou se dále rozkládat. Oxypolymerací methylesteru kyseliny linolenové vzniká široké spektrum dimerů a oligomerů, které obsahují konjugované dieny, trieny, dihydroperoxidy. Dimery vznikají při teplotě do 40 °C, kdežto oligomery při teplotě 150 °C. Triacylglyceroly obsahující oxidované polynenasycené mastné kyseliny podléhají dimerizaci a oligomeraci za vysokých teplot nad 210 °C (Frankel E.N., 2012; Khor Y.P. a kol., 2019)

2 PODMÍNKY ÚDRŽNOSTI LIPIDŮ

Vzhledem k tomu, že při oxidaci lipidů vzniká celá řada oxidační produktů, které jsou potencionálně zdraví škodlivé a také senzoricky negativní, je snaha udržet lipidy a především potraviny na lipidy bohaté v iniciační fázi autooxidace (obr. 21). Existuje celá řada faktorů, které ovlivní údržnost lipidů v potravinách (kap. 6). Mezi nejdůležitější z nich patří koncentrace přirozeně se vyskytujících antioxidantů, převážně tokoferolů. Vyčerpáním, tedy oxidací tokoferolů a dalších antioxidantů, končí iniciační fáze autooxidace. Jelikož ale změna obsahu tokoferolů na obr. 21 není, indikuje se tato skutečnost obvykle prudkým vzestupem obsahu hydroperoxidů (Gordon M.H., 2004; Choe E. a Min D.B., 2007).

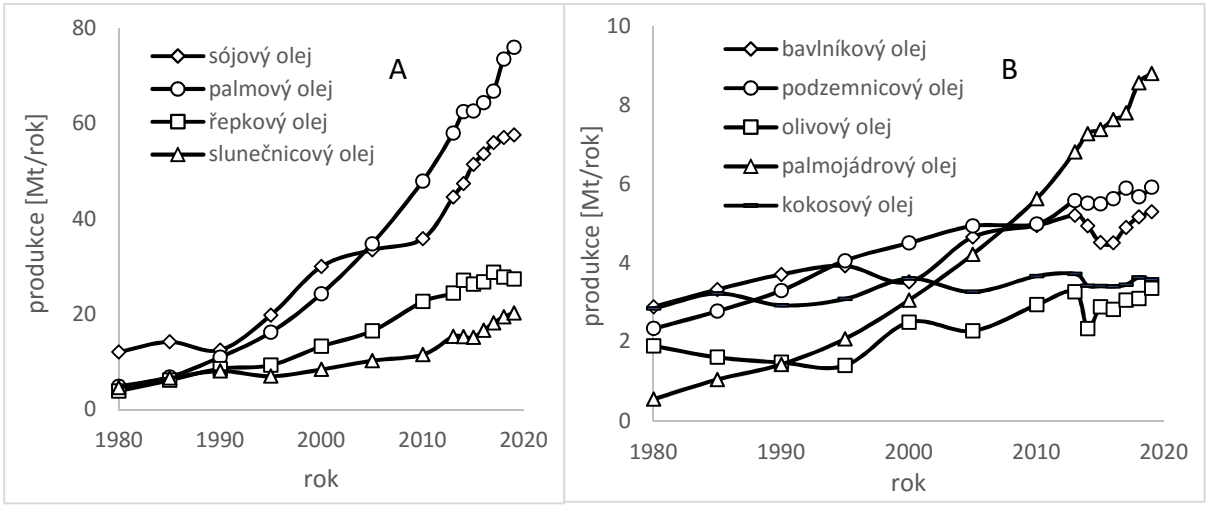


Obr. 21: Chemické a fyzikální změny lipidů během oxidace (podle Choe E. a Min.D.B., 2007)

3 SVĚTOVÁ PRODUKCE OLEJŮ

Oxidace lipidů a možnosti jejího oddálení patří k nejdiskutovanějším reakcím v oblasti lipidů i z toho důvodu, že během posledních čtyřiceti let mimořádně vzrostla produkce rostlinných olejů. Ve skupině 4 majoritních olejnin (obr. 22A) se vyskytují 3 (sójový, slunečnicový a řepkový olej), které obsahují ve vysokých koncentracích polynenasycené mastné kyseliny, a tedy velmi snadno podléhají oxidaci. Z produkce dalších 5 olejnin (obr. 22B) je z pohledu oxidace lipidů zajímavý bavlníkový olej, popř. podzemnicový olej, který ale obsahuje pouze do 20 % polynenasycených kyselin.

Produkce živočišných tuků a olejů se trvale relativně snižuje z pohledu podílu na světové produkci olejů a tuků.



Obr. 22: Světová produkce 1980 – 2019: 4 majoritních olejnin (A); dalších 5 olejnin (B) (Index Mundi, 1980 - 2019)

4 POŽADAVKY NA OLEJE A TUKY V POTRAVINÁŘSKÉM PRŮMYSLU

Pro oleje a tuky používané v potravinářském průmyslu existují mezinárodně uznávané standardy (doporučení), tzv. Codex Alimentarius. Tyto standardy stanovují určité parametry potravin tak, aby byly bezpečné. Problematiku olejů a tuků upravuje Codex Alimentarius CXS 210-1999 Standard for named vegetable oils, CODEX STAN 33-1981 Standard for olive oils and pomace oils a CXS 329-2017 Standard for fish oils.

Peroxidové číslo je obecně pro rafinované oleje stanoveno do 10 mekv.akt.O kg^{-1} a pro oleje lisované za studena a oleje panenské je to hodnota do 15 mekv akt.O kg^{-1}.

Na olivový olej se vztahuje speciální úprava a zde záleží na typu olivového oleje, pokud se jedná o panenský olivový olej, peroxidové číslo je stanoveno na hodnotu do 20 mekv.akt.O kg^{-1}, pro olivový olej a olivový olej z pokrutin je to hodnota do 15 mekv.akt.O kg^{-1} a pro rafinovaný olivový olej a rafinovaný olivový olej z pokrutin je to hodnota do 5 mekv.akt.O kg^{-1}.

Standard pro rybí oleje je stanoven na maximální hodnotu peroxidového čísla (PČ) do 5 mekv.akt.O kg^{-1}, zároveň jsou i dány další parametry, jako maximální hodnota *p*-anisidinového čísla (AČ) do 20 a vypočtený parametr - totální oxidační hodnota (TOTOX) maximálně do 26 (výpočet TOTOX = 2·PČ + AČ).

Vedle standardů Codex Alimentarius existuje v Německu přísnější a rozšířenější norma – Pokyny pro jedlé tuky a oleje, kterou vydalo německé Ministerstvo výživy a zemědělství (Bundesministerium fuer Ernaehrung und Landwirtschaft, 2020). V těchto pokynech nejsou uvedeny pouze maximální hodnoty peroxidového čísla (pro nerafinované jedlé tuky a oleje do 10 mekv.akt.O kg^{-1}, pro rafinované jedlé tuky a oleje do 5 mekv.akt.O kg^{-1}), ale také TOTOX (pro rafinované jedlé tuky a oleje rostlinného původu do 10, pro jedlé tuky a oleje rostlinného původu a lisované za studena do 20) a obsah polymerů jako důkaz tepelné zátěže pro jedlé tuky a oleje rostlinného původu a lisované za studena maximálně 0,1 %.

Použití pouze peroxidového čísla jako ukazatele oxidace lipidů není příliš vhodné z toho důvodu, že se hydroperoxidy rozkládají na sekundární oxidační produkty a nízká hodnota peroxidového čísla může vypovídat o vzorku to, že je buď v počátečních fázích oxidace, nebo naopak už ve velmi pokročilém stavu oxidace, kdy už došlo k rozštěpení vzniklých hydroperoxidů (obr. 21). Je to tedy významný, ale ne vše vypovídající parametr. Zároveň je ale stanovení peroxidového čísla jednoznačné, oproti stanovení obsahu tokoferolů, jež o vzorku více vypovídá. Stanovení obsahu tokoferolů je ale komplikované ve srovnání se stanovením obsahu hydroperoxidů.

Vypočtená hodnota TOTOX je svým způsobem pokus o popsání složitých jevů relativně jednoduchými nástroji, což je snadno použitelné v praxi. Z matematického hlediska je TOTOX korelační vztah, protože obsah hydroperoxidů vyjadřuje látkové množství, kdežto obsah karbonylů vyjádřený pomocí *p*-anisidinového čísla má jednotku [1], jelikož je stanovována absorbance látek s různým molárním absorpčním koeficientem.

Použití TOTOX hodnoty není úplně vhodné, jelikož deodorací (fyzikální rafinací) oxidovaného tuku/oleje dojde rozkladu hydroperoxidů a k odstranění těkavých látek ze vzorku, tedy i těkavých 2-alkenalů, a vzorek vykazuje nižší TOTOX.

Pro potravinářskou praxi je nutné použít kombinaci několika metod - peroxidové číslo, *p*-anisidinové číslo, stanovení OSI (Oil Stability Index – AOCS Cd 12b-92), který se u vzorku po oxidaci a následné deodoraci významně sníží. OSI je možné měřit komerčně dostupnými přístroji Rancimat Metrohm Ltd. (kap. 5.6) a Oxidative Stability Instrument Omnion Inc. Stanovení polárních látek se jeví také jako možné, jelikož to nejsou těkavé sloučeniny a ve vzorku zůstávají i po deodoraci (Shahidi F. a Zhong H.J., 2020).

5 METODY PRO STANOVENÍ OXIDACE LIPIDŮ

Z kapitoly 1.1 je zřejmé, že během autooxidace lipidů dochází k tvorbě rozmanitých produktů a neexistuje pouze jedna standardní metoda ke stanovení všech oxidačních změn pro různé potravinářské systémy.

Obecně mohou být metody pro stanovení oxidačních změn lipidů rozděleny do pěti skupin – metody stanovující absorpci kyslíku jako hlavního reaktantu, metody pro stanovení ztrát výchozích surovin, metody stanovení tvorby volných radikálů, tvorby primárních a sekundárních oxidačních produktů. Přístup k jejich stanovení může být různý, buď klasickými analytickými metodami nebo instrumentálními metodami (Shahidi F a Zhong H.J., 2020).

Významnou roli v metodách pro stanovení oxidace lipidů hrají i zrychlené metody pro určení oxidační stability z důvodu predikce údržnosti potravin.

5.1 STANOVENÍ ABSORPCE KYSLÍKU

K metodám, jež sledují absorpci kyslíku, patří přímá metoda stanovení změny tlaku kyslíku v plynné fázi nad vzorek nebo nepřímá metoda stanovení zvýšení hmotnosti vzorku v počátečních fázích oxidace. Důležité je omezení na počáteční fázi oxidace, jelikož vznikající hydroperoxidy se rozkládají na těkavé oxidační produkty a na změně hmotnosti vzorku se podílí jak vznikající hydroperoxidy, tak i vznikající těkavé sekundární oxidační produkty, které hmotnost vzorku naopak snižují. Princip této metody je využit u komerčně dostupných přístrojů Oxidograf a Oxipres, které stanovují oxidační stabilitu při zvýšené teplotě, kap. 5.6 (Antolovich M. a kol., 2002).

5.2 STANOVENÍ ZTRÁT VÝCHOZÍCH SUROVIN

Změnu obsahu výchozích sloučenin (obsah nenasycených mastných kyselin) je možné stanovit plynovou chromatografií s krokem předúpravy vzorku, ve kterém proběhne převedení triacylglycerolů na těkavější methylestery mastných kyselin. Během oxidace lipidů se přednostně degradují nenasycené mastné kyseliny v závislosti na počtu dvojných vazeb v molekule, dochází ke konjugaci a *cis/trans* izomeraci (Cihelková K. a kol., 2009). Druhou možností je přímá analýza triacylglycerolového složení vzorků kapalinovou chromatografií s reverzí fází. Koncentrace triacylglycerolů, které obsahují nenasycené mastné kyseliny, se

během oxidace snižuje a zároveň vznikají tzv. polární látky, obr. 21 (Ruiz-Gutiérrez V. a Barron L.J.R., 1995; Choe E. a Min.D.B., 2007).

5.3 STANOVENÍ VOLNÝCH RADIKÁLŮ

Během iniciační fáze oxidace lipidů jsou generovány volné radikály, které je možné stanovit elektronovou paramagnetickou rezonancí. Tato metoda je relativně nová a je založena na paramagnetických vlastnostech nepárových elektronů v radikálech. U vzorku umístěného do magnetického pole je měřena absorpce elektromagnetického záření (Andersen M.L. a Skibsted L.H.,2002).

5.4 STANOVENÍ PRIMÁRNÍCH OXIDAČNÍCH PRODUKTŮ

Primární oxidační produkty, hydroperoxidy, jsou nejčastěji stanovovaným oxidačním produktem, a to pomocí peroxidového čísla. Jak již bylo uvedeno, peroxidové číslo se používá jako jeden z hlavních ukazatelů kvality (z hlediska oxidace) potravinářských tuků a olejů. Použití peroxidového čísla jako jediného ukazatele rozsahu oxidace lipidů není správné z toho důvodu, že hydroperoxidy se dále mění na sekundární oxidační produkty, jeho použití má smysl pouze v počáteční fázi oxidace (obr. 21). Stanovení peroxidového čísla je standardní IUPAC i ISO metoda založená na jodometrické titraci. Obvykle používané jednotky peroxidového čísla jsou milielikvivalenty aktivního kyslíku (O) na 1 kg oleje (mekv.akt.O kg^{-1}). Je možné použít i SI jednotky – milimoly aktivního kyslíku na 1 kg oleje (mmol akt.O kg^{-1}), tato hodnota je poloviční než hodnota vyjádřená v mekv.akt.O kg^{-1}. Další možností je vyjádření v miligramech aktivního kyslíku na 1 kg oleje (mg akt.O kg^{-1}), což je 8 násobek hodnoty peroxidového čísla vyjádřeného v mekv.akt.O kg^{-1} (IUPAC 2.501; ISO 3960:2007(E)).

Spektrofotometrickým stanovením konjugovaných dienů a trienů (IUPAC 2.206) se sleduje vznik konjugovaného systému dvojných vazeb z přirozeně se vyskytujícího pentadienového systému dvojných vazeb. Během vzniku hydroperoxidů dochází k polohové izomeraci dvojných vazeb, s ní je spojená i prostorová *cis/trans* izomerace. V iniciační fázi oxidace, u systémů bohatých na kyselinu linolovou, koreluje obsah konjugovaných dienů s hodnotou peroxidového čísla (Marmesat S. a kol., 2009).

5.5 STANOVENÍ SEKUNDÁRNÍCH OXIDAČNÍCH PRODUKTŮ

Z primárních oxidačních produktů vzniká široké spektrum sekundárních oxidačních produktů, které jsou směsí těkavých, netěkavých a polymerních sloučenin, které zahrnují aldehydy, ketony, uhlovodíky, alkoholy, těkavé organické kyseliny, epoxysloučeniny, cyklické substituované ethery s pěti- a šestičlenným kruhem atd.

Do klasických analytických metod patří stanovení *p*-anisidinového, thiobarbiturového čísla nebo obsah karbonylových sloučenin. Tyto metody jsou založené na spekrotofotometrickém stanovení barevných látek vzniklých po reakci aldehydů (především 2-alkenalů) s *p*-anisidinem (*p*-methoxyanilín) v případě *p*-anisidinového čísla (ISO 6885:2016), v případě thiobarbiturového čísla (AOCS Cd 19-90) dochází k reakci malonaldehydu s kyselinou thiobarbiturovou a v případě stanovení obsahu karbonylových sloučenin reagují aldehydy a ketony s 2,4-dinitrofenylhydrazinem (Endo Y. a kol., 2001).

TOTOX číslo (*1*) je pouze matematickým modelem, je to pokus o vyjádření tzv. totální oxidace, v němž jsou zahrnuty jak primární (peroxidové číslo – PČ), tak i sekundární (*p*-anisidinové číslo – AČ) oxidační produkty. Je nutné zopakovat, že toto číslo nemá vědecký základ, že je to pouze kombinace dvou parametrů, které jsou navzájem neslučitelné. Nicméně citované německé standardy (kap. 4) ukazují, že se jedná o silný nástroj pro definování kvality olejů (Shahidi F. a Zhong H.J., 2020).

$$\text{TOTOX} = 2 \cdot \text{PČ} + \text{AČ} \qquad (1)$$

Instrumentálními metodami je možné stanovení těkavých sekundárních oxidačních produktů ať už přímo ze vzorku nebo v parní fázi nad vzorkem metodou plynové chromatografie (solid phase microextraction - SPME/GC nebo headspace sampler - HSS/GC). Netěkavé *n*-oxokyseliny vázané na triacylglyceroly je možné stanovit přímo jako polární látky kapalinovou chromatografií s reverzí fází (AOCS Ce 5b-89, Hrádková I. a kol.,2008) nebo po předúpravě vzorku převedením na methylestery pomocí plynové chromatografie po derivatizaci nebo chromatografické separaci. Polymery vzniklé při oxidaci lipidů je možné stanovit gelovou permeační kapalinovou chromatografií (Cihelková K. a kol., 2009; AOCS Cd 22-91).

Senzorická analýza je jednou z hlavních metod pro rozhodnutí, zda potravina obsahující lipidy je stále ještě vhodná ke konzumaci či nikoli. U oxidovaných olejů se při senzorickém popisu vzorků používají pojmy spojené s chutí/vůní jako máslová, oříšková, trávová nebo rybí

a pojmy spojené se zpracováním jako hydrogenovaný, oxidovaný nebo zatuchlý (Shahidi F. a Zhong H.J., 2020).

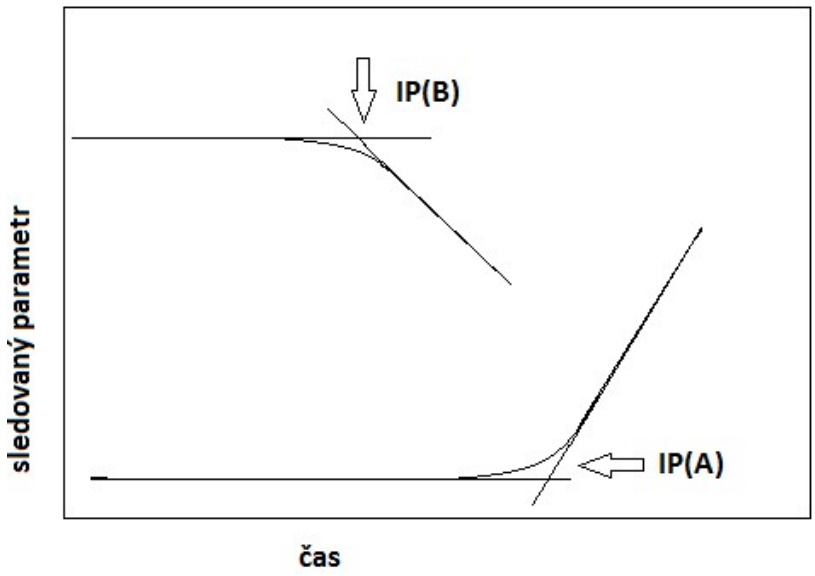
5.6 ZRYCHLENÉ METODY PRO PREDIKCI ÚDRŽNOSTI LIPIDŮ, STANOVENÍ OXIDAČNÍ STABILITY

Metody pro stanovení rozsahu oxidace lipidů uvedené v kapitole 5.1 až 5.5 popisují stav potravin/vzorků obsahujících lipidy v určitém okamžiku, ale nevypovídají o tom, jaká bude udržitelnost výrobku při dlouhodobém skladování nebo při tepelné zátěži (např. pečení, fritování nebo smažení). Z tohoto důvodu se používají tzv. zrychlené metody pro stanovení oxidační stability, které probíhají za zvýšených až vysokých teplot a za zvýšeného nebo intenzivního přístupu kyslíku, tak aby analýza trvala řádově několik hodin. Stanovuje se indukční perioda, což je čas od začátku analýzy až do doby, kdy dochází k významné tvorbě oxidačních produktů nebo poklesu tlaku kyslíku nad vzorkem.

Metody pro stanovení oxidační stability lipidů se mohou rozdělit do dvou skupin. První typ metod je založen na stanovení vzniklých oxidačních produktů (obr. 23- IP(A)) a druhý typ na stanovení spotřeby kyslíku na vzorkem (obr. 23- IP(B)).

Schaalův test je řazen do první skupiny metod a je založen na skladování vzorku při teplotě 60 °C a průběžného monitorování peroxidového číslo, senzorické analýzy nebo těkavých oxidačních produktů plynovou chromatografií (AOCS Cg 5-97). Metodou AOM („Active Oxygen Method“) se stanovuje peroxidové číslo vzorku při teplotě 98 – 100 °C a za současného probublávání vzduchem (AOCS Cd 12-57). Rancimat (firma Metrohm, Švýcarsko) je komerčně dostupný přístroj, který vyhřívá vzorek na teplotu 80 °C a více (ve většině případů se používá teplota 110 °C nebo 120 °C) a zároveň jej probublává vzduchem. Těkavé látky (především kyselina mravenčí a octová) jsou unášeny z oxidovaného vzorku vzduchem do destilované vody, kde se rozpouští a zvyšují vodivost destilované vody. Výsledkem je indukční perioda, tedy čas, kdy nedochází ke změnám vodivosti destilované vody (AOCS 12b-92).

Do druhé skupiny metod je řazena metoda „Oxygen bomb“, která je založená na monitorování poklesu tlaku kyslíku nad vzorkem v uzavřené nádobě při teplotě kolem 100 °C. Komerčně dostupné přístroje s automatickým ohřevem a záznamem tlaku jsou Oxidograf a Oxipres firmy Mikrolab Aarhus A/S, Dánsko (Nogala-Kalucka M. a kol., 2005; Trojáková L. a kol., 2001).



Obr. 23: Stanovení indukční periody – IP(A) – vznik oxidačních produktů, IP(B) – spotřeba kyslíku

Stanovení indukční periody (IP) při určité teplotě je hodnota, která vypovídá o vzorku pouze při dané teplotě. K predikci dlouhodobé údržnosti lipidů se vychází z principů rovnovážné termodynamiky, aplikuje se závislost reakční rychlosti (v tomto případě indukční periody - IP) reakce 1. řádu na teplotě podle Arrheniova vztahu (A – frekvenční faktor, E_a – aktivační energie, T – teplota) a předpokládá se platnost lineárního vztahu (*2*). Sledovaný vzorek je nutné stanovit při dvou různých teplotách a pomocí Arrheniovy rovnice extrapolovat výsledky na teplotu skladování, což je pouze orientační hodnota. Častěji se ale indukční perioda stanoví pouze při jedné teplotě (např. 120 °C) a indukční perioda při teplotě skladování se vyhodnotí na základě databanky výsledků.

$$\ln IP = \ln A - \frac{E_a}{RT} \qquad (2)$$

Ukazuje se, že aplikace principů nerovnovážné termodynamiky vede k přesnějšímu vztahu mezi hodnotou IP za zvýšené teploty (např. 120 °C) a hodnotou IP při skladování (Malvis A. a kol., 2019).

6 OVLIVNĚNÍ OXIDACE LIPIDŮ

Na rychlost oxidace lipidů ve vzorcích mohou mít vliv různé faktory, mezi nejdůležitější patří typ použitého tuku nebo oleje, a tedy z toho vyplývající zastoupení mastných kyselin, které oxidaci podléhají; obsah přirozeně se vyskytujících minoritních látek, především antioxidantů, které naopak oxidaci lipidů oddalují; typ vzorku, zda se jedná o jednofázový nebo vícefázový systém, například emulze; a v neposlední řadě i přítomnost dalších přidaných látek jako například emulgátorů, antioxidantů nebo prooxidantů. Důležitou roli hrají i podmínky skladování a dostupnost kyslíku. U dostupnosti kyslíku se nejedná pouze o typ balení výrobku, ale také o počáteční podmínky - obsah rozpuštěného kyslíku a o tzv. oxidační historii – v jakém rozsahu oxidace lipidů proběhla během výroby a zpracování olejů.

6.1 TYP SUROVINY

Dle typu tuku nebo oleje lze predikovat i jeho údržnost. Jak bylo zmíněno v kapitole 1.1.1, s počtem dvojných vazeb mastných kyselin obsažených ve vzorku roste i rychlost oxidace lipidů. Zastoupení přirozeně se vyskytujících antioxidantů je také velmi důležité, čím vyšší koncentrace těchto látek v surovině, tím je průběh oxidace pomalejší. Přirozeně vyšší obsah antioxidantů je u surovin s vyšším obsahem polynenasycených mastných kyselin (např. sójový olej) a naopak nižší obsah antioxidantů u surovin s nasycenými mastnými kyselinami (např. hovězí lůj, kokosový tuk), tab. II. Srovnání obsahu tokoferolů u různých vzorků v odlišných publikacích je ovšem problematické, pokud není uveden postup izolace lipidů. Existují totiž různé metody pro izolaci lipidů ze vzorků, které se liší šetrností při manipulaci se vzorkem s ohledem na velice oxidačně labilní antioxidanty.

Během skladování semen, výroby a zpracování olejů a tuků dochází ke ztrátám antioxidantů a k iniciaci oxidace lipidů. Rozsah změn vypovídá o šetrnosti těchto kroků. Z kvalitní počáteční suroviny pro výrobu oleje nebo tuku, šetrného získání a zpracování se získá olej nebo tuk s vysokým obsahem antioxidantů a minimální iniciací oxidace lipidů (Frankel E. N., 1998)

Tab. II: Obsah nasycených (SFA), mononenasycených (MUFA) a polynenasycených mastných kyselin (PUFA) a přirozený obsah tokoferolů (TO) ve vybraných olejích (Codex Alimentarius CXS 210-1999[a]; Choe E. a Lee J., 1998[b]). Obsah tokoferolů stanoven metodou AOCS Ce 8-89.

	Obsah mastných kyselin [%]			**Obsah tokoferolů [mg kg^{-1}]**		
Olej/tuk	**ΣSFA**	**ΣMUFA**	**ΣPUFA**	**α-TO**	**β- + γ-TO**	**δ-TO**
Hovězí lůj	44,7[b]	46,0[b]	4,9[b]	30[b]	4[b]	ND[b]
Kokosový tuk	81,0-90,0[a]	5,0-10,0[a]	1,0-2,5[a]	0-17[a]	0-28[a]	ND[a]
Slunečnicový olej	8,0-14,0[a]	14,0-39,0[a]	49,0-74,0[a]	403-935[a]	0-79[a]	0-7[a]
Sójový olej	10,0–19,0[a]	17,0-30,0[a]	52,5-70,0[a]	9-352[a]	89-3239[a]	154-932[a]
				37[b]	459[b]	ND[b]

ND – nedetekováno

6.2 ROLE MEZIFÁZOVÉHO ROZHRANÍ A TYP VÝROBKU

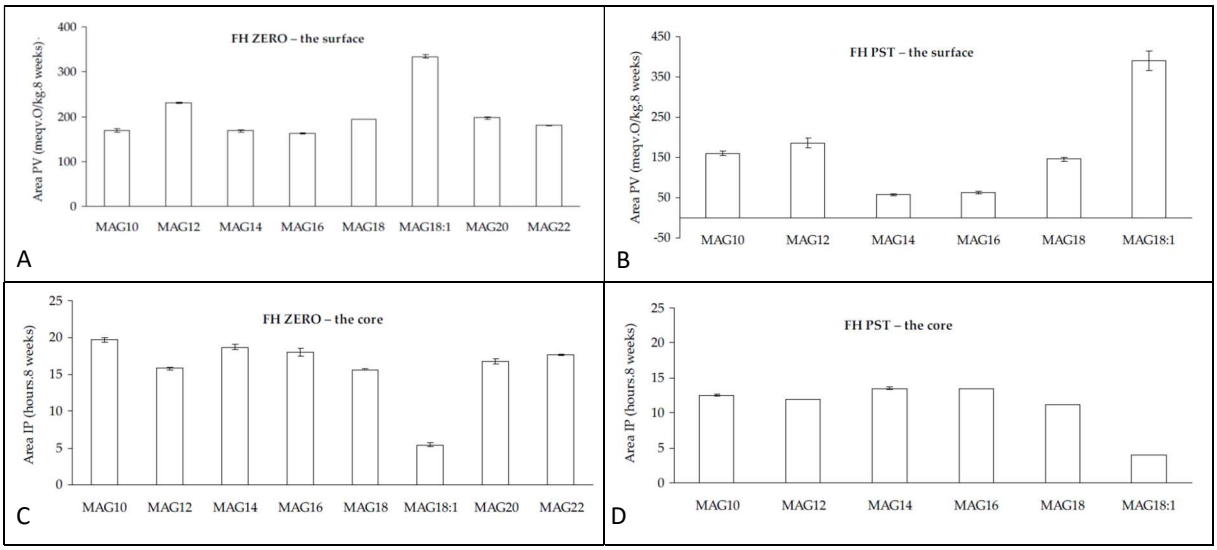
U jednofázových výrobků typu olej je popis difúze kyslíku jako hlavního reaktantu jednoduchá, vyskytuje se zde pouze jedno fázové rozhraní olej/vzduch a směr difúze kyslíku je ze vzduchu do oleje. Pokud se v systému vyskytuje více mezifázových rozhraní, např. emulze nebo disperze tukových krystalů v oleji, difúze kyslíku probíhá přes několik fázových rozhraní. Skutečnost, že se emulgátor samovolně hromadí, přesněji dochází k orientované adsorpci molekul povrchově aktivní látky, a vzniká tak vrstva emulgátoru, která nakonec odděluje obě fáze, je důsledkem snahy snížit povrchovou energii mezifázového rozhraní ($\Delta G_{(\sigma)} < 0$) a dochází k poklesu mezifázového povrchového napětí $(\partial\sigma/\partial c)_T < 0$ (Atkins P. a de Paula J., 2013)

6.2.1 Disperze tukových krystalů v oleji

Za disperzi tukových krystalů v oleji jsou považovány shorteningy a tukové fáze margarínů, tedy tukové násady. Krystaly tuku v oleji mohou být různě uspořádány, buď se mohou vyskytovat samostatně (obvykle polymorfy β, které jsou větší a minimálně asociují), nebo menší krystaly (obvykle polymorfy β´) se schopností asociovat, vytvářet tak limitně druhou spojitou fázi – oleogel. Pokud je do systému přidán emulgátor, hromadí se přednostně na fázovém rozhraní krystaly tuku/olej a tuková disperze/vzduch (Chawla P. a kol., 1990).

Při výrobě shorteningů/pokrmových tuků se používá monoacylglycerolový emulgátor (MAG) jako modulátor krystalizace. Standardně se používá směs mono- a diacylglycerolů. Doba spotřeby těchto výrobků se odvíjí od rychlejšího ze dvou dějí – rekrystalizace a oxidace lipidů.

Byl zkoumán vliv délky acylu MAG emulgátoru na rychlost oxidace lipidů v modelové tukové násadě s definovaným složením krystalů tukové disperze. Pokud jsou tukové krystaly disperze tvořeny plně ztuženým bezerukovým řepkovým olejem (FH ZERO s TAG složením - tristearoylglycerolem 80 % mol. a palmitoyldistearoylglycerolem 15,5 % mol.) nebo plně ztuženým palmstearinem (FH PST s TAG složením - dipalmitoylstearoylglycerol 42,5 % mol., palmitoyldistearoylglycerol 23,6 % mol, tristearoylglycerol 23,6 % mol., tripalmitoylglycerol 4,5 % mol.) jsou trendy vzniku hydroperoxidů a hodnoty indukční periody podobné. Kyslík difunduje tukovou násadou obsahující monooleoylglycerol (MAG18:1) nejrychleji ze všech zkoumaným MAG emulgátorů (maximální vznik hydroperoxidů – obr. 24A-B a minimální hodnota indukční periody – obr. 24C-D). MAG18:1 obsahuje jednu *cis* dvojnou vazbu. Přítomnost této vazby způsobí ohyb jinak přímého acylového řetězce o 42 °, tím naruší těsné uspořádání acylů nasycených mastných kyselin na mezifázovém rozhraní, dojde tak k rychlejší difúzi kyslíku tukovou násadou. Těsnější uspořádání emulgátoru na mezifázových rozhraní a vytvoření účinné bariéry koresponduje s minimálním rozsahem oxidace, jak bylo zjištěno u monoacylglycerolů s nasyceným acylovým řetězcem (především u MAG14 a MAG16). S rostoucím acylovým řetězcem (MAG18, MAG20 a MAG22) již rychlost oxidace neklesá (Spěváčková V. a kol., 2009; Spěváčková V. a kol., 2012 ; Patino J.M.R. a kol., 1993; Patino J.M.R. a kol., 2001).



Obr. 24: Plocha pod křivkou peroxidového čísla modelové tukové násady se strukturním tukem plně hydrogenovaný bezerukový řepkový olej – FH ZERO (A), plně hydrogenovaný palmstearin – FH PST (B); plocha pod křivkou indukční periody modelové tukové násady se strukturním tukem plně hydrogenovaný bezerukový řepkový olej (C), plně hydrogenovaný palmstearin (D) (Spěváčková V. a kol., 2009)

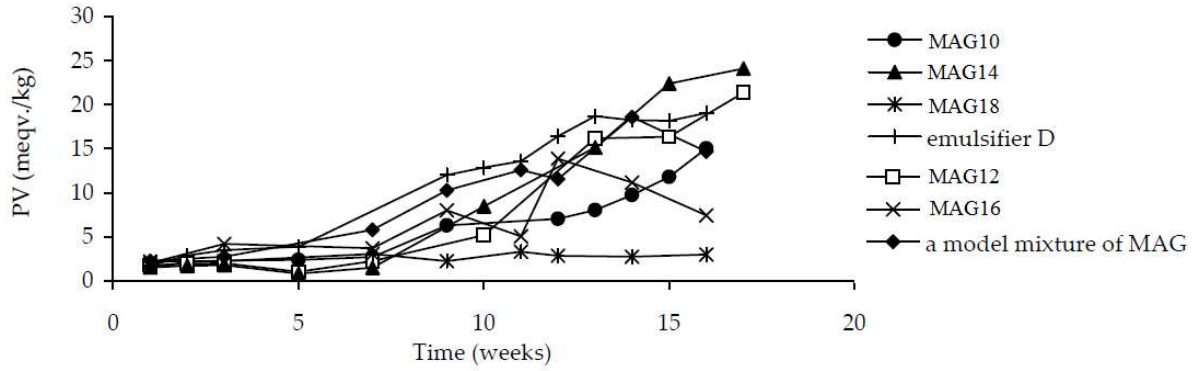
6.2.2 Mikrodisperzní soustava margarínového typu

Disperzní soustavy jsou obecně rozdělovány dle velikosti částic na analytické disperze (velikost částic menší než 1 nm), koloidně disperzní soustavy (velikost částic 1 – 1000 nm) a hrubé disperzní soustavy, které se dále dělí na mikrodisperze (velikost částic 1000 nm – 50000 nm) a makrodisperze (velikost částic větší než 50000 nm). U margarínové emulze, která je typu voda v oleji (v/o) se vyskytují 3 různé fáze – vodná fáze jako disperzní podíl vůči olejové fázi se střední velikosti části 4 – 6 μm, v tomto případě se jedná o mikrodisperzi; tuhá fáze ve formě krystalů TAG s velikosti částic 0,1 – 1 μm (ideálně polymorf β´) a sférulity, jehlice nebo klastry s velikostí částic větší než 1 μm, tedy opět mikrodisperzní soustava, ideálně oleogel, tedy spojitá fáze uvnitř olejové fáze. Její obsah lze v závislosti na teplotě měřit jako „obsah tuhých podílů“. V případě tvorby oleogelu tuhou fází TAG jsou omezeny změny velikosti kapek vodné fáze, dochází tedy ke stabilizaci disperzního podílu emulze. Emulgátor se vyskytuje v těchto systémech jak na fázovém rozhraní krystaly TAG/kapalný olej, tak i na mezifázovém rozhraní olej(tuk)/voda (Silva T.J. a kol, 2020; Bayés-García L. a kol., 2011; Nguyen V. a kol., 2020; Chawla P. a kol., 1990).

Miura S. a kol. (2002a, b) se zabývali stabilitou emulzí typu olej ve vodě v závislosti na délce acylu použitého MAG emulgátoru. Zjistili, že v závislosti na délce nasyceného acylu

dochází k různému hromadění výše tajicích triacylglycerolů kolem emulgátoru, který se vyskytuje na fázovém rozhraní olej/voda a dochází tak i destabilizaci emulze, u monooleoylglycerolu (nenasyceného MAG emulgátoru) k tomuto jevu nedocházelo. Destabilizace emulze závisela i na použité tukové fázi emulze.

Mikrodisperzní soustavy margarínového typu (voda v oleji) byly zkoumány z hlediska vlivu délky acylového řetězce MAG emulgátoru na rychlost oxidace lipidů (Pokorná I. a kol., 2004). Ze závislosti peroxidového čísla na čase (obr. 25) byl zjištěn nejvyšší nárůst hydroperoxidů u emulzí se směsí monoacylglycerolů v komerčním emulgátoru D (směs palmitoyl-, stearoyl- , oleoyl- a linoylglycerolu). Přítomnost oleylglycerolu a v menší míře i linoylglycerolu s *cis* dvojnými vazbami má za následek to, že na mezifázových rozhraních nedochází k tak těsnému uspořádání jako v případě použití pouze 1 typu emulgátoru a s nasyceným acylovým řetězcem. Nejúčinnější bariéru vůči difúzi kyslíku (minimální oxidace) vytvořil monostearoylglycerol, tedy jeho předpokládaná monomolekulární vrstva, která tvoří mezifázové rozhraní.

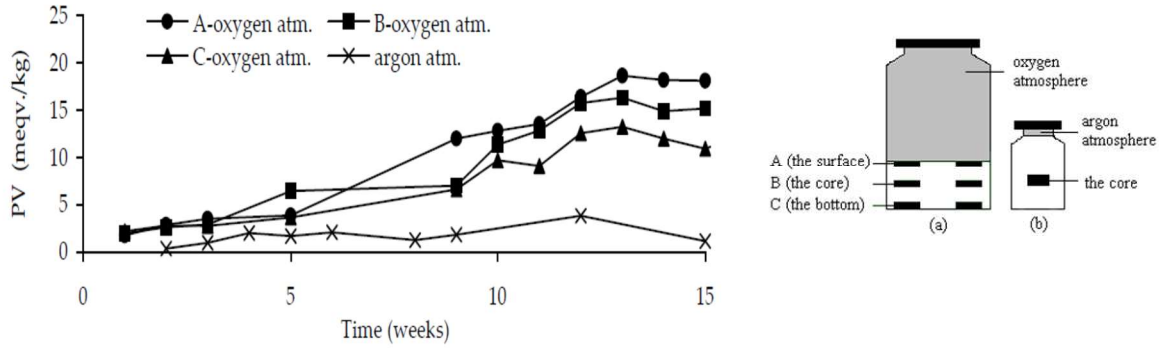


Obr. 25: Peroxidové číslo margarínové emulze obsahující MAG emulgátor s různou délkou acylu (C10-C18, emulsifier D – směs MAG16, MAG18 a MAG18:1, a model mixture of MAG – směs MAG10, MAG12 a MAG14), teplota skladování 15 °C (Pokorná I. a kol., 2004)

6.3 PODMÍNKY SKLADOVÁNÍ

Oleje a tuky nebo výrobky oleje a tuky obsahující jsou různě dlouhou dobu skladovány. Aby rozsah oxidace ve výrobcích byl minimální, musí se zvolit vhodné podmínky při výrobě (minimalizace rozpuštěného kyslíku ve výrobku) a především vhodné podmínky skladování (zamezení přístupu kyslíku a světla).

Jelikož je kyslík hlavním reaktantem při oxidace lipidů a vyskytuje se přirozeně v atmosféře, je tedy snaha výrobců o jeho minimalizaci ve výrobku vytvořením inertní atmosféry a volbou vhodného obalu. Atmosféra nad výrobkem významně ovlivní rychlost oxidace. Při srovnání skladování margarínové emulze v kyslíkové atmosféře a inertní (argonové) atmosféře (obr. 26) bylo zjištěno, že vzorek skladovaný pod kyslíkovou atmosférou oxidoval významně rychleji. Ze závislosti peroxidového čísla na čase je zřejmé, že dochází k postupné spotřebě rozpuštěného kyslíku ve vzorku (do 5. týdne skladování), není významný rozdíl mezi jednotlivými vrstvami výrobku. V dalším průběhu skladování je zaznamenána snižující se tendence tvorby hydroperoxidů směrem od vrchní ke spodní vrstvě v důsledku postupné difúze kyslíku přes rozhraní atmosféra/výrobek a mezi jednotlivá mezifázová rozhraní ve výrobku. Na oxidaci výrobku skladovaného v interní atmosféře se podílí pouze kyslík rozpuštěný ve výrobku, oxidace lipidů je v tomto případě minimální a zůstává v iniciační fázi (Pokorná I. a kol., 2004).

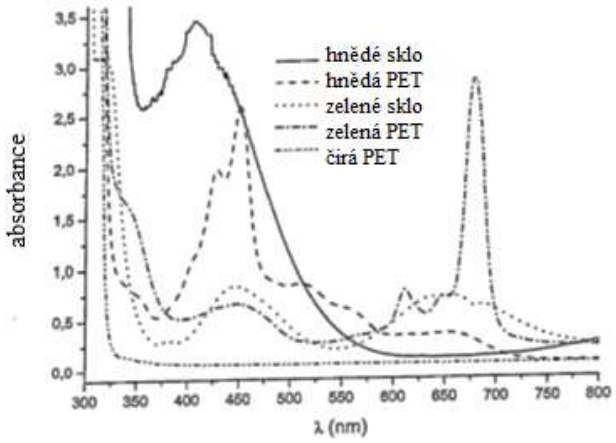


Obr. 26: Peroxidové číslo margarínové emulze s komerčním emulgátorem D skladované v kyslíkové a argonové atmosféře při teplotě 15 °C (Pokorná I. a kol., 2004)

Spolu s interní atmosférou je významným faktorem pro difúzi kyslíku do výrobku i materiál použitého obalu. Sklo je z hlediska propustnosti kyslíku ideální obalový materiál, jeho propustnost je minimální. Plastové obaly jsou v mikroměřítku sítí, která pro kyslík velice dobře propustná. Různé povrchové úpravy platových obalů je možnost, jak propustnost kyslíku obalem snížit, ale nevyrovnají se bariérovým vlastnostem skla (Michiels Y. a kol., 2017). Vedle kvality obalového materiálu hraje také důležitou roli jeho barva (obr. 27). Hnědé sklo absorbuje v oblasti 350 – 400 nm maximálně, vliv zelené barvy je minimální (Sekretár S. a kol., 2010).

Světlo v kombinaci s fotosenzibilizátory, které se přirozeně vyskytují v potravinách, se podílí na vzniku singletového kyslíku (viz kapitola 1.2). Rychlost oxidace vzorků skladovaných

ve tmě 8 – 12krát pomalejší oxidaci než u vzorků skladovaných na světle (Sekretár S. a kol., 2008).



Obr. 27: UV-VIS spektra obalových materiálů (podle Sekretár S. a kol., 2010)

6.4 ANTIOXIDANTY

Jednou z možností, jak oddálit oxidaci lipidů, přesně prodloužit indukční periodu, je použití antioxidantů. Za posledních 70 let se zájem o použití antioxidantů v potravinách mnohonásobně zvýšil, jelikož se častěji při výrobě potravin používají tuky a oleje rostlinného původu se zvýšenými obsahy polynenasycených mastných kyselin a zároveň použití syntetických antioxidantů je limitováno. Od devadesátých let 20. století spotřebitelé začínají vnímat aditivní látky v potravinách s určitou obavou z hlediska bezpečnosti potravin. Především syntetické aditivní látky, včetně syntetických antioxidantů, byly a jsou vnímány negativně. Z toho důvodu byl potravinářský průmysl motivován k hledání přírodních alternativ nebo k přípravě jejich modifikovaných analogů (Brewer M.S., 2011).

Obecně jsou antioxidanty definovány jako jakákoli látka, která když je přítomna v nízké koncentraci, ve srovnání s oxidovaným molekulami, může oddálit nebo zabránit oxidaci molekul, ať už sama tato látka nebo její oxidační produkty anebo chránit organismus před škodlivými účinky oxidativního stresu. Oxidaci mohou podléhat lipidy, sacharidy, DNA, RNA a proteiny (Senanayake S.P.J.N. a kol., 2020).

6.4.1 Výživová doporučení a antioxidanty v potravinářství z hlediska legislativy

Antioxidanty v potravinách (*in vitro*) prodlužují jejich trvanlivost a zároveň, pokud nejsou spotřebované během skladování, zvyšují i nutriční kvalitu potravin, jelikož řada z nich se uplatní *in vivo* jako vitamíny (příkladem jsou vitamín C – kyselina askorbová a vitamín E - tokoferoly).

Podle vědeckého stanoviska Evropského úřadu pro bezpečnost potravin (EFSA) se doporučuje pro muže denní příjem vitamínu C zvýšit až na 110 mg den^{-1} (jako tzv. PRI – Population Reference Intake), tento příjem je odhadován jako dostačující pro pokrytí potřeb téměř všech zdravých jedinců (97 – 98 %), pro ženy je to 95 mg den^{-1}. V tomto doporučeném denním příjmu je zahrnuta potřeba udržení koncentrace askorbátového aniontu v krevní plasmě na hodnotě 50 μmol l^{-1}, metabolická ztráta 50 mg den^{-1}, 80% gastrointestinální absorpce v organismu, vyloučení močí vitamínu C z 25 % a navýšení této potřeby o 10 % (EFSA, 2013). U vitamínu E, za který se považuje pouze α-tokoferol, je přiměřený denní příjem (AI – Adequate Intake) stanoven na 13 mg pro muže a 11 mg pro ženy. Tato doporučení vychází ze stravovacích návyků zdravé populace bez zjevného nedostatku vitamínu E (EFSA, 2015).

Pro občany České republiky Společnost pro výživu uvádí na svých internetových stránkách výživová doporučení z 6. dubna 2012 pro vitamín C 100 mg den^{-1} a pro vitamín E pouze zvýšení jeho příjmu (Dostálová J., 2012).

Výživová doporučení pro USA platná pro roky 2015 – 2020 uvádějí doporučený denní příjem pro vitamín C 90 mg pro muže a 75 mg pro ženy, vitamínu E 15 mg jako α-tokoferol denně pro obě pohlaví (Dietary Guidelines for Americans 2015-2020).

Z výživových doporučení je zřejmý trend navyšování denního příjmu vitamínu C.

Z hlediska legislativy je přesně dáno, které látky se smí použít jako antioxidanty (primární nebo sekundární) v potravinách a zároveň i jejich maximální množství v daném typu potraviny. V tabulce III je uveden přehled všech antioxidantů, které mohou být použity v potravinách (Nařízení Evropského parlamentu a Rady (ES) č.1333/2008). V kosmetických přípravcích upravuje možnost použití antioxidantů a jejich maximální koncentrace Nařízení Evropského parlamentu a Rady (ES) č. 1223/2009.

Tab. III: Přehled primárních a sekundárních antioxidantů, které mohou být použity v potravinách

Název	E-kód	Název	E-kód
Kyselina askorbová	E 300	Dodecylgallát	E 312
Askorbát sodný	E 301	Kyselina erythorbová (isoaskorbová)	E 315
Askorbát vápenatý	E 302	Erythorban sodný	E 316
Estery mastných kyselin s kyselinou askorbovou	E 304	Butylhydroxyanisol	E 320
Extrakt s obsahem tokoferolů	E 306	Butylhydroxytoluen	E 321
Alfa-tokoferol	E 307	Lecithiny	E 322
Gama-tokoferol	E 308	Mléčnan sodný	E 325
Delta-tokoferol	E 309	Mléčnan draselný	E 326
Propygallát	E 310	Kyselina citrónová	E 330
Oktylgallát	E 311	Kyselina vinná	E 334

6.4.2 Rozdělení antioxidantů

Antioxidanty se dělí dle původu na přírodní a syntetické a dle účinku na primární, které přerušují řetězovou reakci oxidace, a sekundární, které mají ochranný (preventivní) účinek. Některé antioxidanty mohou vstupovat do oxidace lipidů několika různými mechanismy, z toho důvodu se pak mluví o multifunkčních antioxidantech (Senanayake S.P.J.N. a kol., 2020).

Významnou charakteristikou je pak hydrofilita a lipofylita antioxidantů. Tokoferoly jako primární lipofilní antioxidanty *in vivo* chrání v prvé řadě lipidy buněčných membrán, jejichž povrch ale komunikuje s okolním hydrofilním prostředím a vniklé radikály tokochromanolů jsou reparovány reakcemi s hydrofilními antioxidanty jako je kyselina askorbová nebo glutathion. Tato interakce se využívá *in vitro* typicky v emulzích, kde lipofilní antioxidant je obsažen v tukové fázi (v emolientu kosmetického přípravku) a hydrofilní antioxidant ve vodném prostředí, ať již se jedná o emulze o/v nebo v/o. K popisované interakci dochází na mezifázovém rozhraní (Frankel E.N., 2007).

V zásadě je možné po derivatizaci převést antioxidant z formy hydrofilní na lipofilní a naopak. Prvním případem jsou estery mastných kyselin s kyselinou askorbovou (E304). Tato derivatizace má ovšem za následek, že látky vykazují povrchovou aktivitu, jsou to neionické emulgátory (mají charakteristické hodnoty HLB), což může být v emulzních systémech výhodné (Kim T.S. a kol., 2012; Budilarto E. S. a Kamal-Eldin A., 2015).

Analogicky je možné hydrofilizovat tokoferoly, protože ale při derivatizaci dojde k reakci fenolového hydroxylu, tak tato derivatizace slouží k vnesení vitaminu E do přípravku, ale tokoferol ztrácí funkci antioxidantu. Provádí se to např. acylací se sukcinylanhydridem za vzniku monoesteru tokoferolu. Vlastní hydrofylizace se provádí reakcí karboxylu s oxiranem tak, aby vznikl neionický emulgátor (solubilizátor) s vysokou hodnotou HLB (Yushkova Y.V. a kol., 2014).

Primární antioxidanty

Primární antioxidanty (Ant) zasahují do iniciační, propagační a v některých případech i do terminační fáze oxidace lipidů. V iniciační fázi reagují se vzniklými radikály za vzniku původního lipidu a radikálu antioxidantu (Ant·) (obr. 28A). V iniciační a propagační fázi oxidace lipidů reagují antioxidanty s peroxylovými radikály (ROO·) za vzniku hydroperoxidů (ROOH) nebo alkoxylovými radikály (RO·) za vzniku stabilních hydroxysloučenin a radikálu antioxidantu (obr. 28B-C). Radikál antioxidantu je stabilní, jeho standardní redukční potenciál je menší než 500 mV a jeho stabilita je zapříčiněna delokalizací elektronů na aromatickém kruhu u fenolových antioxidantů, u endiolových antioxidantů vzniká mezomerní stav – delokalizovány jsou π vazby oxoskupin a molekulový radikál je stále stabilizován γ-laktonovým kruhem (Tu Y.J. a kol., 2017; Senanayake S.P.J.N. a kol., 2020).

Radikál antioxidantu reaguje přednostně s dalším peroxylovým, alkoxylovým radikálem nebo radikálem antioxidantu než s další nenasycenou mastnou kyselinou (obr. 28D-F), (Frankel E.N., 2007).

$$R\cdot + Ant\text{-}H \rightarrow RH + Ant\cdot \quad (A)$$

$$ROO\cdot + Ant\text{-}H \rightarrow ROOH + Ant\cdot \quad (B)$$

$$RO\cdot + Ant\text{-}H \rightarrow ROH + Ant\cdot \quad (C)$$

$$Ant\cdot + ROO\cdot \rightarrow ROOAnt \quad (D)$$

$$Ant\cdot + RO\cdot \rightarrow ROAnt \quad (E)$$

$$Ant\cdot + Ant\cdot \rightarrow Ant\text{-}Ant \quad (F)$$

Obr. 28: Vznik (A, B) a reakce radikálu antioxidantu (C-F)

Primární antioxidanty jsou nejúčinnější, pokud jsou přidány k oxidovaným lipidům co nejdříve, tedy v iniciační, popř. na začátku propagační fázi oxidace. Jakmile je v lipidickém systému molárně více vzniklých radikálů, primární antioxidanty rychle odreagují, nelze již hovořit o potenciálu antioxidantu ve vztahu k lipidickému systému jako celku. Jakmile dojde k vyčerpání veškerého primárního antioxidantu, dochází k prudkému nárůstu vzniklých hydroperoxidů (Senanayake S.P.J.N. a kol, 2020).

Za určitých podmínek mohou některé primární antioxidanty působit i prooxidačně. Radikály antioxidantů reagují s další mastnou kyselinou za vzniku jejího radikálu nebo s hydroperoxidem za vzniku peroxylového radikálu (obr. 29). Prooxidační reakce antioxidantů jsou popisovány při jejich vysoké koncentraci, za vysokých teplot nebo v přítomnosti kovových katalyzátorů a dalších prooxidantů. Tyto skutečnosti tedy poměrně jednoznačně vymezují koncentrační interval, resp. rozsah teplot, kdy se látka chová ještě jako antioxidant (Martin-Rubio A.S. a kol., 2018; Senanayake S.P.J.N., 2018).

$$Ant\cdot + R\text{-}H \rightarrow Ant\text{-}H + R\cdot$$

$$Ant\cdot + ROOH \rightarrow Ant\text{-}H + ROO\cdot$$

Obr. 29: Prooxidační reakce radikálu antioxidantu

Přírodní primární antioxidanty zahrnují flavonoidy, fenolové kyseliny, karotenoidy, tokoferoly a tokotrienoly, do skupiny syntetických primárních antioxidantů jsou řazeny butylhydroxyanisol (BHA), butylhydroxytoluen (BHT), propylgallát (PG) a terciální butylhydrochinon (TBHQ).

O tom, jak bude antioxidant účinný během oxidace lipidů, s jakými sloučeninami bude reagovat a zda je možnost regenerace radikálu antioxidantu zpět na antioxidant, vypovídá tzv. standardní redukční elektrodový potenciál ($E^{0'}$), (tab. IV). Oxidovaná forma sloučenin z redox páru je schopna přijmout vodíkový atom pouze od redukované formy sloučeniny, která má nižší redox potenciál. Například radikál nenasycené mastné kyseliny může přijmout vodíkový atom od tokoferolu, ovšem už ne od hydroperoxidu mastné kyseliny. Standardní redox potenciál je závislý na pH, koncentraci a teplotě. Při srovnávání jednotlivých standardních redox potenciálů

musí být podmínky stejné. Bruettner G.R. (1993) uvádí sice hodnotu pH = 7, ale další parametry uvedeny v práci nejsou.

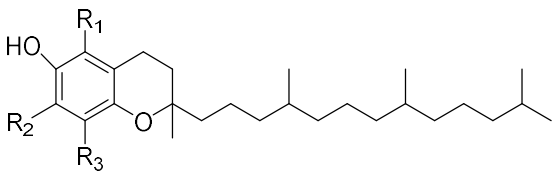
Tab. IV.: Standardní redukční elektrodové potenciály pro vybrané radikálové páry při pH = 7 (Buettner G.R., 1993)

Redox pár	**$E^{0'}$ [mV]**
HO·, H·/ H_2O	2310
RO·, H·/ROH	1600
ROO·, H·/ROOH	1000
R·, H·/RH[1]	600
α-TO·, H·/ α-TOH[2]	500
k. askorb·, H·/k. askorb.[3]	330

[1]nenasycená mastná kyselina, [2] α-tokoferol, [3]kyselina askorbová

Tokoferoly patří k často se přirozeně vyskytujícím lipofilním antioxidantům, stejně tak i k často používaným aditivním látkám do potravinářských i kosmetických výrobků.

Z chemického hlediska je jedná o fenolové antioxidanty a jednotlivé formy se od sebe liší stupněm methylace chromanového kruhu (obr. 30). Biologická aktivita (*in vivo*) tokoferolů klesá v pořadí α > β > γ > δ a antioxidační aktivity v olejích a tucích (*in vitro*) klesají v pořadí δ > γ > β > α. V souvislosti s hodnotami redox potenciálů tokoferol přednostně reaguje s peroxylovým radikálem, dále pak také s hydroxylovým radikálem nebo radikálem nenasycené mastné kyseliny (Kalman-Eldin A. a Appelqvist L.A., 1996).

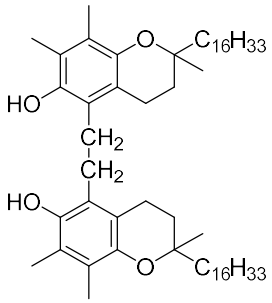


Tokoferol	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3

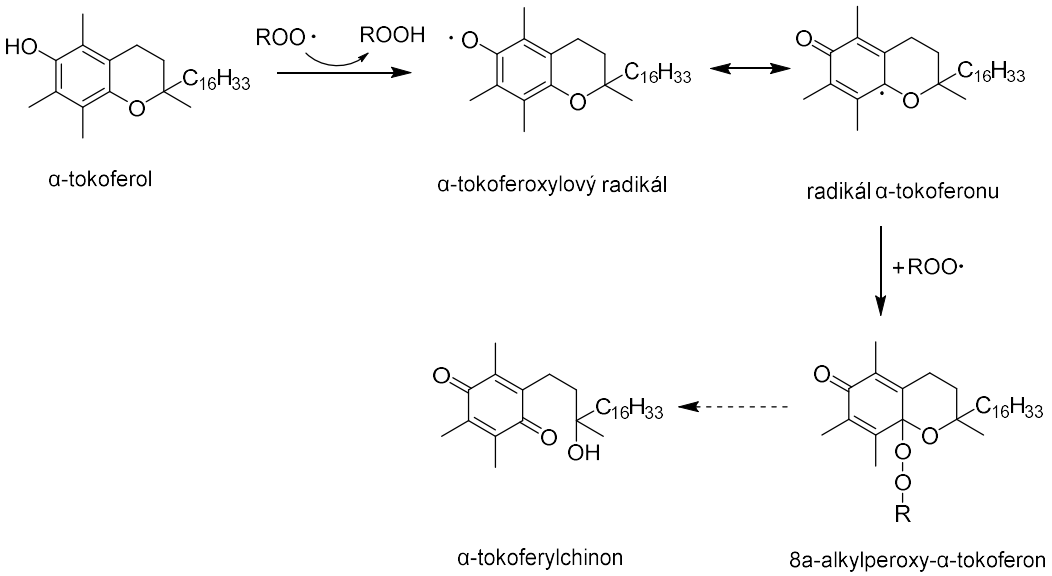
Obr. 30: Chemická struktura tokoferolů

Z tokoferolu může vznikat celá řada oxidačních produktů. Nejjednodušší variantou je odtržení vodíkového atomu (jednoelektronová oxidace) a vznik tokoferoxylového radikálu. Tento radikál může být zpětně redukován na tokoferol nebo může reagovat s peroxylovým, alkoxylovým radikálem (obr. 28D-E), radikálem mastné kyseliny nebo dalším tokoferoxylovým radikálem (obr. 28F) a dochází ke vzniku stabilních sloučenin, tokoferol je již pro další oxidačně/redukční mechanismy zablokován. V případě reakce dvou tokoferoxylových radikálů vznikají dimery α-tokoferolu (obr. 31) (Krol E.S. a kol., 2001).

Dalšími možnými produkty oxidace α-tokoferolu jsou 8a-alkylperoxy-α-tokoferon (nebo také označovaný jako 8a-(alkyldioxy)-tokoferon) a stabilní α-tokoferylchinon. V tomto případě se jedná o 2 elektronovou změnu α-tokoferolu na α-tokoferylchinon (obr. 32) (Tanno R. a kol., 2020; Senanayake S.P.J.N. a kol., 2020).

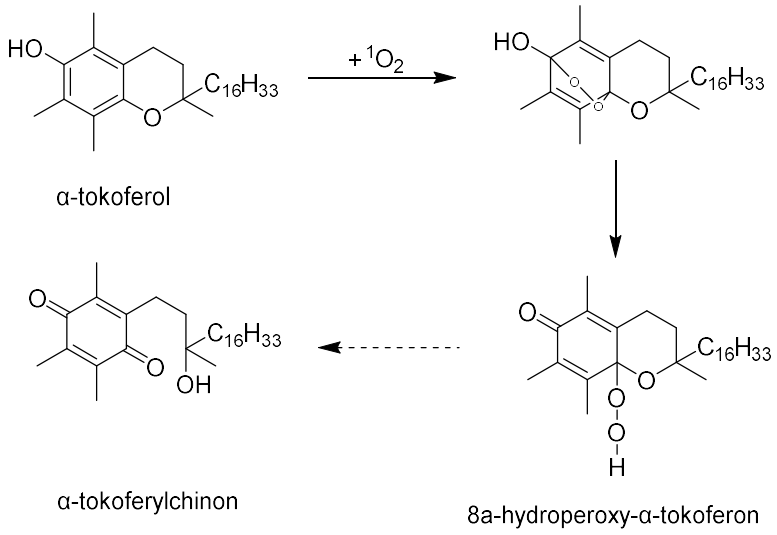


Obr. 31: Dimer α-tokoferolu



Obr. 32: Vznik 8a-alkylperoxy-α-tokoferonu a α -tokoferylchinonu

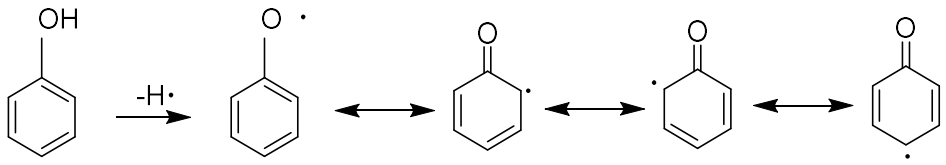
Fotooxidací, která představuje přímo reakci tokoferolu se singletovým kyslíkem, z α-tokoferolu vzniká 8a-hydroperoxy-α-tokoferon a z něj následně α-tokoferylchinon, obr. 33 (Tanno R. a kol., 2020). Možností, jak může α-tokoferol v potravinách reagovat, je mnohem více a je možné říct, že zatím všechny mechanismy nejsou plně objasněny (Liebler D.C. a Burr J.A., 1995; Verleyen T. a kol., 2001)



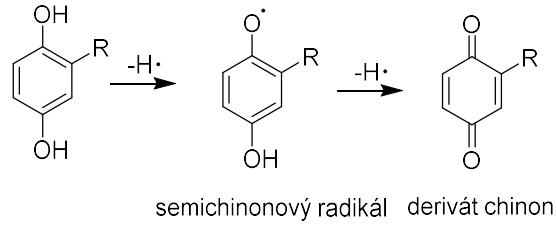
Obr. 33: Vznik 8a-hydroperoxy- α-tokoferonu a α-tokoferylchinonu

Dalšími rozšířenými přírodními antioxidanty jsou fenolové kyseliny a jejich deriváty, které se vyznačují i antimikrobiálními a antikancerogenní účinky (Merkl R. a kol., 2010; Senawong T. a kol., 2014; Wang J. a kol., 2014).

U monohydroxy fenolových sloučenin dochází k odtržení jednoho atomu vodíku a vzniká buď fenoxylový radikál nebo je radikál rezonančně stabilizován v *ortho* nebo *para* poloze na benzenovém jádře (obr. 34). U dihydroxyfenolových sloučenin je možné odtržení jednoho nebo dvou atomů vodíku a následně vzniká buď semichinonový radikál nebo stabilní chinon (obr. 35). Dihydroxyfenolové sloučeniny jsou zpravidla silnější antioxidanty než monohydroxyfenolové sloučeniny (Frankel E.N., 2007; Buettner O. R., 1993; Choe E. a Min D.B., 2009).

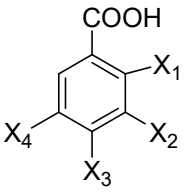


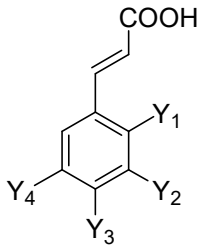
Obr. 34: Vznik stabilních radikálů monohydroxyfenolových antioxidantů



Obr. 35: Mechanismus antioxidační aktivity dihydroxybenzenových sloučenin

Fenolové kyseliny a jejich deriváty mohou být rozděleny do dvou základních skupin dle struktury – deriváty kyseliny benzoové a deriváty kyseliny skořicové (obr. 36).





Kyselina	X_1	X_2	X_3	X_4
Benzoová	H	H	H	H
p-Hydroxybenzoová	H	H	OH	H
Gallová	H	OH	OH	OH
Protokatechuová	H	H	OH	OH
Syringová	H	OCH_3	OH	OCH_3
Vanilová	H	OCH_3	OH	H
Gentisová	OH	H	H	OH

Kyselina	Y_1	Y_2	Y_3	Y_4
Skořicová	H	H	H	H
Kávová	H	OH	OH	H
p-Kumarová	H	H	OH	H
Ferulová	H	OCH_3	OH	H
Sinapová	H	OCH_3	OH	OCH_3

Obr. 36: Chemické struktury některých fenolových kyselin

Přítomnost skupiny –CH=CH-COOH v molekule derivátů kyseliny skořicové zvyšuje jejich antioxidační účinek tím, že dvojná vazba se účastní stabilizace vzniklého radikálu.

Jovanovic S. V. a kol. (1994) vypočetli redox potenciál pro kyselinu protokatechuovou $E^{0'}$ = 580 mV, pro kyselinu kávovou $E^{0'}$ = 550 mV (při pH = 7 a teplotě 20 °C). Z těchto dat vyplývá, že účinnějším antioxidantem bude kyselina kávová, tedy derivát kyseliny skořicové.

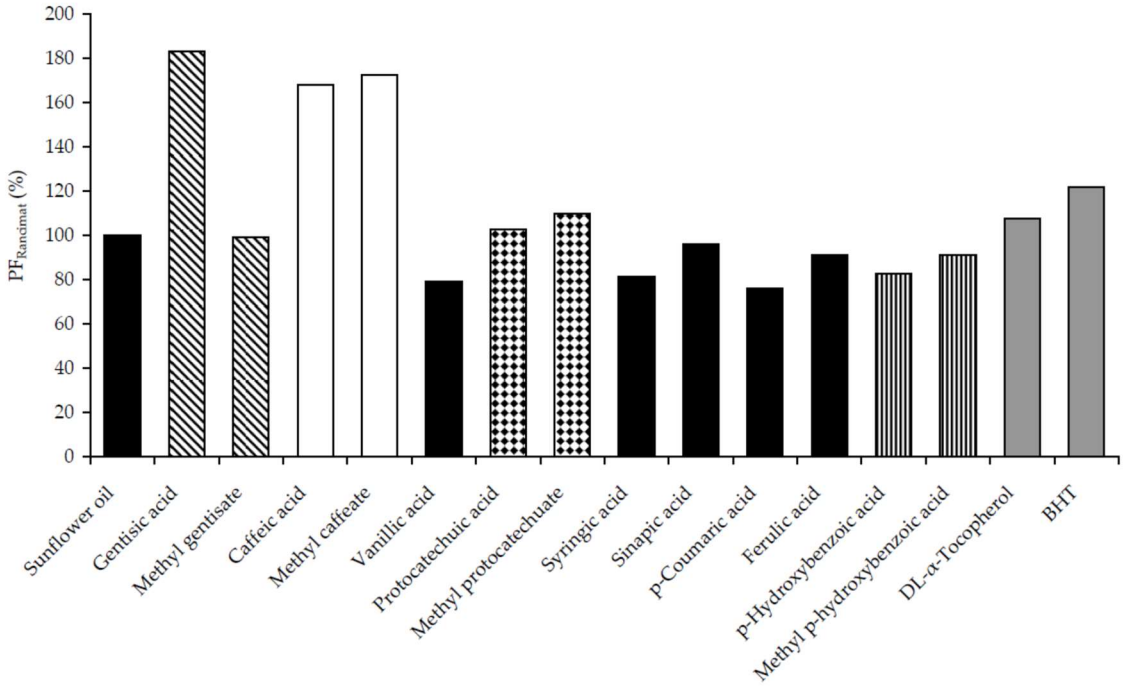
Při stanovení antioxidační aktivity vybraných fenolových kyselin a jejich methylestů vyplývá (obr. 37), že významný antioxidační účinek ve slunečnicovém oleji (celkový obsah tokoferolů 150 mg kg^{-1}, majorita α-tokoferol) vykazovala kyselina gentisová, kávová a její methylester. Protokatechuová kyselina a její ester vykazovaly také antioxidační účinek, který byl srovnatelný s účinkem α-tokoferolu. Je také patrné, že esterifikací kyseliny (snížením polarity) se zvýšila rozpustnost fenolové sloučeniny v nepolárním oleji, a tedy i antioxidační účinek připravených antioxidantů. Výjimkou je kyselina gentisová, jejíž methylester už antioxidační účinek nevykazuje. Struktura molekuly fenolové kyseliny nebo jejího esteru vypovídá o jejím antioxidačním účinku – deriváty kyseliny skořicové jsou účinnější než deriváty kyseliny benzoové, dihydroxykyseliny jsou účinnější než monohydroxykyseliny a methylestery kyselin jsou účinnější než odpovídající kyseliny (Hrádková I. a kol., 2009).

Se zvyšující se koncentrací fenolových sloučenin roste jejich antioxidační účinek měřený hodnotou protekčního faktoru (tab. V) a sice v rozsahu molárního poměru fenolové kyseliny (esteru) vůči α-tokoferolu 0,37 – 9,32 pro kyselinu gentisovou, 0,32 – 7,97 pro kyselinu kávovou a 0,30 – 7,39 pro methylester kyseliny kávové. Přibližně platí, že do hodnoty molárního poměru cca 6 - 7 je nárůst hodnoty protekčního faktoru pro všechny 3 látky přibližně lineární a koncentrace fenolových látek je max. 2 – 2,6 mmol kg^{-1} (0,004 % w/w). Tyto molární koncentrace odpovídají cca obsahu 900 - 1100 mg kg^{-1} α-tokoferolu v oleji, což jsou hodnoty pro nerafinovaný slunečnicový olej. To lze také interpretovat tak, že v jednofázovém lipidickém systému, který obsahuje nedostatečný obsah α-tokoferolu, je možné jej nahradit fenolovými kyselinami, resp. jejich estery. V takovém systému rovněž nedochází k disociaci karboxylové skupiny a esterifikace zvyšuje jejich lipofylitu a rozpustnost. (Hrádková I. a kol., 2009; Wang J. a kol., 2014).

Sekundární antioxidanty

Do skupiny sekundárních antioxidantů jsou řazeny chelatační látky (např. kyselina citrónová, vinná nebo jablečná, lecitin), zhášeče singletového kyslíku (karotenoidy), lapače kyslíku a redukující látky (např. kyselina askorbová, askorbylpalmitát nebo kyselina erythorbová a její sodná sůl). Mechanismus působení sekundárních antioxidantů se liší od

primárních v tom, že sekundární antioxidanty nezasahují do radikálové řetězové reakce oxidace lipidů, chelatují kovové ionty a tak je deaktivují; převádí singletový kyslík do základního tripletového stavu (kapitola 1.2) nebo přednostně reagují s tripletovým kyslíkem. Kyselina askorbová a askorbylpalmitát působí jako primární i sekundární antioxidanty (Shahidi F. a Zhong Y., 2010).



Obr. 37: Protekční faktor fenolových kyselin a vybraných methylesterů fenolových kyselin ve srovnání se α-tokoferolem a BHT ve slunečnicovém oleji (koncentrace 0,05 % (*w/w*)) (Hrádková I. a kol., 2009)

Tab. V: Hodnota protekčního faktoru (PF) v závislosti na koncentraci antioxidantu (Hrádková I., a kol., 2009)

Concentration (% w/w)		0.002	0.004	0.006	0.010	0.020	0.030	0.040	0.050
PF(%)	gentisic acid	107.7	110.9	116.1	116.1	137.1	159.3	181.9	183.1
	caffeic acid	102.8	104.8	106.5	108.1	119.8	124.6	137.9	171.0
	methyl caffeate	100.8	100.8	110.5	114.9	138.3	146.4	164.9	179.4

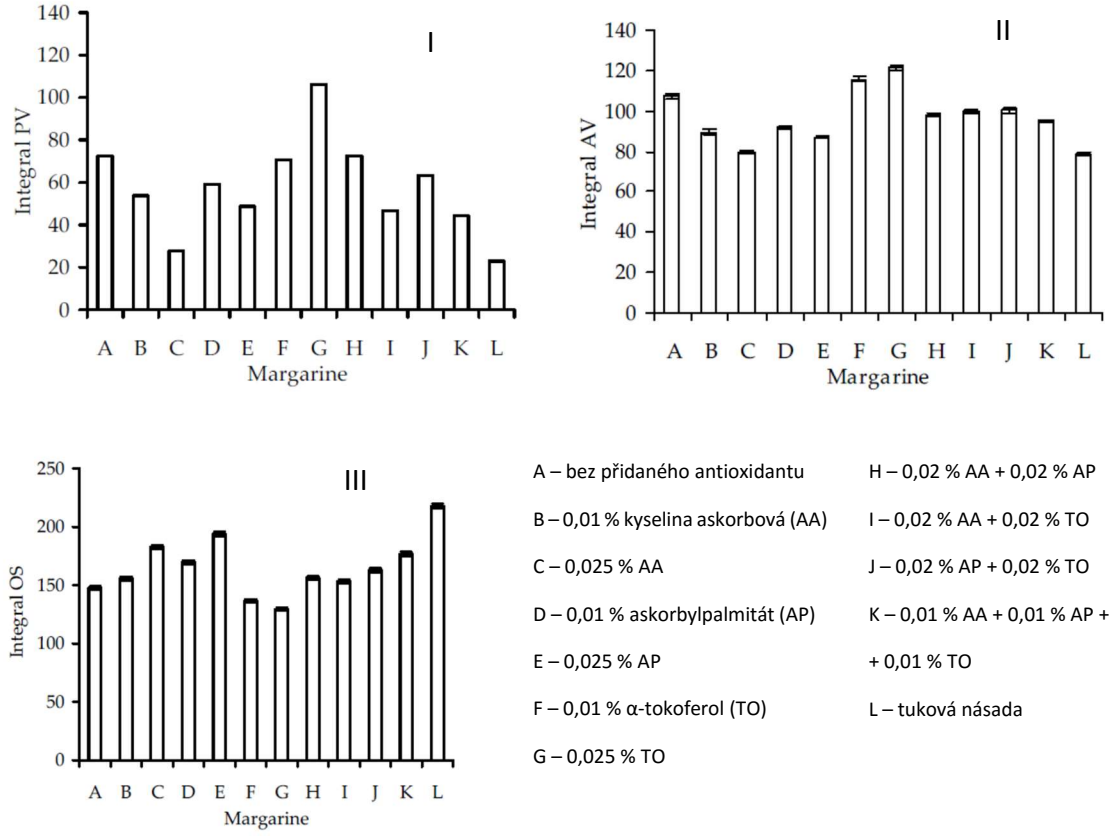
6.4.3 Polární paradox

Účinek antioxidantů je závislý i na prostředí, ve kterém se vyskytují, tento jev je nazýván jako tzv. polární paradox. Na mnoha studiích byl pozorován vyšší antioxidační účinek nepolárních (lipofilních) antioxidantů anebo povrchově aktivních antioxidantů s nízkou hodnotou HLB (hydrophylic-lipophilic balance) v polárních systémech (emulze typu olej ve vodě), zatímco účinek polárních (hydrofilních) antioxidantů anebo povrchově aktivních antioxidantů s vysokou hodnotu HLB je vyšší v nepolárních systémech (olej nebo emulze voda v oleji). Tuto hypotézu podporují studie s polárními antioxidanty (polárním analogen α-tokoferolu – troloxem – 6-hydroxy-2,5,7,8-tetramethylchroman-2-karboxylovou kyselinou, kyselinou askorbovou, gallovou, ferulovou nebo kávovou), které jsou účinnější v samotném oleji a studie s lipofilními antioxidanty (BHA a BHT), které jsou účinnější v emulzích typu olej ve vodě než v olejích. Stejně tak estery kyseliny gallové nebo kávové, které mají dlouhý alkylový řetězec (jsou nepolární), vykazují vyšší účinek v emulzích než v samotném oleji, ve kterém jsou účinnější estery těchto kyselin s kratším alkylovým řetězce (jsou polárnější), (Porter W. L. a kol., 1989; Shahidi F. a Zhong Y., 2011; Hrádková I. a kol., 2013).

Polární paradox je způsobený rozdílným chováním a distribucí antioxidantů ve dvoufázovém systému v závislosti na jejich polaritě a polaritě prostředí. Polární antioxidant se v nepolárním prostředí oleje nebo v emulzi typu voda v oleji vyskytuje na rozhraní olej/vzduch nebo na mezifázovém rozhraní olej/voda, kde dochází k iniciaci oxidace, a tak účinně chrání nepolární prostředí před oxidativními změnami, na rozdíl od nepolárních antioxidantů, které jsou rozpuštěny v nepolárním prostředí. V polárním prostředí emulze typu olej ve vodě dochází k přednostnímu hromadění nepolárních antioxidantů na mezifázovém rozhraní voda/olej a tak vzniká ochranná bariéra vůči iniciaci oxidace lipidů, polární antioxidanty jsou rozpouštěny ve vodné fázi. V některých případech ovšem hypotéza polárního paradoxu antioxidantů nefungovala a bylo zjištěno, že je to pouze jeden z možných úhlů pohledu na tuto problematiku. Účinek antioxidantu v polárních systémech ovlivní i struktura a velikost molekuly, koncentrace antioxidantu apod. (Shahidi F. a Zhong Y., 2011).

Z výsledků Filip V. a kol. (2009) je zřejmé, že v margarínové emulzi typu voda v oleji je chování antioxidantů s různou polaritou v souladu s polárním paradoxem (je nutné upřesnit, že tuková násada emulze obsahovala bázi přirozeně se vyskytujících tokoferolů a vždy se tedy jednalo interakci přidaného antioxidantu s tokoferoly). Nejúčinnější z hlediska primárních oxidačních produktů (peroxidové číslo), sekundárních oxidačních produktů (*p*-anisidinové číslo) a také oxidační stability byla polární kyselina askorbová při koncentraci 0,025 % (vzorek

C). Askorbylpalmitát, lipofilní analog kyseliny askorbové, je již méně účinný, nicméně se zvyšující se koncentrací se zvyšuje i jeho antioxidační účinek. Lipofilní α-tokoferol působí v margarínových emulzích jednoznačně prooxidačně, se zvyšující se koncentrací se prooxidační účinek zvyšuje ve všech zkoumaných parametrech (obr. 38), tato skutečnost zřejmě také souvisí s celkovou koncentrací tokoferolů v margarínu (tokoferoly přidané a tokoferoly přirozeně se vyskytující), která překročila koncentraci prooxidačního chování tokoferolu.



Obr. 38: Integrál změny peroxidového čísla v čase (I), integrál změny *p*-anisidinového čísla v čase (II) a integrál změny oxidační stability v čase (III) margarínových emulzí s různými antioxidačními systémy (Filip V. a kol., 2009)

6.4.4 Synergický a antagonický účinek antioxidantů

Potraviny, kosmetické přípravky anebo farmaceutické přípravky jsou ve většině případů mnohasložkové systémy, ve kterých může docházet k různým interakcím. Antioxidanty přítomné ve výrobcích/přípravcích se mohou vyskytovat přirozeně v jednotlivých surovinách nebo mohou být přidávány do výrobků/přípravků s cílem zvýšit jejich oxidační stabilitu. Pokud dojde ke zvýšení celkové oxidační stability lipidického systému výrobku/přípravku, který obsahuje různé antioxidanty, hovoří se o synergickém účinku jednotlivých antioxidantů. Pokud

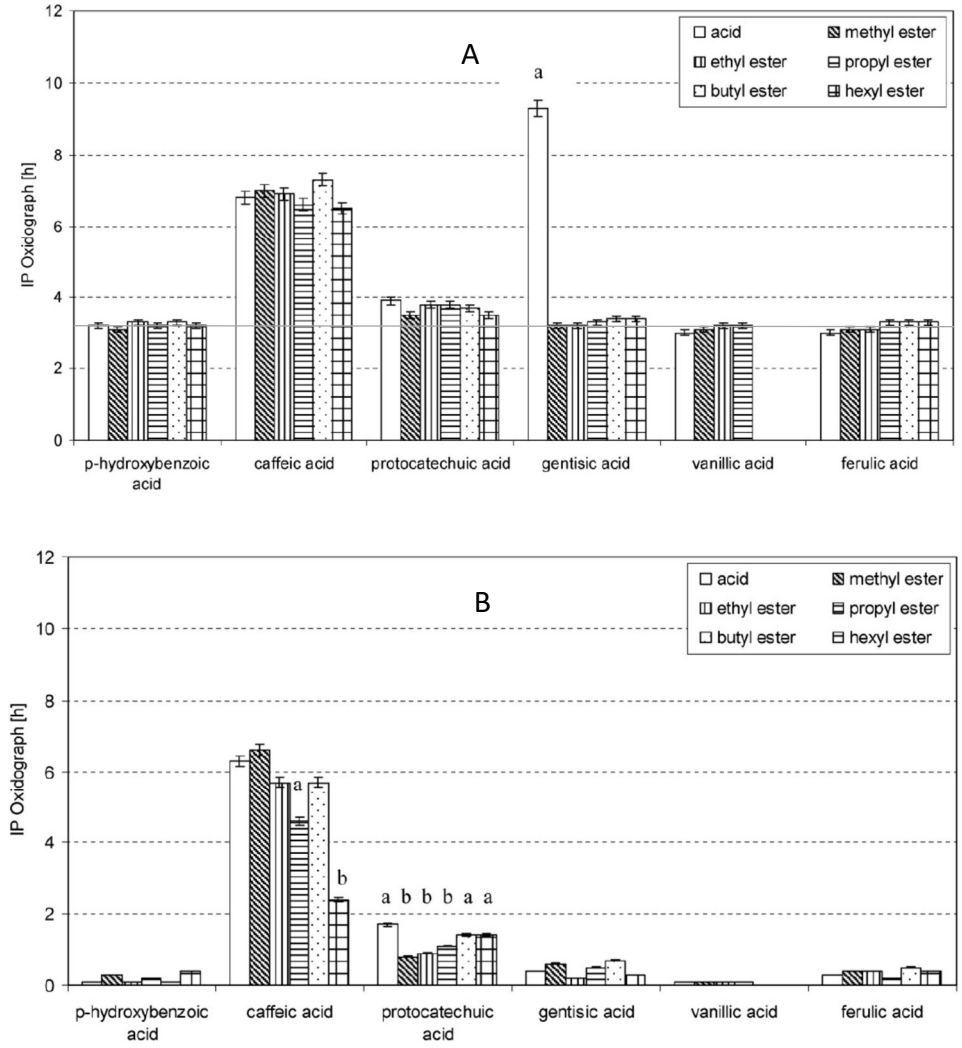
dojde ke snížení oxidační stability lipidického systému výrobku/přípravku, jedná se o antagonický účinek jednotlivých antioxidantů, který je z hlediska výrobce i zákazníka nežádoucí.

Jsou známy tři druhy synergického účinku antioxidantů - homosynergismus, heterosynergismus a autosynergismus. Homosynergické působení vzniká mezi antioxidanty, které mají stejný mechanismus působení, např. zabránění řetězové reakce oxidace lipidů. Příkladem je kombinace α-tokoferolu a kyseliny askorbové, kdy α-tokoferoxylový radikál je regenerován kyselinou askorbovou o nižším redox potenciálu. Heterosynergismus se vyskytuje u sloučenin s různým antioxidačním účinkem. Příkladem je směs tokoferolů s kyselinou citrónovou, která působí proti iniciaci oxidace kovovými ionty. Autosynergismus se týká jednoho antioxidantu, u kterého je popisováno více funkcí, například flavonoidy působí na volné radikály, deaktivují kovové ionty tvorbou komplexů, inhibují enzymatickou tvorbu volných radikálů atd. (Filip V. a kol., 2009; Hajimehdipoor H. a kol., 2014; Frankel E.N., 2007).

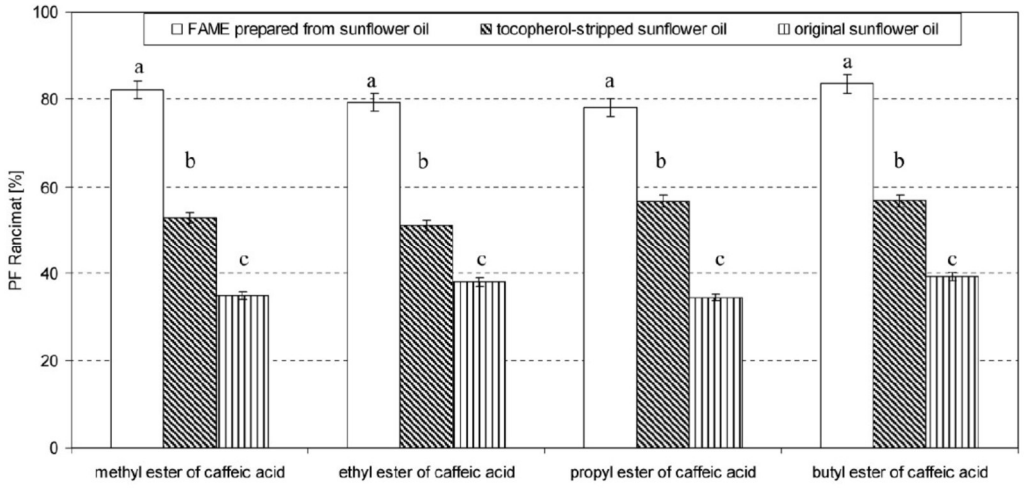
Antioxidační účinek byl pozorován u přirozeně se vyskytujících tokoferolů slunečnicového oleje (149 mg kg^{-1}, majorita α-tokoferol) v kombinaci s fenolovými kyselinami a jejich alkylestery. Z obr. 39A vyplývá, že kyselina kyselina kávová a protokatechuová a jejich alkylestery, stejně tak i kyselina gentisová v porovnání s ostatními fenolovými kyselinami a jejich alkylestery zvyšují významně oxidační stabilitu slunečnicového oleje (indukční perioda slunečnicového oleje bez přidaných antioxidantů byla 3,3 h).

Methylestery mastných kyselin (FAME) slunečnicového oleje po vakuové destilaci se používají jako vhodné médium pro oxidaci, u něhož nejsou detekovány přirozeně se vyskytující tokoferoly, neřeší se účinek kombinace antioxidantů přirozeně se vyskytujících a přidaných, ale pouze účinek jednoho konkrétního přidaného antioxidantu. Na obr. 39B je stanovena indukční perioda fenolových kyselin a jejich alkylderivátů v prostředí FAME slunečnicového oleje (samotné FAME slunečnicového oleje vykazovaly hodnotu indukční periody 0,1 h). Významný antioxidační účinek je zachován u kyseliny kávové a jejích alkylesterů, nižší účinek u kyseliny protokatechuové a jejích alkylesterů. Kyselina gentisová (obr. 39 A a B) spolu s přirozeně se vyskytujícími tokoferoly slunečnicového oleje působí synergicky, ovšem její alkylestery již synergický účinek nevykazují. Ostatní antioxidanty vykazovaly minimální antioxidační aktivitu. Vzájemné působení methyl-, ethyl-, propyl- a butylesteru kyseliny kávové (obr. 40) s přirozeně se vyskytujícími tokoferoly slunečnicového oleje (byla použita oxidační média s různým obsahem přirozeně se vyskytujících tokoferolů) lze popsat jako antagonické, se

zvyšující se koncentrací tokoferolů dochází ke snížení protekčního faktoru (Hrádková I. a kol., 2013).



Obr. 39: Indukční perioda měřená na přístroji Oxidograf (110 °C) vzorků fenolových kyselin a jejich alkylesterů (3 mmol kg^{-1}) ve slunečnicovém oleji, obsah tokoferolů 149 mg kg^{-1} (A); v methylesterech mastných kyselin slunečnicového oleje, obsah tokoferolů - nedetekováno (B) (Hrádková I. a kol., 2013)



Obr. 40: Protekční faktor alkylesterů kyseliny kávové (3 mmol kg^{-1}) v různých oxidovaných médiích – methylestery mastných kyselin slunečnicového oleje (FAME, obsah tokoferolů – nedetekováno), slunečnicový olej po odstranění tokoferolů kolonou chromatografií (obsah tokoferolů 8,7 mg kg^{-1}), původní slunečnicový olej (obsah tokoferolů 149 mg kg^{-1}), (Hrádková I. a kol., 2013).

7 ZÁVĚR

Oxidace lipidů, i když je to téma studované již řadu desítek let, nabízí stále nové směry výzkumu z toho důvodu, že zahrnuje velké množství reakcí. Stálý zájem o oxidaci lipidů je způsoben i tím, že roste produkce olejů s vyšším obsahem polynenasycených mastných kyselin a je snaha minimalizovat oxidační změny lipidů během zpracování olejů/tuků a během následné výroby potravin, kosmetických a farmaceutických přípravků.

Praktickým výsledkem celého výzkumu je prodloužení doby spotřeby výrobků za definovaných podmínek.

Bylo dokázáno, že monoacylglycerolové emulgátory mohou ovlivnit nejen fyzikální stabilitu mikrodisperzního systému, ale i jeho oxidační stabilitu, kdy záleží na těsnosti uspořádání monoacylglycerolů na fázovém rozhraní.

Dosažitelnost kyslíku jako hlavního reaktantu při oxidaci lipidů je důležitý parametr. Bylo zjištěno, že hlavním zdrojem kyslíku pro oxidaci lipidů ve výrobcích je především atmosféra nad vzorkem, počáteční množství rozpuštěného kyslíku ve výrobku hraje minoritní roli.

Polární paradox u antioxidantů je většinou zkoumán v prostředí emulze olej ve vodě. V našich výzkumech jsme potvrdili polární paradox i u emulzí typu voda v oleji (margarínového typu).

Fenolové kyseliny a jejich estery jsou dnes často studovanými látkami pro jejich multifunkční účinek. Byl u nich potvrzen antioxidační, antimikrobiální i antikancerogenní účinek. Z výsledků provedených experimentů vyplývá, že jejich antioxidační účinek souvisí se strukturou molekuly – deriváty kyseliny skořicové jsou účinnější než deriváty kyseliny benzoové, dihydroxykyseliny jsou účinnější než monohydroxykyseliny a estery jsou účinnější než odpovídající kyseliny. Bylo pozorováno také synergické a antagonické chování fenolových kyselin a jejich esterů s přirozeně se vyskytujícími tokoferoly.

Další výzkum v této problematice by měl být orientován na studium oxidačních produktů antioxidantů, které není zatím detailně propracované a pomůže ujasnit, jak probíhají synergické a antagonické interakce antioxidantů.

8 LITERATURA

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9 PŘÍLOHY

V této kapitole jsou uvedeny vybrané autorské nebo spoluatorské vědecké práce přímo související s tematikou „Oxidace lipidů a použití antioxidantů“, které byly publikované v mezinárodních časopisech s recenzním řízením.

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Changes of Sunflower Oil Polyenoic Fatty Acids under High Temperatures

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Abstract: Heat induced *cis-trans* isomerisation of sunflower oils depending on temperature, reaction time and original content of linoleic acid was investigated. The content of isomeric fatty acids was determined by gas chromatography and the content of polymers by gel permeation high-performance liquid chromatography. The content of *trans* fatty acids increased with time and with temperature and a rate of *cis-trans* isomerisation and polymerisation depends on the temperature according to Arrhenius equation. The content of polymers was significantly lower in sunflower oil with high content of oleic acid because of the low concentration of linoleic acid in oil. In both oils the content of conjugated linoleic acid initially increased depending on time and temperature, however after certain time the stationary state occurred. Polymerisation of polyenoic fatty acids takes place directly with heat induced *cis-trans* isomerisation.

Keywords: *cis-trans* isomerisation; polymerisation; *trans* fatty acid; conjugated linoleic acid

INTRODUCTION

Double bonds of unsaturated fatty acids (FA) can be either subject to geometrical isomerisation with forming *trans* isomers of fatty acids (TFA) or positional isomerisation with shifting of double bond in carbon chain. Dienoic FA usually provide a conjugated isomers (Velíšek 2002). Conjugated isomers of octadecadienoic acid are collectively known as conjugated linoleic acid (CLA) (Larqué *et al*. 2001). Isomerisation of double bonds of FA are effectively induced by heating up 200°C, so the TFA are formed especially by physical refining and deodorisation of plant oils. Their presence negatively affects a final nutritious quality of refined oils (Ackman *et al*. 1974; O'Brien 2004; Čmolík *et al*. 2007).

Under high temperatures also polymerisation and cyclisation of FA probably occurs. Generating polymers and cyclic FA are together with TFA deleterious. Therefore, the thermal isomerisation and polymerisation of FA should be more studied in term of technological and in particular of nutritional aspect.

The aim of this study was to investigate heat induced *cis-trans* isomerisation and polymerisation of standard sunflower oil (SSO) and high oleic acid sunflower oil (HOASO) depending on temperature, reaction time and original content of linoleic acid in oil.

MATERIALS AND METHODS

For experiments were chosen two refined sunflower oils differing in content of oleic and linoleic acid and not containing linolenic acid. SSO was obtained from Setuza, a.s. (Czech Republic), production 2007 (Σ C18:2 61.35%, *t* C18:2 1.65%, linoleic acid 59.69%; Σ C18:1 19.53%, *t* C18:1 0.00%; polymers 0.00%). HOASO was obtained from Palma-Tumys, Bratislava, a.s. (Slovakia), production 2007 (Σ C18:2 9.78%, *t* C18:2 0.20%, linoleic acid 9.58%; Σ C18:1 77.78%, *t* C18:1 0.00%; polymers 0.00%).

Laboratory isomerisation. Samples of investigated plant oils (ca 40 g) were parallel heated repeatedly twice in couple of glass laboratory reactors under argon atmosphere in hot-flue thermostat at

temperatures 200, 220, 230, 240 and 250°C for time lasting up to 170 hours. Reactors were equipped by air-leak tubes for gas intake. Argon flow was just minimal for a weak agitation of heated oils. Accurate temperature in thermostat was scanned by a digital thermometer F200 for the whole time of isomerisation according to computer programme F200E. Samples for analyses were taken of heated oils in certain intervals (twice per day).

Analysis of composition of fatty acids isomers. Contents of individual isomeric FA were determined by capillary gas chromatography (Agilent Technology, 6890N) with flame ionisation detection (CGC/FID) according to ISO 15304:2002. Fatty acid methyl esters (FAME) were prepared by standard method (ISO 5509:2000), pentadecanoic acid was used as internal standard to obtain content of FA in absolute values (% w/w). For analysis the capillary column SP^{TM} 2560 (Supelco, Bellefonte), 0.25 mm × 100 m, film thickness 0.2 μm was used. The conditions of analysis were: hexane solution of FAME (1%) was used for the injection (1 μl), split injection (1:50) at 220°C; flow of carrier gas (He) 1 ml/min; analysis at 175°C for 120 min; FID detection at 250°C, flow of H_2 40 ml/min, air flow 450 ml/min and make-up gas (N_2) flow 45 ml/min.

Determination of content of polymeric lipids. Polymers of FA were determined by gel permeation high-performance liquid chromatography (Agilent 1100 Series, Agilent Technologies) with evaporative light scattering detection (GP-HPLC/ELSD). Sample of heated oil (10 mg) was diluted in 10 ml of tetrahydrofuran (THF) and 5 μl of the solution was injected on column PLgel 5 μm, 10 nm, 300 × 7.5 mm (Agilent) with separation capacity up to 4000 g/mol. THF was used as a mobile phase with flow 0.6 ml/min. Analysis was running at 25°C for 15 min, ELSD detection at 50°C. Results were introduced as a sum of all polymer lipids without resolution of polymerisation degree in % rel.

RESULTS AND DISCUSSION

The content of individual TFA increased with the time and with the temperature in both investigated oils, as it was supported by expressed degree of isomerisation (DI) depending on reaction time (Figure 1). DI of octadecenoic (C18:1), resp. octadecadienoic acid (C18:2) was expressed as a ratio between total content of *trans* isomers and total content of all isomers of C18:1 (*c-* and *t-*), resp. C18:2 (Kemény *et al*. 2001). In both oils the $DI_{C18:2}$ was significantly higher then the $DI_{C18:1}$. $DI_{C18:2}$ were same in both oils, but $DI_{C18:1}$ was lower in HOASO. Range of *cis-trans* isomerisation of C18:2 thus is not depend on original content of linoleic acid, but the higher is original content of oleic acid, the lower is range of isomerisation of C18:1 in oil. The main products of *cis-trans* isomerisation of original linoleic acid are mono-*trans* isomers (9*c*,12*t*- and 9*t*,12*c*-).

Dependance of content of *trans* C18:2 (*c,t-*, *t,c-*, *t,t-*) and CLA and *trans* C18:1 on reaction time was linear in all temperatures with average correlation coefficients explicited in Table 1. This dependance was linear only at the start of reaction but after a certain time a stationary state occurs at temperatures 240 and 250°C. It can be caused by determined retardation of original linoleic

Table 1. Correlation coefficients (*R*) for linear dependance of contents of *trans* isomers on reaction time for SSO and HOASO

		200°C	220°C	230°C	240°C	250°C
SSO	*cis-trans* C18:2[a]	1.00	1.00	1.00	1.00	1.00
	trans-cis C18:2	1.00	1.00	1.00	1.00	1.00
	trans-trans C18:2	0.92	0.98	0.95	0.99	0.99
	conjugated C18:2	0.93	0.96	0.96	0.98	0.94
	trans C18:1[b]	–	0.90	0.69	0.98	0.99
HOASO	*cis-trans* C18:2	1.00	0.99	1.00	0.99	0.99
	trans-cis C18:2	1.00	0.99	1.00	0.99	0.99
	trans C18:1	0.79	0.88	0.96	0.95	1.00

[a]octadecadienoic acid, [b]octacedenoic acid

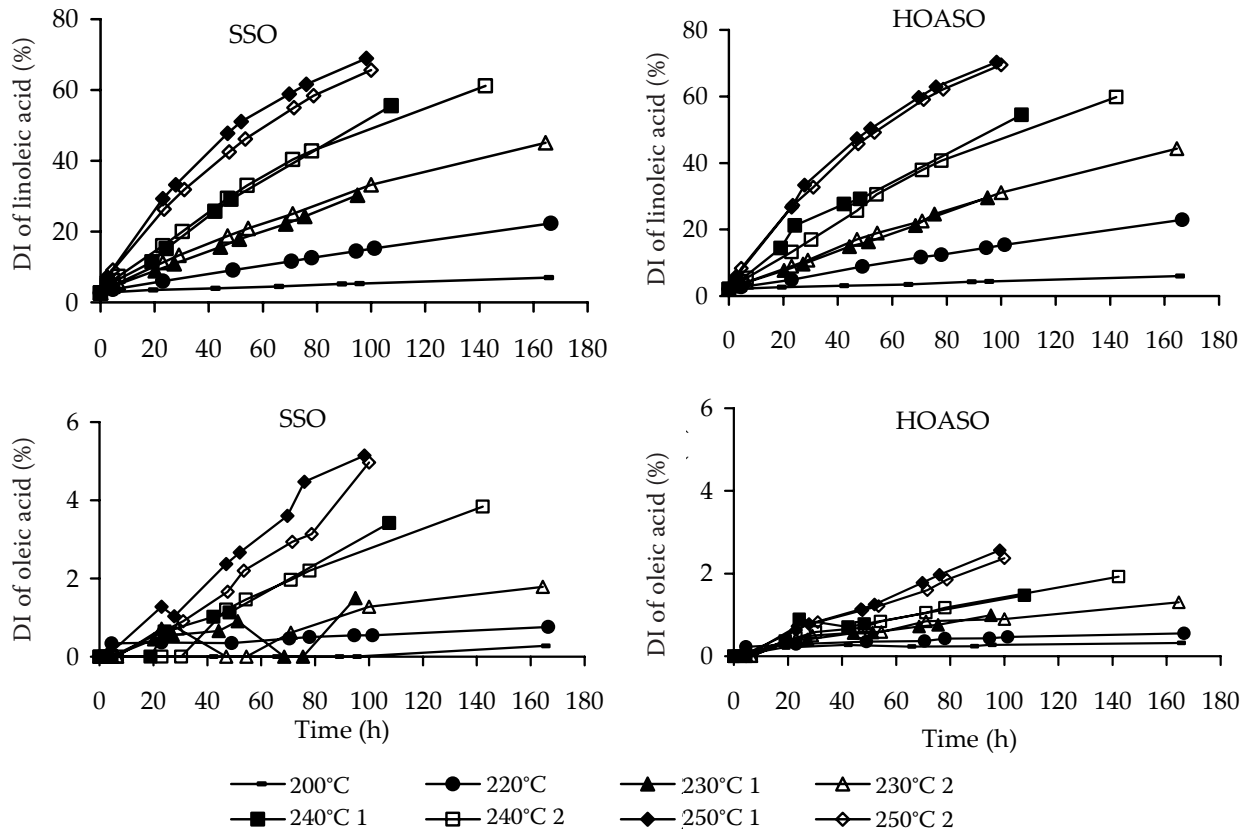

Figure 1. Time course of the degree of isomerisation (DI) of linoleic and oleic acid in SSO and HOASO at controlled temperatures (200–250°C)

acid decrease. To verify this hypothesis a dependance between linoleic acid content and $DI_{C18:2}$ was examined. Obtained dependance was linear with correlation coefficients 0.11, 0.93, 0.99, 0.99, 1.00 for SSO and 0.46, 0.96, 0.99, 0.98, 1.00 for HOASO in order of temperature (200, 220, 230, 240, 250°C) and thus the hypothesis about correlation between $DI_{C18:2}$ and it is content in oil was supported. Regarding the decrease of original C18:2 the *cis-trans* isomerisation corresponds with the kinetics of the first order (Wolff 1993; Hénon *et al*. 1999), but regarding the increase of TFA the isomerisation is close to zero order.

The content of CLA increased with time at all of investigated temperatures in both oils (Figure 2A). This increase was initially a linear function of time, but after a certain time the stationary state occurred because the forming conjugated isomers were probably consumed in the polymerisation reaction. The higher is temperature, the faster is forming of CLA.

The rate and the degree of *cis-trans* isomerisation increase with the heating time and temperature according to Arrhenius equation (Wolff 1993; Hénon *et al*. 1999).

$$k = Ae^{-E/RT}$$

In order to verify an Arrhenius formula an exponential dependance of rate constant of isomerisation on reciprocal absolute temperature ($1/T$) was investigated for all isomeric FA and polymers. Values of rate constants were found out of time dependance of contents of individual isomeric acids and polymers. Values of correlation coef-

Table 2. Correlation coefficients (R) for exponencial dependance of rate of an increase of isomeric FA and polymers content on isomerisation temperature

	SSO	HOASO
cis-trans C18:2[a]	1.00	1.00
trans-cis C18:2	1.00	1.00
trans-trans C18:2	1.00	–
conj. C18:2	0.99	0.47
trans C18:1[b]	0.98	0.96
Polymers	0.95	–

[a]octadecadienoic acid, [b]octacedenoic acid

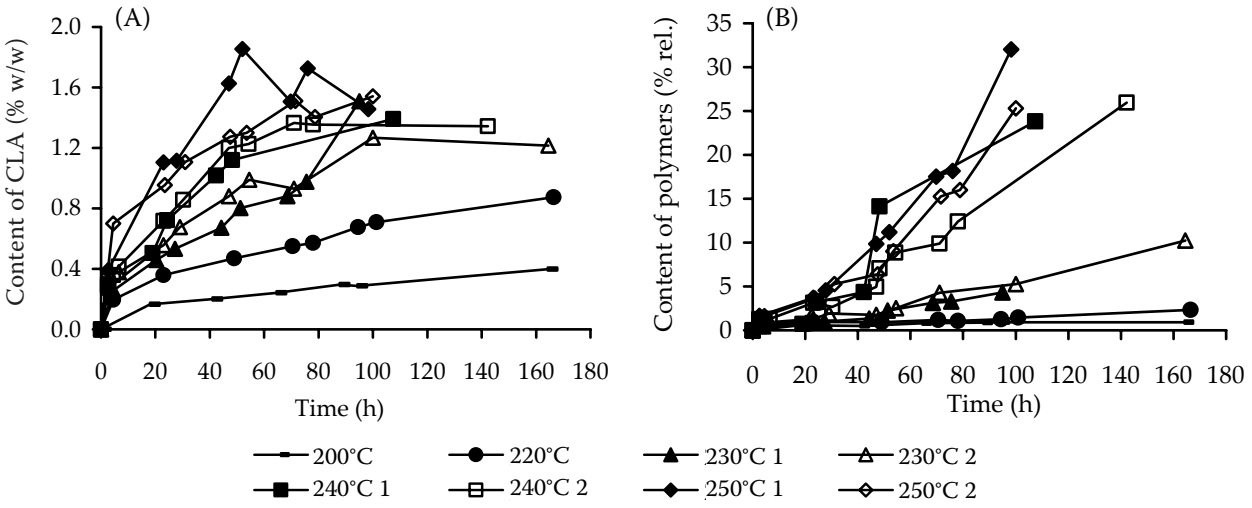

Figure 2. Time course of the content of CLA (A) and polymers (B) at controlled temperatures (200–250°C) for SSO

ficients obtained from exponential functions of temperatures are introduced in Table 2. Arrhenius equation was validated for rate of forming of all *trans* isomers, CLA and polymers in SSO. In HOASO this dependance was proved only for *c,t* and *t,c* C18:2 and *t* C18:1 because of the lower isomerisation and polymerisation degree.

Range of polymerisation increased with time (Figure 2B) in particular in SSO. In HOASO the content of polymers was significantly lower because of the low concentration of original linoleic acid in oil. The content of polymers initially linearly increased according to content of CLA. When forming of CLA comes to a stationary phase, the content of polymers begins to increase rapidly probably because of consuming of a newly formed conjugated isomers for polymerisation.

CONCLUSIONS

Content of TFA, CLA and polymers increased with the time and temperature. A linear correlation between $DI_{C18:2}$ and its content in oil was obtained. Polymerisation of polyenoic FA takes place directly with *cis-trans* isomerisation with temperature dependance according to Arrhenius equation like all of TFA and CLA.

Acknowledgements: This work was supported by funding from the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046137305).

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Quantitative and qualitative analysis of high molecular compounds in vegetable oils formed under high temperatures in the absence of oxygen

Klara Cihelkova, Andreas Schieber, Daise Lopes-Lutz, Iveta Hradkova, Jan Kyselka & Vladimir Filip

European Food Research and Technology
Zeitschrift für Lebensmittel-Untersuchung und -Forschung A

ISSN 1438-2377

Eur Food Res Technol
DOI 10.1007/s00217-013-2015-9

Springer

Eur Food Res Technol
DOI 10.1007/s00217-013-2015-9

ORIGINAL PAPER

Quantitative and qualitative analysis of high molecular compounds in vegetable oils formed under high temperatures in the absence of oxygen

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Received: 3 December 2012 / Revised: 26 April 2013 / Accepted: 8 May 2013

Abstract Conditions of deodorization/physical refining of sunflower oil were simulated by high temperature heating in laboratory at 240, 250, and 260 °C. Oxygen atmosphere was excluded by argon atmosphere. Influence of temperature, initial oxidation of incoming oil (peroxide value 2–30 mmol ½ O_2/kg) on geometrical, positional isomerization, and polymerization was measured. The level of initial oxidation of the oil has a significant influence on rate of isomerization, polymerization, and duration of induction period of polymerization reactions. Induction period of polymerization is dependent linearly on temperature at constant hydroperoxide content and is extended with decreasing temperature and lower peroxide value of oil. Significant conjugation took place with geometrical isomerization and resulted in all-*trans* diene formation. All-*trans* dienes are incoming reactants for polymerization according to the Diels–Alder mechanism. Suggested "propagation" phase of polymerization took place later. Induction period was observed only in the case of polymerization reactions of triacylglycerols. This is the confirmation of the hypothesis that the *cis*/*trans* isomerization, positional isomerization, and polymerization are consecutive reactions in ascending order. Identification of molecular peaks and confirmation of fragments were possible by connecting HPSEC with APCI-MS. Dimers of triacylglycerols (TAG) dominated in studied system. Remaining compounds may have been formed from di-, monoacylglycerols, and other minor constituents of sunflower oil.

Keywords APCI-MS · Conjugation · Dimer · Deodorization · Physical refining · Polymerization · Sunflower oil · Triacylglycerol · HPSEC

Introduction

Unsaturated fatty acids of lipids, especially triacylglycerols, are exposed to a higher temperature of 160–200 °C and oxygen access during culinary treatment (such as frying and deep-frying) [1]. Geometrical isomerization of double bonds of unsaturated fatty acids that started during deodorization/physical refining of oils (temperature >200 °C) can continue under these conditions [2, 3]. Volatile compounds such as aldehydes and mainly fatty acids [4] are distilled with water vapor from edible oils during deodorization/physical refining (low absolute pressure 3–10 hPa, temperature 200–250 °C, and reaction time several hours) [5, 6].

Numerous side reactions can occur during deodorization/physical refining. Of these, the geometrical isomerization of double bonds, especially of polyunsaturated acids (linoleic and linolenic acids), has been studied most of all. This type of reaction is known as heat-induced *cis*/*trans*

This paper is based on a presentation given at the conference Chemical Reactions in Foods VII, Prague, November 16–18, 2012.

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Published online: 02 June 2013

isomerization [2]. It is a non-catalyzed, kinetically controlled process. Dependence of the rate constant on temperature is determined by the Arrhenius equation, and the reactivity of fatty acids significantly increases in the order monoenes < dienes < trienes [3, 5, 7].

It is interesting that studies dealing with *cis*/*trans* isomerization of double bonds of fatty acids at higher temperature without oxygen access [1–7] did not discuss possible formation of conjugated double bonds including Tsuzuki [8], who studied *cis*/*trans* isomerization of triolein, trilinolein, and trilinolenin. The most often used technique for determination of *cis*/*trans* fatty acid isomers [1–8] is gas chromatography of fatty acid methyl esters on fused silica capillary columns coated with cyanopropyl polysiloxane phase, length 50 m (recently even 100 m). This column allows separation of both geometrical isomers of dienes and trienes, but also positional isomers, particularly isomers with double bonds in conjugation [9]. In particular, Sebedio et al. [10] studied isomerization and conjugation of linoleic acid esters at temperature 275 °C, without air access. They found that after 12 h of isomerization, conjugated dienes *cis*,*trans* and *trans*,*cis* are formed in positions Δ9,11, Δ10,12, Δ11,13 a Δ12,14, while *trans*,*trans* isomers are formed in positions Δ9,11, Δ10,12, Δ11,13. Destaillats and Angers [11] proposed a theoretical explanation of the mechanism of double bond conjugation of linoleic acid in the absence of air, that is, without participation of peroxyl, alkoxyl, and hydroxyl radicals: "[1,3]-Sigmatropic reaction appears to be responsible for the formation of Δ9,11, Δ10,12 conjugated octadecadienoic acid (CLA) isomers."

The primary reactants for polymer formation are conjugated dienes. The substituted cyclohexenic oligomers of fatty acids are formed by Diels–Alder mechanism at zeolite catalysis and without air access. Triacylglycerol polymers are formed already in raw oil [12] by radical mechanism for oxygen access. These compounds are oxopolymers, especially dimers (in concentrations of 0.2–0.6 %), because they are found in the polar fraction together with oxidized triacylglycerols. During deodorization/physical refining of soybean oil, their content slightly increased up to 3.26 % [13]. It is not well explained how polymers can be formed under the conditions of deodorization/physical refining in the medium without oxygen.

The aim of this study was to monitor several processes that take place under the conditions simulating deodorization/physical refining of triacylglycerols without oxygen participation. In this study, deep vacuum was replaced by argon atmosphere. As a result, geometrical isomerization and double bond conjugation of fatty acids and subsequent polymerization took place. The reaction time used was longer than the typical time during oil refining to improve our understanding of current processes.

Experimental

Materials

Common odd and even fatty acids and corresponding methylester standards were purchased from Sigma-Aldrich Company. Tripalmitin, tristearin, triolein, and trilinolein were from Sigma-Aldrich as well. Only HPLC or reagent grade solvents were used: acetonitrile, acetone, methanol, tetrahydrofuran, and hexane (Sigma-Aldrich). TAG/ECN 38 (*sn*-1,2-didecanoyl-3-tetradecanoylglycerol) and isomers of *cis*,*trans*-octadeca-9,11-dienoic acid and *trans*,*cis*-octadeca-10,12-dienoic acid were not commercially available.

Standard refined sunflower oil (SSO; peroxide value: 2.06 mmol ½ O_2/kg, acidity value: 0.20 mg KOH/g) was used and purchased from a local food store (Czech Republic). Fatty acid composition determined by capillary gas–liquid chromatography (CGC) is shown in Table 1. Sunflower oil was oxidized under defined conditions at reaction vessels (glass tube, height 160 mm, inner diameter 45 mm) equipped with gas exhaust in the upper part and porous frit S4 at the bottom through which oxygen (oxygen 5.0; purity 99.999 %) was slowly bubbled (20 l/h) at 60 °C. Samples with different peroxide values (PV) were collected for the laboratory polymerization experiments (PV1 = 2.61 mmol ½ O_2/kg, PV2 = 7.12 mmol ½ O_2/kg, PV3 = 11.53 mmol ½ O_2/kg, PV4 = 27.68 mmol ½ O_2/kg).

Table 1 FA composition of standard SSO

Saturated FA[a]	Content (% ww)	Unsaturated FA	Content (% ww)
C8:0	0.01	*cis*-9-C16:1	0.12
C14:0	0.07	*cis*-9-C18:1	**31.69**
C16:0	**6.04**	*cis,cis*-9,12-C18:2	**57.04**
C17:0	0.10	*cis,cis,cis*-9,12,15-C18:3	0.06
C18:0	**3.32**	*cis*-9-C20:1	0.15
C20:0	0.25	*trans*-9-C18:1	0.07
C22:0	0.73	*cis*,*trans*-9,12-C18:2, *trans*,*cis*-9,12-C18:2	0.23
C24:0	0.29	*trans*,*trans*-9,11-C18:2, *trans*,*trans*-10,12-C18:2	0.03

FA composition of standard SSO, main fatty acids are highlighted in bold

[a] Individual FA are listed in abbreviation: carbon atom number: double bond number

Alkali-isomerized methyl linoleate: preparation of standards

Positional and geometrical isomers of methyl linoleate were not commercially available. Catalytic alkali isomerization was used to prepare standards. Conjugation of methyl linoleate double bonds was performed as previously described [14] with slight modification. In brief, 25 g of potassium hydroxide ethylene glycol solution (6.5–6.6 %, m/m) was placed in the glass isomerization tube (glass tube equipped with glass capillary reaching to the bottom of the vessel, height 254 mm, inner diameter 25 mm). Current of argon (Ar 4.8; purity 99.998 %, oxygen content <3 ppm) passed through the reagent solution by means of capillary to remove remaining air and to agitate ethylene glycol alkaline solution gently at 150 °C. After addition of 250 mg of methyl linoleate into isomerization tubes, temperature increased to 180 °C. The resulting mixture was heated for an additional 20 min at 180 °C. The reaction mixture was allowed to cool to room temperature. Isomers of *cis*,*trans*-octadeca-9,11-dienoic acid and *trans*,*cis*-octadeca-10,12-dienoic acid were partitioned between hexane and acidified water phase to neutralize ethylene glycol alkaline solution. Hexane extract was washed with water to neutral reaction, dried over anhydrous sodium sulfate, and concentrated. Fatty acid composition of isolated methyl linoleate isomers was determined by capillary gas–liquid chromatography. The elution order of geometrical isomers on the column SP^{TM} 2560 (Supelco, Bellefonte) corresponded with published data [9].

Laboratory high temperature experiments

Polymerization experiments were performed in the same isomerization tubes (glass tube equipped with glass capillary reaching to the bottom of the vessel, height 254 mm, inner diameter 25 mm) used for the isomerization of methyl linoleate designed according to IUPAC standard method [14]. Sunflower oil (25 g) was heated in glass isomerization tubes placed in a hot thermostat (a modified oven of gas chromatograph Chrom 4, LP Praha) at temperatures 240, 250, and 260 °C up to 170 h under an argon atmosphere (Ar 4.8; purity 99.998 %, oxygen content <3 ppm). Exact temperature was scanned by a digital thermometer F200 (Automatic Systems Laboratories, UK). Samples of the heated oils for analyses were taken in appropriate intervals. Acid values of the collected samples were estimated. Sunflower oil samples oxidized under defined conditions with different peroxide values (PV1 = 2.61 mmol ½ O_2/kg, PV2 = 7.12 mmol ½ O_2/kg, PV3 = 11.53 mmol ½ O_2/kg, PV4 = 27.68 mmol ½ O_2/kg) were polymerized under the same conditions as previous samples.

Determination of positional and geometrical unsaturated fatty acid isomers by capillary gas–liquid chromatography (CGLC) and ultraviolet spectrophotometry

Positional and geometrical unsaturated fatty acid isomers were determined according to IUPAC and AOCS Official methods [15–17] by capillary gas–liquid chromatography (CGC). Pentadecanoic acid added before the methylation stage [18] was used as an internal standard for accurate determination of absolute concentration of fatty acids converted to corresponding fatty acid methyl esters (FAME). Correction factors for the internal standard were used before conversion of peak areas into mass percentages of the individual fatty acids. Analysis was performed on gas chromatograph 6890 N (Agilent Technologies) with capillary column SP^{TM} 2560 (Supelco, Bellefonte), 0.25 mm × 100 m, film thickness 0.2 μm. The conditions of analysis were as follows: hexane solution of FAME (1 %) was used for the injection (1 μl), split injection (1:50) at 220 °C; flow of carrier gas (He) 1 ml/min; analysis at 175 °C for 120 min; FID detection at 250 °C, flow of H_2 40 ml/min, air flow 450 ml/min and make-up gas (N_2) flow 45 ml/min. Determinations were performed minimally in triplicate. The error bars in scatter plots represented standard deviations. Detection limit was estimated.

Percentage content (m/m) of total conjugated dienes was determined according to IUPAC standard method [14]. Absorbance measurement of hexane solution at 233 nm was performed on Varian Cary 50 UV–Vis spectrophotometer (Agilent Technologies) in triplicate.

Quantitative analysis of triacylglycerol composition

Content of individual triacylglycerols (TAG) in oils before and after heating was determined by reversed phase high-performance liquid chromatography with evaporative light-scattering detector (RP-HPLC/ELSD) in accordance with equivalent chain number (ECN) values [19]. The analysis was carried out using an HPLC Chromatograph Agilent 1100 Series (Agilent Technologies) with pre-column SEPARON ^{TM}SGX C18 5 μm (30 × 3.3 mm) and column Nucleosil 120-5-C18 (250 × 4 mm, 5 μm; WATREX). Samples for analysis were dissolved in acetone with addition of TAG/ECN 38 (*sn*-1,2-didecanoyl-3-tetradecanoylglycerol; purity >98 %) as an internal standard close to the material to be analyzed. Solvent mixture of acetone:acetonitrile:methanol (4:2:1) was used as a mobile phase. Parameters of analysis were as follows: injection volume 50 μl, mobile phase with flow 1 ml/min, temperature 40 °C, and time 55 min. Instead of RID, ELSD detector was used, temperature of detection 90 °C. Linear

range of detector response for selected triacylglycerol standards with different degrees of unsaturation was measured (tripalmitin, tristearin, triolein, trilinolein; purity >98 %) [20]. Each sample was run in triplicate. The error bars in scatter plots represented standard deviations. Detection limit was estimated.

Quantitative analysis of triacylglycerol polymers

The total content of TAG polymers was determined using high-pressure size exclusion chromatography (HPSEC)/evaporated light-scattering detector analysis according to the ISO method [21]. The analysis was performed on an HPLC Chromatograph Agilent 1100 Series (Agilent Technologies) with a column PLgel, (7.5 × 300 mm with the capability to separate molecules of MW <4,000 Da, 5 μm; Agilent Technologies). Tetrahydrofuran (THF) was used as a mobile phase with flow 0.6 ml/min. Analysis was performed at 25 °C for 15 min. Instead of refractive index detector, ELSD detector was used (temperature 50 °C, air as a carrier gas). Samples of oils were dissolved in the THF to the final concentrations 3–5 mg/ml. Stearic acid was added as an internal standard instead of glycerol suggested by Burkow et al. [22]. Determinations were performed minimally in triplicate. The error bars in scatter plots represented standard deviations. Detection limit was estimated.

Qualitative analysis of triacylglycerol polymers

Mass spectrometry (MS) analysis of TAG polymers was performed both as direct injection of samples to MSD using atmospheric-pressure chemical ionization (APCI) source of ionization and as HPSEC/APCI-MS analysis after previous separation of molecules on HPSEC column, as for quantitative polymer content analysis. The measurements were made using mass spectrometer 4000 QTRAP LC–MS/MS System (Applied Biosystems, Streetsville, ON, Canada). In the case of direct MS analysis, the samples were injected by a syringe to the ion source using automatic syringe pump (Harvard Apparatus) with flow rate 50 μl/min. All MS measurements were processed using Analyst 1.5 program (Applied Biosystems).

For direct APCI-MS in the positive ion mode, the conditions were as follows: ion source—heated nebulizer with high-purity nitrogen (99.995 %) with values 15 (GS1), 0 (GS2), curtain gas 10 psi, nebulizer current 3 kV, temperature of source 400 °C, unit mass resolution for Q1 and Q3, and scan range 50–2800 m/z in 3 s. The fragmentation values as CE, DP, EP, and CCexP were 10–60 eV, 115, 10, and 10 V, respectively.

HPSEC/MS (LC/MS) analyses were performed using ultra-performance liquid chromatograph UFLC XR prominence (Schimadzu). The optimized analysis conditions for TAG polymer samples were following: volume of injection 2 μl, mobile phase (THF) with flow rate 0.3 ml/min, ambient temperature (pressure ca 1.55 MPa), and analysis time 20 min. The optimal concentration for the injection of samples based on empirical experience was 2–3 mg/ml for polymerized oils and 1–1.5 mg/ml for unpolymerized oils (oils before heating). Samples were dissolved only in THF to corresponding concentration. MS parameters were adjusted as follows: curtain gas 70 kPa, source temperature 400 °C, GS1 25, GS2 30, and nebulizer current 3 kV. The spectra were obtained over a scan range 50–2000 m/z in 3 s. Linear ion trap (LIT) fill time was set at 20 ms, and the IDA threshold was set at 100 cps for collecting enhanced product ion spectra (EPI) from the eight most intense peaks. The EPI scan rate was 4,000 Da/s. The enhanced MS (EMS) scan rate was 1,000 Da/s. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas. The CE was 30 eV, DP 115 V, EP 10 V, CE 30 eV, and CCexP 20 V.

Data analysis: nonlinear regression analysis and calculation of induction period of polymerization

Nonlinear regression analysis of increase in total triacylglycerol polymers and decrease in linoleic acid during laboratory high temperature experiments of sunflower oil samples with different peroxide values was performed by software Statistica 9 (Statsoft data mining and statistical analysis software) and Origin 8.0 (OriginLab data analysis and graphing software). In the case of increasing triacylglycerol content, inflection points were determined according to the second derivative changes of the best-fit mathematical equations of the model. Optimal mathematical model was assessed according to the 95 % CIs, coefficients of determination, and the standard deviation of the residuals. Decrease in linoleic acid was found to be the first-order reaction. Reaction rate constants were calculated.

Results and discussion

Analysis of geometrical and positional isomers of octadecadienoic acids

Quantitative analysis of absolute concentrations of individual fatty acids was based on the internal standard method. Therefore, previous determination of the relative mass response factors between selected fatty acid methyl-ester (f_i^m) present in sunflower oil and pentadecanoic acid methyl ester was of primary importance. Moreover, retention factors can be instrument dependent. Following response factors were determined: 0.99 for palmitic acid;

0.98 for stearic acid; and 0.97 for oleic and linoleic acid. Detection limit of the determination of positional and geometrical unsaturated fatty acid isomers by capillary gas–liquid chromatography (CGC) was 0.05 % of the total area.

Conjugated isomers of *cis*,*trans*-octadeca-9,11-dienoic and *trans*,*cis*- octadeca-10,12-dienoic were prepared by alkali isomerization of linoleic acid in ethylene glycol [14]. This method was found to be suitable as other catalytic isomerization techniques, for example, iodine, selenium, and light isomerization. Selective catalyst for geometrical isomerization is *p*-toluene sulfinic acid.

The sequence of *trans*,*trans*-, *cis*,*trans*-, *trans*,*cis*- and *cis*,*cis*-octadeca-9,12-dienoic acid methyl ester isomers corresponds to elution order on column SP^{TM} 2560 and is in agreement with published data [9]. Approximately 6–9 isomers of conjugated *cis*,*trans*- and *trans*,*cis*-octadecadienoic acids were formed. Sum of these minor compounds represent up to 50 % of total conjugated dienes in the final stage of laboratory high temperature experiments. Conjugated all-*trans*-octadecadienoic acids were identified by ECN values. Retention time of these isomers is very similar; therefore, they were eluted in one peak. They represent residual conjugated dienes present in the samples [9]. Figure 1 illustrates a typical GC/FID chromatogram of sunflower oil fatty acid methyl esters.

Influence of initial oxidation of the lipid matrix on isomerization and polymerization processes

High temperature heating of sunflower oil caused geometrical and positional isomerization—conjugation and polymerization of unsaturated fatty acids. Oxopolymerization can be excluded because of absence of oxygen in the argon atmosphere. To explain the influence of matrix oxidative stability, sunflower oil was oxidized under defined conditions. Samples with different peroxide values were prepared (PV1 = 2.61 mmol ½ O_2/kg, PV2 = 7.12 mmol ½ O_2/kg, PV3 = 11.53 mmol ½ O_2/kg, PV4 = 27.68 mmol ½ O_2/kg). Increase in total triacylglycerol polymers during laboratory high temperature experiments of oxidized samples was monitored. Inflection points that symbolized induction periods of polymerization of the best nonlinear regression results of curve fitting were determined as the second derivative changes (Fig. 2, 3). Numerical values are shown in Table 2. Optimal mathematical model was assessed according to narrow 95 % CIs and high coefficients of determination.

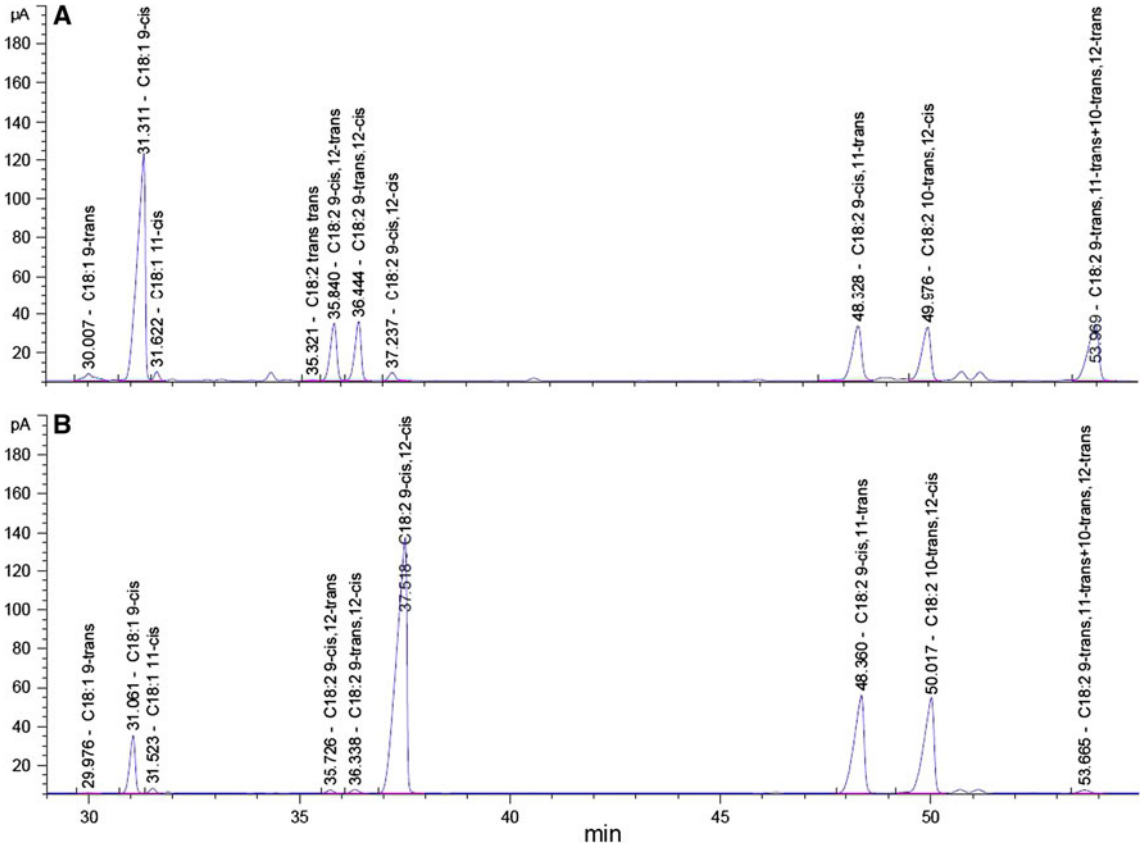

Fig. 1 GC/FID chromatograms of sunflower oil after deodorization/physical refining simulation at 260 °C for 52 h (chromatogram A) and after controlled conjugation experiment in ethylene glycol with KOH (chromatogram B)

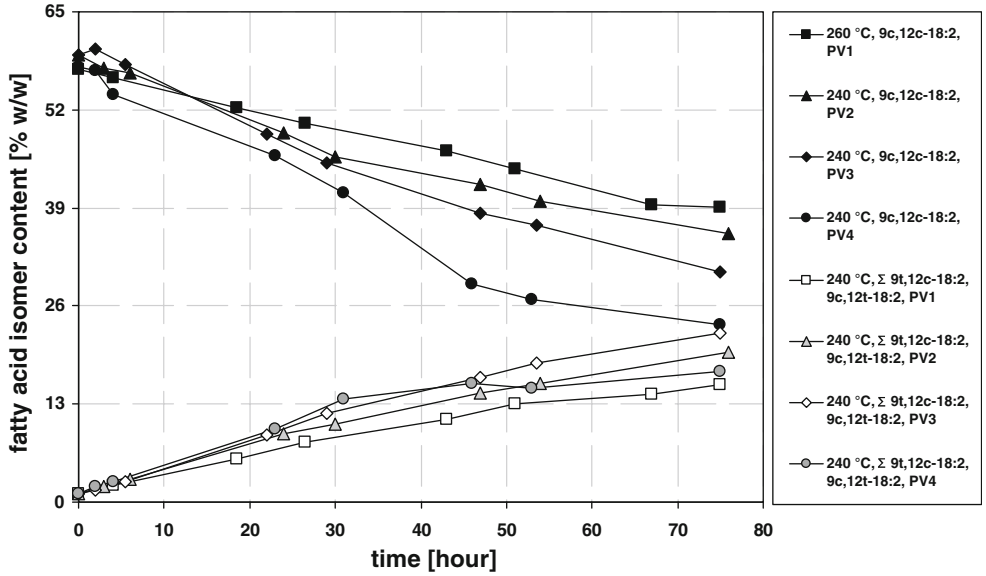

Fig. 2 Increase in selected geometrical isomers: *cis*,*trans*-9,12-octadecadienoic acid (9*c*,12*t*-18:2) and *trans*,*cis*-9,12-octadecadienoic acid (9*t*,12*t*-18:2) and decrease in linoleic acid before and during deodorization/physical refining simulation at 240 °C (time 0–75 h), affected by different peroxide values

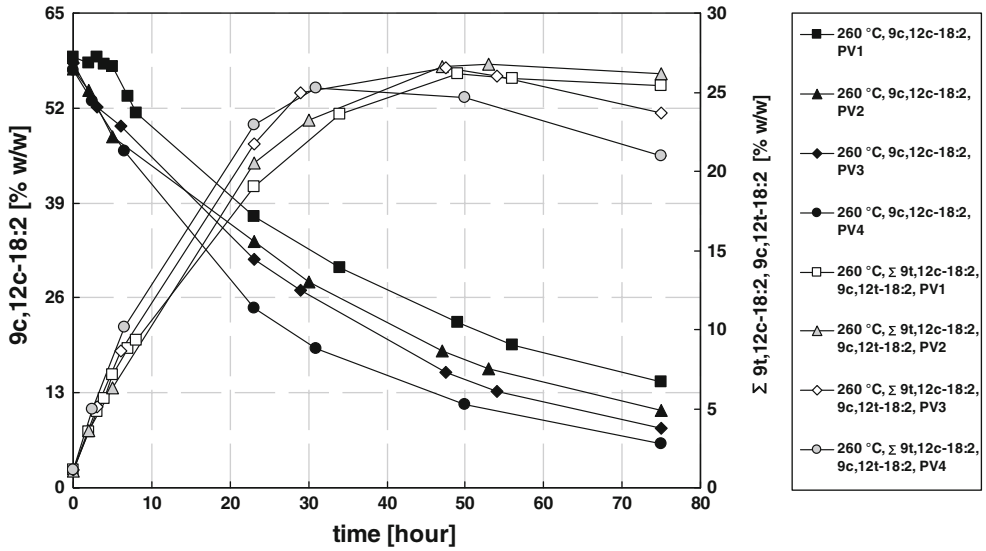

Fig. 3 Increase in selected geometrical isomers: *cis*,*trans*-9,12-octadecadienoic acid (9*c*,12*t*-18:2) and *trans*,*cis*-9,12-octadecadienoic acid (9*t*,12*t*-18:2) and decrease in linoleic acid before and during deodorization/physical refining simulation at 260 °C (time 0–75 h), affected by different peroxide values

Reaction rate of isomerization (Fig. 2, 3) and polymerization (Fig. 4, 5) increased with higher peroxide value of initial oils. It was observed that the higher peroxide values of sunflower oil samples lowered the induction period of polymerization of heat-induced polymerization. The level of initial oxidation of the oil has a significant influence on the rate of isomerization, polymerization (Table 2), and duration of induction period of polymerization reactions in systems without propagation step of autoxidation (e.g., low partial pressure of oxygen during edible oil deodorization or argon atmosphere during model laboratory high temperature experiments). Induction period of polymerization was temperature dependent at constant hydroperoxide content of origin sunflower oil (SSO; peroxide value: 2.06 mmol ½ O_2/kg), and there was a strong negative correlation between induction period of polymerization and temperature. Exact values of induction periods of polymerization under different temperatures are as follows: 220 °C/107.47 h; 230 °C/83.53 h; 240 °C/75.47; 250 °C/31.71 h; and 260 °C/20.03 h.

Table 2 Influence of initial oxidation of the lipid matrix on TAG polymerization

Temperature (°C)	Peroxide value (mmol ½ O_2/kg)	IP of polymerization[a] (h)	k[b] (s^{-1})
240	2.61	16.69	0.0053
	7.12	15.09	0.0069
	11.53	12.72	0.0092
	27.68	9.25	0.0130
260	2.61	16.47	0.0198
	7.12	15.91	0.0227
	11.53	9.50	0.0266
	27.68	5.83	0.0305

[a] Induction periods of polymerization reactions

[b] Reaction rate constants of linoleic acid decrease—first-order reactions

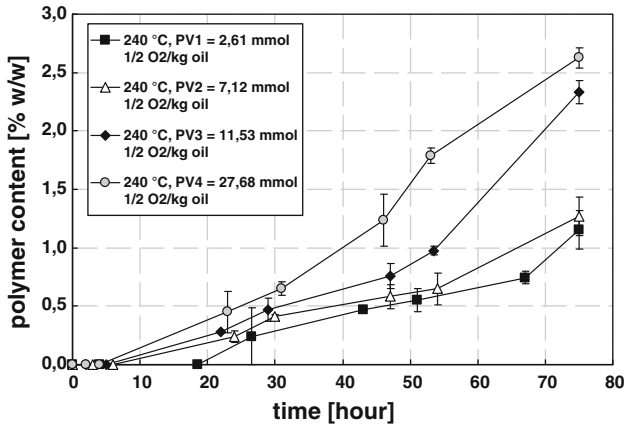

Fig. 4 Increase in total polymer triacylglycerol concentration before and during deodorization/physical refining simulation at 240 °C (time 0–75 h), affected by different peroxide values

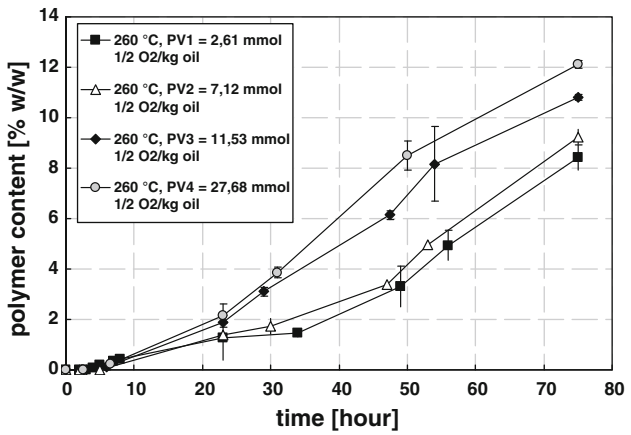

Fig. 5 Increase in total polymer triacylglycerol concentration before and during deodorization/physical refining simulation at 260 °C (time 0–75 h), affected by different peroxide values

At high temperature, hydroperoxides are decomposed to form peroxyl and alkoxyl radicals. Alkoxyl radicals are preferred due to lower dissociation energy of RO–OH (184 kJ/mol) in comparison with dissociation energy of ROO–H (377 kJ/mol) [23, 24]. Hydroperoxides are a source of radicals in the initial phase of thermally induced isomerization, cyclization, and polymerization of unsaturated fatty acids [25, 26]. This factor is important in addition to high temperature and plays a key role during homolytic splitting of C–H bonds. Thus, generated radicals and their mesomeric forms enter into the above mentioned reactions.

Thermally induced geometrical and positional isomerization

In the studied system, geometrical isomerization proceeds preferentially [2, 3, 5]. This type of isomerization has critical influence on decrease in the content of original *all-cis* isomers (Fig. 6) mainly of linoleic acid pentadienic system. In the case of acid-catalyzed geometrical isomerization (in equilibrium), formation of *trans* isomers with lower energy ($\Delta H^0_{\text{hydrog}} = -4.0$ kJ/mol [27]) occurs. In the studied system of sunflower oil, a presence of acid catalyst is not expected. Reaction under heat-induced conditions is not in equilibrium because the formed *cis*/*trans* isomers proceed to next reactions. Geometrical isomerization is preferred above positional isomerization or conjugation. The main products of geometrical isomerization are *cis*,-*trans*- and *trans*,*cis*-octadeca-9,12-dienoic acids that show a temperature-dependent maximum (Fig. 7). *trans*,*trans*-Octadeca-9,12-dienoic acid is a minor product and is formed subsequently from the two previous mentioned isomers.

The content of conjugated dienes was determined by two methods. Higher amounts of conjugated diene contents were determined by the spectrophotometric rather than the CGC method. Differences of the two methods, which may be caused by the occurrence of conjugated dienes in structures of high molecular weight compounds that may not be determined by the CGC method, are illustrated in Fig. 7. Although conjugated dienes have lower energy and thus are more energetically stable due to the bonding interaction than dienes with isolated double bonds ($\Delta H^0_{\text{hydrog}} = -16.0$ kJ/mol [28]), the extent of conjugation was significantly lower than geometrical isomerization (Fig. 8). Conjugated diene content was higher at both monitored temperatures than the content of *trans*,*trans*-octadeca-9,12-dienoic acids. *all-trans*-Octadecadienoic conjugated acids were formed from the beginning of the process. Their content increased and had a maximum at 260 °C that was shifted in time from the maximum of *cis*,*trans*-octadeca-9,12-dienoic acids. *all-trans*-Octadeca-dienoic conjugated acids participate in the other reactions because they are essential precursors for polymer formation by the Diels–Alder mechanism.

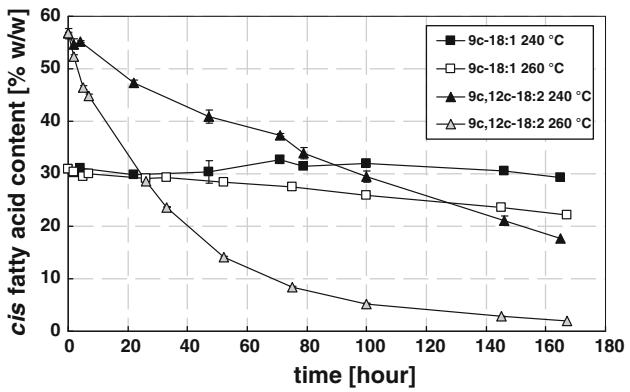

Fig. 6 Decrease in total *cis*-9-octadecenoic acid (9*c*-18:1), *cis,cis*-9,12-octadecadienoic acid (9*c*,12*c*-18:2) concentration before and during deodorization/physical refining simulation at two temperatures, 240 and 260 °C (time 0–167 h)

Polymerization of TAG

Quantitative analysis of triacylglycerol composition was based on the internal standard method. Previous determination of linear range of detector response for selected triacylglycerol standards was of primary importance [20]. It was found out that the detector response of pure standards of tripalmitin, tristearin, triolein, and trilinolein was linear over the concentration range 2–3 mg/ml (Fig. 9). Coefficients of determination of the calibration curves in reported concentration range were following: 0.9921 for tripalmitin, 0.9918 for tristearin, 0.9950 for triolein, and 0.9952 for trilinolein (Fig. 9). Detection limit of the method for unsaturated TAG standards was 0.03 mg/ml. Decrease in individual triacylglycerols by RP-HPLC/ELSD was estimated. Concentration changes of total and selected

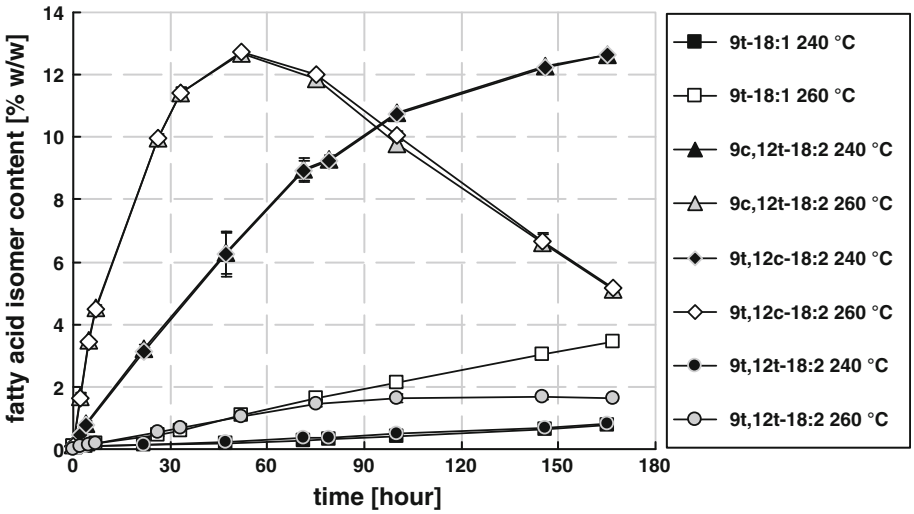

Fig. 7 Changes in total *trans*-9-octadecenoic acid (9*t*-18:1), *cis*,*trans*-9,12-octadecadienoic acid (9*c*,12*t*-18:2), *trans*,*cis*-9,12-octadecadienoic acid (9*t*,12*t*-18:2) and *trans*,*trans*-9,12-octadecadienoic acid (9*t*,12*t*-18:2) concentration before and during deodorization/physical refining simulation at two temperatures, 240 and 260 °C (time 0–167 h)

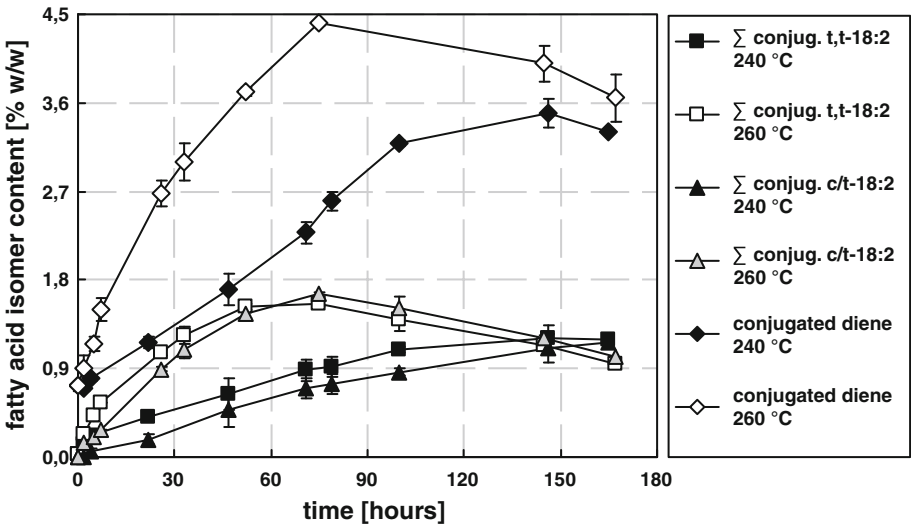

Fig. 8 Changes in *trans*,*trans*-9,11-octadecadienoic acid, *trans*,*trans*-10,12-octadecadienoic acid (∑conjug. *t*,*t*-18:2), *trans*,*cis*-9,11-octadecadienoic acid, *trans*,*cis*-10,12-octadecadienoic acid, *cis*,*trans*-9,11-octadecadienoic acid, *cis*,*trans*-10,12-octadecadienoic acid (∑conjug. c/*t*-18:2) and total conjugated diene concentration before and during deodorization/physical refining simulation at two temperatures, 240 and 260 °C (time 0–167 h)

triacylglycerols according to equivalent carbon number during deodorization/physical refining simulation are shown in Fig. 10 and 11. Quantitative analysis of TAG polymers on HPSEC column was based on the internal standard method with stearic acid instead of glycerol suggested by Burkow et al. [20, 22]. Free fatty acids, estimated as acid values of corresponding samples, coeluted in one peak with internal standard, were corrected.

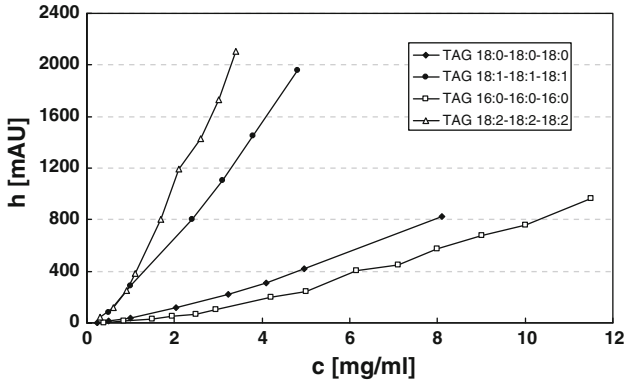

Fig. 9 Linear range of the detector response of pure analytical standards of tripalmitin, tristearin, triolein, trilinolein

Polymerization in connection with conditions of deodorization/physical refining is usually not mentioned [1–7]. If polymerization discussed, it appears that the polymers originate mainly from raw oil and are formed with oxygen participation [12, 13]. Decreasing content of monomeric TAG was determined by two methods: HPSEC/ELSD and RP-HPLC/ELSD. HPSEC/ELSD allowed estimating of total content of triacylglycerols, whereas RP-HPLC/ELSD enabled to determine individual triacylglycerols according to ECN values. Direct estimation of triacylglycerol species confirmed that trilinolein (ECN 42) and dilinoleoyl-oleoyl glycerol (ECN44) participated in the polymerization reactions. The less reactive was trioleoylglycerol (ECN48) as it is shown in Figs. 10 and 11.

Polymeric reactions have an induction period. If peroxides are absent in the system, the induction period of polymerization significantly increases. Increasing hydroperoxide content accelerates polymeric reactions and thus

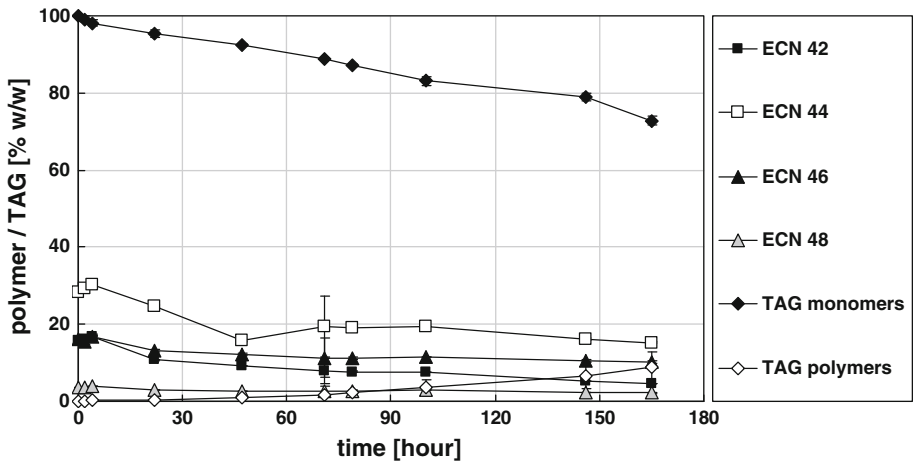

Fig. 10 Decrease in total and selected triacylglycerol (according to equivalent carbon number) concentrations before and during deodorization/physical refining simulation at 240 °C (time 0–167 h)

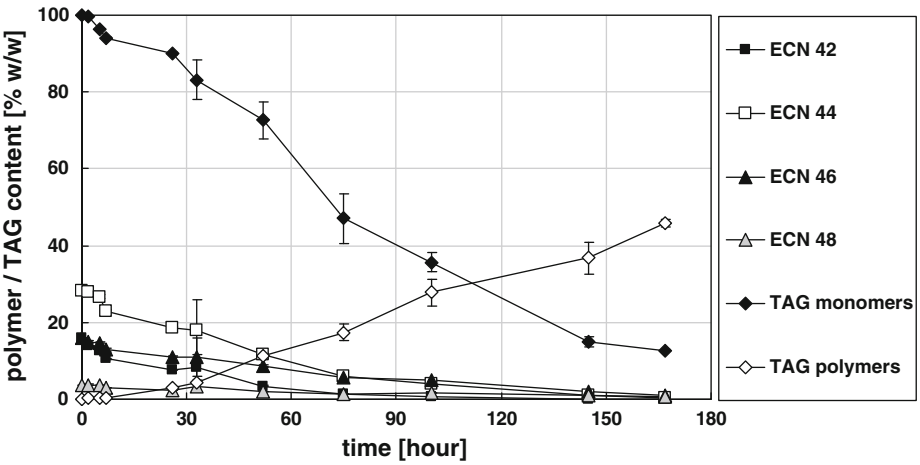

Fig. 11 Decrease in total and selected triacylglycerol (according to equivalent carbon number) concentrations before and during deodorization/physical refining simulation at 260 °C (time 0–167 h)

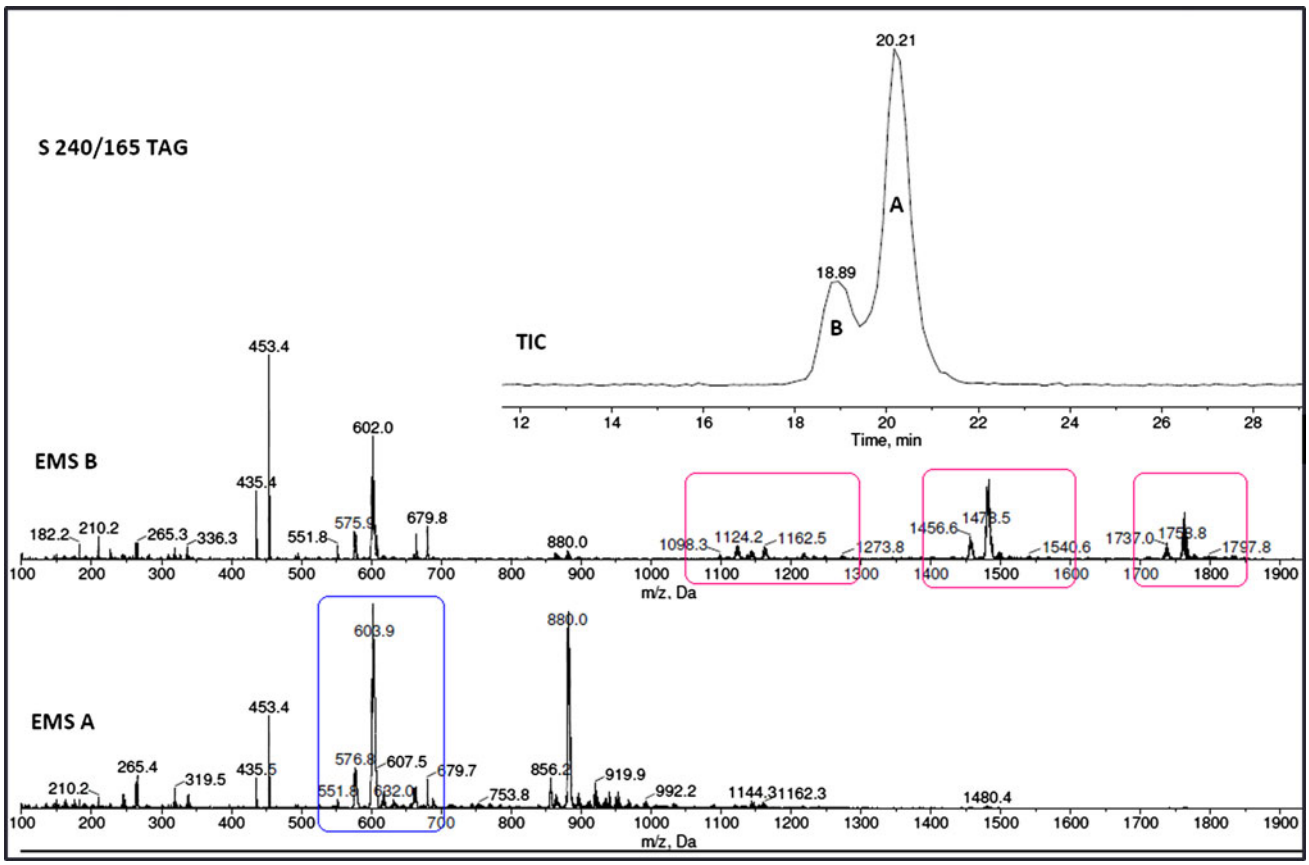

Fig. 12 APCI-MS spectra of separated triacylglycerol (*peak A*) and polymerized lipid fraction (*peak B*)

decreases calculated induction periods of polymerization. Induction period of polymerization is temperature dependent and decreases linearly with increasing temperature at the same hydroperoxide content (Fig. 10, 11). In the initial phase of the polymerization reaction, the oxygen radicals cause the reaction.

If oxygen radicals are exhausted in initial phase of polymerization, free radical polymerization or Diels–Alder reaction can occur. It is not known, if the thermally induced free radical chain reaction proceeds from increasing polymer content [24, 29].

The main conjugated diene found was all-*trans*-octadecadienoic isomer that is the only one of all conjugated dienes that may enter polymerization reactions with dienophils according to the Diels–Alder mechanism. In previous studies, two submechanisms are discussed—six-centered transition state without intermediate; and a diradical intermediate path with additional cyclization [30, 31]. Induction period was observed only in the case of polymerization reactions of triacylglycerols. This is the confirmation of the hypothesis that the *cis*/*trans* isomerization, positional isomerization, and polymerization are consecutive reactions in ascending order.

Qualitative analysis of acylglycerol polymers by HPSEC/APCI-MS

The qualitative analysis of polymerized compounds was performed with the same column used for quantitative determination of polymer content. Two peaks were detected: The first represented high molecular polymers (peak A) and the second fraction corresponds to TAG monomers (peak B) with enhanced mass spectra of both fractions. The total ion chromatograms (TIC) of both fractions of sunflower oil (SSO) heated at 240 °C for 165 h are listed in Fig. 12. Molecular ions were detected in the fraction A in the following intervals: 1100–1300, 1400–1600, and 1700–1850 m/z. Direct APCI-MS analysis confirmed previous results.

The highest molecular ions in the interval 1700–1800 m/z belonged to specific TAG dimers with different ECN values. Compounds from the other intervals (1400–1600 and 1100–1300 m/z) can be fragments of molecular ions of TAG dimers due to destructive conditions in the MS ion source. Because the partial hydrolysis of TAG was observed, it can be suggested that these high molecular components can be formed after polymerization

of TAG with di- and monoacylglycerols. The proposed molecular weights belong to this range.

Conclusion

Initial oil oxidation influences the rate of isomerization and polymerization during deodorization/physical refining. Conjugation that leads to all-*trans* diene formation is a significant process together with the well-described geometrical isomerization during isomerization reactions. All-*trans* dienes are incoming reactant for polymerization according to the Diels–Alder mechanism. Polymerization takes place in two phases: The initial phase conditions were found to be 10–30 h at 240–260 °C with peroxide content 2–30 mmol ½ O_2/kg. The propagation phase followed later. Identification of molecular peaks and confirmation of fragments were accomplished by connecting HPSEC to APCI-MS. In the studied system, dimers of triacylglycerols (TAG) dominated; remaining compounds were probably formed from di- and monoacylglycerols.

Acknowledgments This work was supported by funding from the Czech Ministry of Education, Youth and Sports (MSMT 6046137305) and Hlávkova foundation.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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Antioxidants in Margarine Emulsions

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Abstract: The lipid oxidation in margarine takes place in continuous liquid oil phase. The extension of fat interfaces in the system – emulsion of water in oil and the dispersion of fat crystals in liquid oil influences on the peroxidation, decomposition of hydroperoxides to aldehydes and the oxidative stability in the comparison with oxidation in the fat blend. Different antioxidants were used in margarine dispersions: *L*(+)ascorbic acid, ascorbyl palmitate and DL-α-tocopherol. Increasing polarity and decreasing molecular size of antioxidants have the positive influence on lipid oxidation: DL-α-tocopherol is the least effective antioxidant of all antioxidants, ascorbic acid is the most effective antioxidant and ascorbyl palmitate possesses similar, however, lesser effect. The combination of all three antioxidants restricts the production of hydroperoxides, the decomposition of hydroperoxides to aldehydes and the increase of oxidative stability was also achieved. Content of antioxidants 0.02% as ascorbic acid or ascorbyl palmitate mostly restrict the extent of lipid oxidation in the margarine dispersion with existent content of naturally present tocopherols in fat blend.

Keywords: antioxidant; ascorbic acid; ascorbyl palmitate; emulsion; margarine; tocopherol

INTRODUCTION

Lipid oxidation in the simplest system takes place in a liquid phase, oxygen diffuses to oil through macroscopic interface air/oil. The situation is more complicated in the case of food dispersions: the lipid oxidation in o/w emulsions takes place in droplets and on their surface. Oxygen diffuses in this case from air through the continuous water phase to the surface of lipid particles. The oxidation in o/w emulsions is relatively well studied; it is very often case in foods. In the margarine emulsion (w/o type) oxygen diffuses from air directly to the continuous oil phase where the oxidation takes place. Oxygen diffusion can be decreased by an interface form: the interface o/w and oil/solid crystal forms, presenting monoacylglycerole emulsifier creates a membrane on both interface and decreases considerably the oxygen diffusion, thus, the oxidation rate (Pokorná *et al*. 2004). A type of used emulsifier determines the decrease of lipid oxidation rate in droplets (Silvestre *et al*. 2000). Emulsifier can also influence antioxidant distribution in the o/w emulsion (Yuji *et al*. 2007).

Antioxidants possess different polarity and solubility in water and in oil; lipophilic antioxidants possess surface activity and together with emulsifier form interface. Interfacial phenomena are keys to a better understanding of antioxidant action in heterogenous foods (Frankel 1996). Tocopherols are surface active substances; therefore they are more effective than Trolox (hydrophilic analog) as inhibitors of hydroperoxide formation and their decomposition (Huang *et al*. 1996). Ascorbic acid acts as an antioxidant in aqueous system, a prooxidant, a metal chelator, a reducing agent of heavy metals or as an oxygen scavenger. The mixture of tocopherols and ascorbic acid exhibits a strong synergistic effect because ascorbic acid reduces tocopherols radicals. This can take place also in w/o olive oil emulsion (Mosca *et al*. 2008a) and efficiency of this synergism depends on specific surface area of aqueous dispersed phase. Ascorbyl palmitate increases the effect of ascorbic acid as antioxidant and also as emulsifier (Mosca *et al*. 2008b). Application of antioxidants depends on initial oxygen content in lipid system and on the type of oxygen supply limitation in lipid system.

MATERIAL AND METHODS

Composition of model margarine (% w/w): Fat blend 70%; solid fat content profile (%): $SFC_{10°C}$ 26.8, $SFC_{20°C}$ 15.1, $SFC_{30°C}$ 5.8, $SFC_{35°C}$ 4.5, $SFC_{40°C}$ 2.5. Fatty acid composition: SFA 23.4%, C18:1 25.1%, C18:2 50.2%, C18:3 0.4%, *trans*-FA 1.6%; content of tocopherols 0.045%. Emulsifier D (mixture of monoacylglycerols) – 0.02%. Sodium chloride 0.10%, lactic acid 0.02% (adjustment of water phase pH on 4.0 ± 0.25), water up to 100% (CaO 100 mg/l). Used antioxidants: L(+) ascorbic acid (99.7%; Merck) – AA, ascorbyl palmitate (> 99.0%; Fluka) – AP, DL-α-tocopherol (> 99.0%; Fluka) – TO. Used antioxidant content – 0.01, 0.02 and 0.025% w/w.

Margarine preparation: Laboratory mixer – Stephan, amount of one bath – 2.5 kg. The emulsification and the crystallisation – under argon atmosphere: agitation 1500 rpm, cooling from 45°C to 20–21°C during 25 minutes. The filling – 250 g of sample to the pot with plastic lid, no air in the package (air could diffuse through the lock between the lid and the pot).

Storage temperature and time – 15°C, 15 weeks. Analytical methods: the peroxide value (PV) of margarine emulsion (ISO3960/1994), the *p*-anisidine value (AV) of isolated fat phase (ISO6885/1994). The oxidation stability (induction period at 100°C – IP) – ML-Oxidograph. The average samples were taken from emulsion. Results are expressed as definite integral of the measured dependence X = X(τ), where X is PV, AV or IP. Primary data are not presented, exception to the oxidation stability. Series of margarine emulsions: A = control emulsion; B = 0.01% of ascorbic acid; C = 0.025% of AA; D = 0.01% of AP; E = 0.025% of AP; F = 0.01% of TO; G = 0.025% of TO; H = 0.02% of AA + 0.02% of AP; I = 0.02% of AA + 0.02% of TO; J = 0.02% of AP + 0.02% of TO; K = 0.01% of AA + 0.01% of AP + 0.01% of TO; L = fat blend.

RESULTS AND DISCUSSION

The extension of fat interfaces in emulsion w/o and the dispersion of fat crystals in liquid oil (the sample A) influences on the rate and on the extent of oxidation, in the comparison with oxidation in the fat blend (the sample L) from the point of view of the formation of hydroperoxides, their decomposition to aldehydes and the oxidative stability. The fat blend is the simple dispersion of fat crystals in liquid oil. The main component, that oxidises, is linoleic acid. The definite integrals were counted from measured dependences of the peroxide value, the *p*-anisidine value and the oxidative stability, because of the induction period could not be determined (Figures 1, 2 and 4).

It is obvious, that increasing polarity and decreasing molecular size of antioxidants have the positive influence on lipid oxidation in the dispersive system of the margarine. DL-α-tocopherol (the samples F and G) is the least effective antioxidant of all antioxidants. The content 0.025% of tocopherol has already prooxidative effect. If natural content of tocopherols in fat blend is added up, the total content of tocopherols (0.057%) exceeds the limit, when tocopherol has antioxidant effect (Frankel 1996). Ascorbic acid is the most effective antioxidant, if it is used only one antioxidant (the samples B and C) (Mosca *et al*. 2008b), the minimal concentration of hydroperoxides and consequently

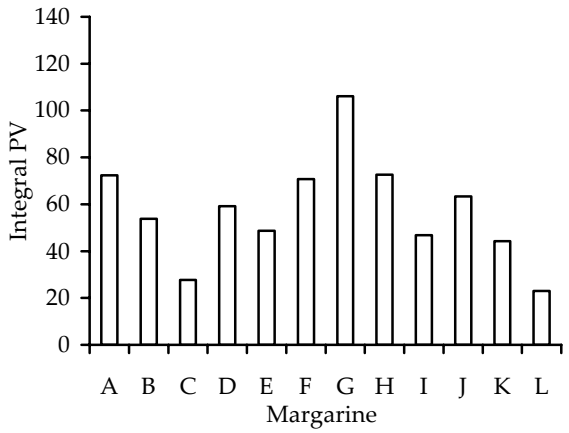

Figure 1. Integral of peroxide value of overall margarine emulsion

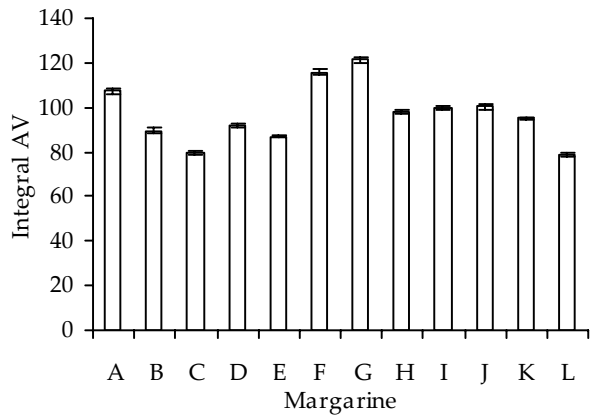

Figure 2. Integral of *p*-anisidine value of fat phase of margarine emulsion

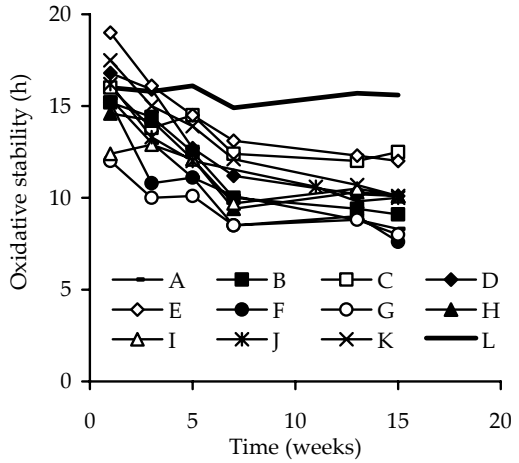

Figure 3. Oxidative stability of fat phase of margarine emulsion

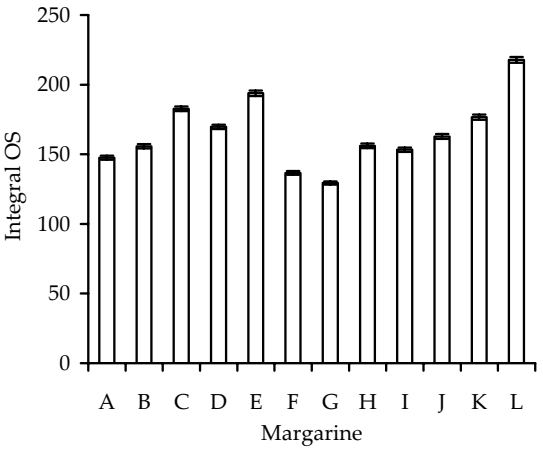

Figure 4. Integral of oxidative stability of fat phase of margarine emulsion

of aldehydes forms at the concentration 0.02%. Ascorbyl palmitate possesses similar, however, lesser effect (the samples D and E). It is interesting, that lower degree of oxidation and higher oxidative stability are not achieved by the combination of ascorbic acid and ascorbyl palmitate (the sample H) as well as ascorbyl palmitate and tocopherol (the sample J), in the comparison with only ascorbic acid and ascorbyl palmitate. The combination of ascorbic acid with tocopherol (the sample I) affects positively the formation of hydroperoxides, that decompose to aldehydes in higher rate (Figures 1 and 2), so the oxidative stability does not increase. The combination of all three antioxidants restricts the production of hydroperoxides, the decomposition of hydroperoxides to aldehydes and the increase of oxidative stability was also achieved (Figures 3 and 4). The increase of oxidative stability of the margarine (Figures 3 and 4) with 0.02% of ascorbic acid (the sample C) and 0.02% of ascorbyl palmitate (the sample E) corresponds to minimal extent of peroxidation and minimal decomposition of hydroperoxides to aldehydes.

CONCLUSION

Antioxidants as ascorbic acid or ascorbyl palmitate mostly restrict the extent of lipid oxidation in the margarine dispersion with existent content of naturally present tocopherols in fat blend.

Acknowledgements: This work was supported by funding from the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046137305).

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Antioxidant Stability of Phenolic Acids and Their Esters

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Abstract: Natural antioxidants found in plants play an important role in food and cosmetics because there is a trend to use this type of antioxidants. Phenolic acids (hydroxy derivatives of benzoic and cinnamic acids) are well soluble in various systems containing water, however, in bulk oils are limited soluble. Esters of phenolic acids are suitable antioxidants for bulk oil systems. The oxidative stability of the bulk oil systems with selected phenolic acids and their esters was determined by method Rancimat and Oxidgraph and compared with α-tocopherol and butylhydroxytoluene (BHT). The antioxidant activity of gentisic acid, caffeic acid and methyl caffeate is the highest and generally inversely proportional to redox potential of phenolic acids.

Keywords: phenolic acid; antioxidant; oxidative stability

Antioxidants are widely used in many foods to prevent lipid oxidation. However, the usage of the synthetic antioxidants is questionable because of possible toxic and carcinogenic components formed during their degradation. Thus, the interest in natural antioxidants and their application has increased in order to avoid such negative influence (Farhoosh 2005; Rousis *et al.* 2008).

Phenolic acids are a subgroup of a large group of secondary plant metabolites that is named phenolics. They usually occur as esters of organic acids, glycosides or are bound to protein and other cell wall polymers. Only minority of phenolic acids exists as free acids (Shahidi & Naczk 2004a).

The antioxidant activity of the phenolic compounds in food systems depends not only on the structure and chemical reactivity of the phenolics but also on other factors such as their physical location and environmental conditions (Sorensen *et al.* 2008). The phenolic acids are able to scavenge of free radicals and chelate and/or reduce metal ions (Chen & Ho 1997; Gülcin 2006). The application of phenolic acids in oil and fat systems is limited because of their hydrophilic nature. Changes of solubility of phenolic acids can be achieved upon their esterification (Kabourne *et al.* 2008).

MATERIAL AND METHODS

Sunflower oil (Setuza, a.s.): the fatty acids composition (CG-FID): C16:0 – 5.91%; C18:0 – 3.84%; C18:1 – 19.53%; C18:2 – 61.35%; C18:3 – 0.29%; C20:0 – 0.25%; C22:0 – 0.67%.

Phenolic acids (Figure 1): gentisic (98%, Aldrich), protocatechuic (≥ 97%, Fluka), vanillic (≥ 97%, Fluka), syringic (≥ 97%, Fluka), caffeic (99%, Alfa Aesar), ferulic (≥ 98%, Fluka), sinapic (≥ 97%, Fluka), *p*-coumaric (≥ 98%, Fluka) and *p*-hydroxybenzoic (99%, Aldrich).

Methyl esters of phenolic acids: the phenolic acids were esterified (70°C, 5 hours) by methanol in the presence of *p*-toluenesulfonic acids.

Commercial antioxidants: dl-α-tocopherol (98.2%, Calbiochem), butylhydroxytoluene (BHT) (99%, Aldrich).

The concentration of antioxidant in sunflower oil was 0.05% w/w, concentration range for phenolic acids with significant antioxidant effect was 0.002–0.05% w/w.

Rancimat method (Rancimat 743, Metrohm): the sample of oil with antioxidant was stored at 120°C and bubbled by air. Volatile secondary oxidative products were carried to the demineralised water

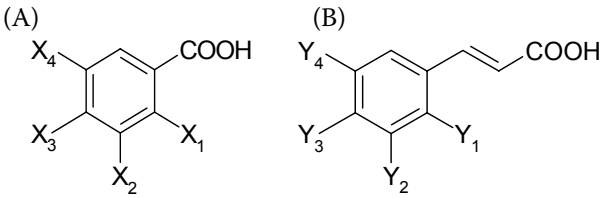

Figure 1. Used phenolic acids derived from benzoic acid (A) and cinnamic acid (B)

gentisic acid: X_1 = -OH, X_4 = -OH; **protocatechuic acid**: X_2 = -OH, X_3 = -OH; **vanillic acid**: X_2 = -OCH_3; X_3 = -OH; **syringic acid**: X_2 = -OCH_3, X_3 = -OH, X_4 = -OCH_3; ***p*-hydroxybenzoic acid**: X3 = -OH; **caffeic acid**: Y_2 = -OH, Y_3 = -OH; **ferulic acid**: Y_2 = -OCH_3, Y_3 = -OH; **sinapic acid**: Y_2 = -OCH_3, Y_3 = -OH, Y_4 = = -OCH_3; ***p*-coumaric acid**: Y_3 = -OH. If it is not mentioned, X and Y = -H;

and a conductivity of water was measured. The induction period was determined as time when the conductivity does not increase – $IP_{Rancimat}$.

Oxidograph method (ML Oxidograph, Mikrolab Aarhus): the sample was stored at 110°C under oxygen atmosphere and the decrease of pressure of oxygen was measured. The induction period is time when oxygen pressure does not decrease – $IP_{Oxidograph}$.

Table 1. The induction periods (IP) in hours of sunflower oil with the antioxidants (0.05% w/w)

Sample	$IP_{Rancimat}$	$IP_{Oxidograph}$
Sunflower oil	2.48	2.55
Gentisic acid	4.54	6.85
Methyl gentisate	2.46	2.65
Caffeic acid	4.17	5.70
Methyl caffeate	4.28	6.25
Vanillic acid	1.97	1.90
Protocatechuic acid	2.55	2.90
Methyl protocatechuate	2.72	3.40
Syringic acid	2.02	2.20
Sinapic acid	2.38	2.35
p-Coumaric acid	1.89	2.10
Ferulic acid	2.26	2.40
p-Hydroxybenzoic acid	2.05	2.40
Methyl *p*-hydroxybenzoate	2.26	2.40
DL-α-Tocopherol	2.67	3.00
BHT	3.02	5.60

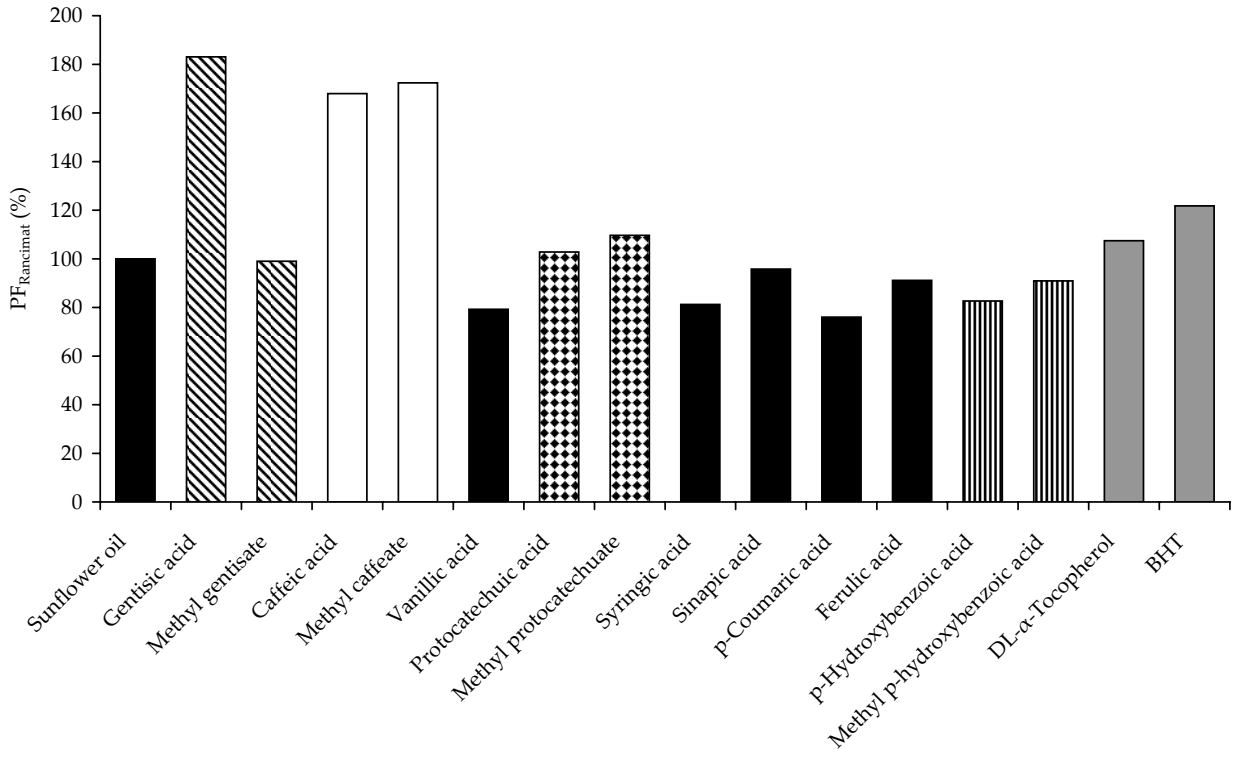

Figure 2. The protection factor (Rancimat method) of the used antioxidants (0.05% w/w)

Table 2. The influence of the induction period on the concentration of the phenolic acids

Concentration (% w/w)		0.002	0.004	0.006	0.010	0.020	0.030	0.040	0.050
PF(%)	gentisic acid	107.7	110.9	116.1	116.1	137.1	159.3	181.9	183.1
	caffeic acid	102.8	104.8	106.5	108.1	119.8	124.6	137.9	171.0
	methyl caffeate	100.8	100.8	110.5	114.9	138.3	146.4	164.9	179.4

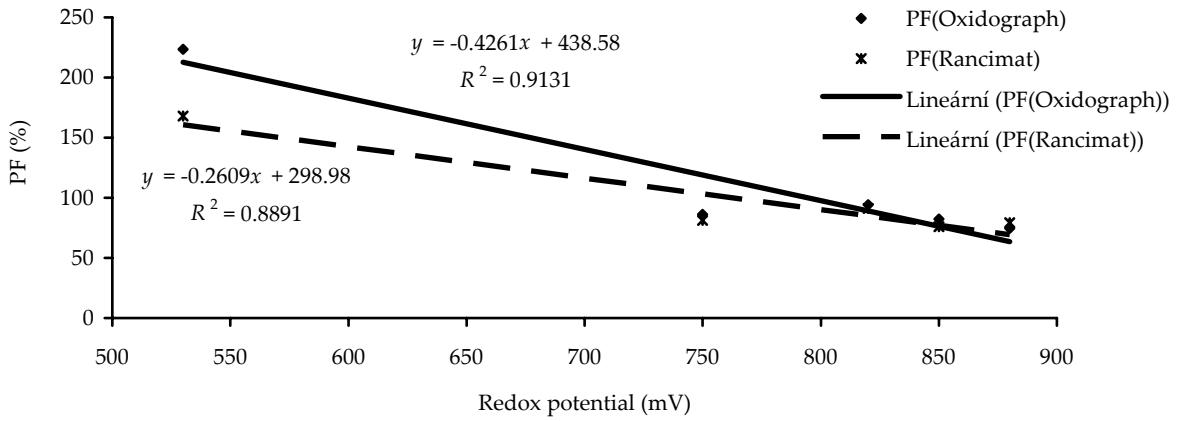

Figure 3. The correlation of the measured protection factor and the redox potential of selected phenolic acids (0.05% w/w)

The protection factor of antioxidants:

$PF = IP_{(oil+antioxidant)}/IP_{(oil)} \times 100$ (%)

RESULTS AND DISCUSSION

Table 1 and Figure 2 show that gentisic acid, caffeic acid and methyl caffeate have higher antioxidant effect in sunflower oil than α-tocopherol and BHT. Protocatechuic acid and methyl protocatechuate have only minor antioxidant activity. Based on the structure of benzoic acid with two hydroxy groups (gentisic and protocatechuic acid), as well as derivative of cinnamic acid (caffeic acid), these phenolic acids increase the protection of oil against lipid oxidation. If any hydroxy group in phenolic acid is substituted by a methoxy group (vanillic acid, ferulic acid), another methoxy group (syringic acid, sinapic acid) or only one hydroxy group (*p*-hydroxybenzoic acid, *p*-coumaric acid) is contained in phenolic acid, antioxidant activity of these substances will not be detected. In the case of the presence of two hydroxy groups in the molecule, the quinone structure can be formed.

The methyl esters of phenolic acids increase their solubility in oil systems. It is obvious that the methyl ester of caffeic and protocatechuic acid has a higher antioxidant activity than relevant acid (Table 1, Figure 2). The increasing content of the antioxidants increases also the protection of oil system against oxidation (Table 2).

The antioxidant effect of phenolic acids is related to their redox potential. Acworth and Phil (2003) reported that antioxidants have typical redox potential about +500 mV and prooxidants more than +600 mV. Thereby it is obvious from redox potential of caffeic (+530 mV), syringic (+750 mV), ferulic (+820 mV), *p*-coumaric (+850 mV) and vanillic acid (+880 mV) that caffeic acid functions as the antioxidant and other mentioned phenolic acids as prooxidants (Shahidi & Naczk 2004b). The protection factor of the phenolic acids is inversely proportional to their redox potential (Figure 3).

Acknowledgements: This work was supported by funding from the Ministry of Education, Youth and Sports of the Czech Republic (Project No. MSM 6046137305).

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Research Article

Antioxidant effect of mono- and dihydroxyphenols in sunflower oil with different levels of naturally present tocopherols

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Antioxidant properties of mono- and dihydroxyphenolic acids and their alkyl esters were examined, with emphasis on the relationship between their molecular structure and antioxidant activity. Test media with different tocopherol level were used for determining the oxidative stability: original refined sunflower oil (total tocopherols 149.0 mg/kg), partially tocopherol-stripped sunflower oil (total tocopherols 8.7 mg/kg) and distilled fatty acid methyl esters (FAME) as a tocopherol-free medium. The chemical reaction of tocopherols with diazomethane tested for the purpose to eliminate their antioxidant activity failed due to the negligible degree of methylation of hydroxyl group in the tocopherol molecule. Caffeic acid and protocatechuic acid (3,4-dihydroxyphenolic acids) and their alkyl esters were found to be more active antioxidants than monohydroxyphenolic acid (*p*-hydroxybenzoic acid), 2,5-dihydroxyphenolic acid (gentisic acid), 3-methoxy-4-hydroxyphenolic acids (vanillic and ferulic acids) and their corresponding alkyl esters. Naturally present tocopherols in refined sunflower oil proved to have a synergistic effect on gentisic acid but not on its alkyl esters. In contrast, tocopherols showed an antagonistic effect on alkyl esters of caffeic acid, because their protection factors decreased with increasing level of tocopherols in the test medium. Moreover, the antioxidant activity of these alkyl esters decreased with increasing length of their alkyl chain in conformity with the polar paradox hypothesis.

Practical applications: Tocopherols as naturally present antioxidants influence considerably the antioxidant activity of other antioxidants added to plant oils used as a test medium. Distilled fatty acid methyl esters prepared from refined sunflower oil may serve as an optimal tocopherol-free test medium. Some alkyl esters of phenolic acids were evaluated to be applicable as natural more lipophilic antioxidants in comparison with phenolic acids.

Keywords: Alkyl esters of phenolic acids / Antioxidant activity / Lipophilic antioxidants / Phenolic acids / Tocopherols

Received: August 16, 2012 / Revised: February 7, 2013 / Accepted: March 22, 2013

DOI: 10.1002/ejlt.201200293

1 Introduction

Lipid oxidation is responsible for a consequent decrease in nutritional and sensory quality of lipid-containing products. Their stability is directly associated with addition of suitable antioxidants. In the last years, there is a growing interest in natural antioxidants found in plants because of the world-wide trend toward the use of natural additives in food and cosmetics [1]. Phenolic acids are frequently studied because of their anti-inflammatory, anti-allergic, antimicrobial, anticarcinogenic, antiviral, and antioxidant effects. Phenolic acids occur in oilseeds (sinapic acid in rapeseed, *p*-hydroxybenzoic and caffeic acid in soya beans, chlorogenic acid in sunflower seed) [2], herbs (ferulic acid in lavender, caffeic acid in oregano, caffeic acid in lemon balm, rosmarinic acid in rosemary) [3], fruit (gallic and caffeic acid in blackberries, *o*-, *m*-, and *p*-hydroxybenzoic acid in cranberries), and vegetable (hydroxycinnamic acid derivatives and *p*-hydroxybenzoic acid in carrot, chlorogenic acid in potato) in unbound or bound forms (as glycosides or esters) [4].

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Abbreviations: FAME, fatty acid methyl esters of sunflower oil; **FID,** flame ionization detector; **IP,** induction period; **PF,** protection factor

Polarity of phenolic antioxidants influences their effect. Potter et al. [5] reported that more polar antioxidants are effective in nonpolar system of bulk oil and more lipophilic antioxidants are effective in polar system of oil-in-water emulsion. Polar antioxidant (such as Trolox and ascorbic acid) accumulates on oil–air interfaces in nonpolar system and thus protects oil against oxidation. In contrast, nonpolar antioxidants (such as α-tocopherol and ascorbyl palmitate) are dissolved in the oil phase. This paradoxical behavior of antioxidants is known as the polar paradox [6].

Phenolic acids act as primary antioxidants, exhibiting antioxidative potential by donating a hydrogen atom for breaking the free radical chain [7]. The molecular structure (position and number of hydroxyl groups) of phenolic acids has a considerable effect on their antioxidative properties. Phenolic acids with two (protocatechuic acid – 3,4-dihydroxybenzoic acid; caffeic acid – 3,4-dihydroxycinnamic acid) and more hydroxyl groups in a molecule (gallic acid – 3,4,5-trihydroxybenzoic acid) are more effective antioxidants than phenolic acids with only one hydroxyl group (3- or 4-hydroxybenzoic acid). The position of hydroxyl groups is also important, for example, 2,5-dihydroxybenzoic acid has lower antioxidant activity than 2,3-dihydroxybenzoic acid. Methylene group (3,4-dihydroxyphenylacetic acid) or ethylene group (caffeic acid) inserted between a phenyl ring and carboxylic group brings about the significant changes in antioxidant activity [8]. Derivatives of benzoic acid (*p*-hydroxybenzoic, vanillic, syringic, protocatechuic acid) have weaker antioxidant properties than the corresponding analogs of cinnamic acid (*p*-coumaric, ferulic, sinapic, caffeic acid) [9].

Alkyl esters of phenolic acids are known not only as efficient antioxidants but also as antimicrobial compounds [10]. Alkyl esters of caffeic and dihydrocaffeic acid have higher scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals than caffeic acid, while dihydrocaffeic acid shows maximal scavenging activity. This can be caused by different conformation of molecules. Dihydrocaffeic acid is connected with an aromatic ring by single bond and phenyl group can rotate flexibly, whereas rotation of phenyl group with esterified carboxylic group can be restrained. Caffeic acid has coplanar conformation [11]. Alkyl esters of rosmarinic and chlorogenic acid show so-called cut-off effect in oil-in-water emulsions. Their antioxidant activity increases with elongation of alkyl chain to critical point in homologous series of alkyl esters. Then, antioxidant activity rapidly decreases with further increase of the alkyl chain length due to a decrease of antioxidant concentration in water phase. Critical point of homologous series of alkyl esters of rosmarinic acid is octyl rosmarinate and chlorogenic acid is dodecyl chlorogenate [12, 13].

Antioxidant effect can be determined by using many accelerated methods. One group of such methods is based on determination of oxidation stability of oil with an antioxidant at higher temperatures (Schaal oven-storage test [14], active oxygen method, Rancimat test [15], Oxidograph test [16]). These tests predict shelf-life of samples and their results provide information about how much the added antioxidant increases their oxidative stability at room temperature.

If plant oil is used as the test medium, there is a problem with the presence of naturally occurring antioxidants (especially tocopherols), which may have interactive effects on the antioxidant activity of the antioxidants studied. Tocopherols can be removed from plant oil by column chromatography (adsorption on silica gel) [17, 18] or by methanolysis of triacylglycerols and subsequent distillation of fatty acid methyl esters (FAME) prepared from plant oil under low pressure [19].

The objective of this study was to prepare an optimal test medium for determination of antioxidant effect of mono- and dihydroxyphenolic acids and their more lipophilic esters and to examine how tocopherols naturally present in sunflower oil may affect the antioxidant activity of the used antioxidants.

2 Materials and methods

2.1 Test media for determining the oxidative stability

Three different lipid matrices were used as a test medium for determining the antioxidant activity of phenolic acids and their alkyl esters (the test media were analyzed by peroxide value [20], *p*-anisidine value [21], fatty acid, and tocopherol composition; Table 1):

Table 1. Peroxide value, *p*-anisidine value, fatty acid and tocopherol composition in original sunflower oil, tocopherol-stripped sunflower oil, and in FAME prepared from original sunflower oil.

	OSO[a)]	TSO[b)]	FAME
Peroxide value (meq.act.O/kg)[c)]	2.1 ± 0.2	2.6 ± 0.1	2.2 ± 0.1
p-Anisidine value [1][c)]	4.4 ± 0.1	4.9 ± 0.2	4.6 ± 0.1
Fatty acid (%)[d)]			
C16:0	6.2	6.8	6.6
C18:0	3.6	4.1	3.7
C18:1	26.4	29.1	26.6
C18:2	62.6	58.8	62.1
C18:3	1.0	0.3	0.8
Tocopherol (mg/kg)			
α	120	2.4	nd
β	10	nd	nd
γ	12	5.6	nd
δ	7	0.7	nd

nd, not detected.
[a)] Original sunflower oil.
[b)] Tocopherol-stripped oil.
[c)] Results are expressed as means ± SD of three samples.
[d)] % of total fatty acids.

2.1.1 Original sunflower oil (Vegetol Gold, Oleofin a.s., Ústí nad Labem, Czech Republic)

2.1.2 Tocopherol-stripped sunflower oil

Tocopherol-stripped sunflower oil was prepared according to the modified method of Waraho et al. [18]. Briefly, a glass chromatographic column (internal diameter 3.0 cm, height 35 cm) was packed with 45 g of activated Silica gel 60 (Merck KGaA, Darmstadt, Germany) dissolved in *n*-hexane. Original sunflower oil (30 g) was dissolved in 30 mL of *n*-hexane and passed through the column by eluting with 270 mL of *n*-hexane. The tocopherol-stripped sunflower oil was obtained by solvent removing with a vacuum rotary evaporator (Buchi Laboratortechnik AG, Flawil, Switzerland) at 37°C. Traces of solvent were removed by flushing with nitrogen.

2.1.3 FAME prepared from original sunflower oil

FAME were prepared from original sunflower oil according to the modified method described by Rashid and Anwar [22]. Original sunflower oil was reacted with methanol in the presence of KOH (25°C, 1 h) in molar ratio 1:10:0.14. The phase containing FAME was separated from glycerol phase and washed by distilled water to neutral reaction of phenolphthalein. FAME were dried under vacuum and distilled at pressure of 3 mbar and temperature at 180°C.

2.2 Methylation (inactivation) of α-, γ-, and δ-tocopherol

α-, γ-, and δ-tocopherols were methylated by diazomethane (CH_2N_2).

Diazomethane was prepared according to Arndt [23, 24]. In the first step *N*-methyl urea reacted with $NaNO_2$ in the presence of H_2SO_4 to form *N*-nitroso-*N*-methyl urea. In the second step *N*-nitroso-*N*-methyl urea was decomposed by KOH solution to diazomethane.

Diazomethane was dosed to 1% solution of DL-α-tocopherol (98.2%, Merck KGaA), D-γ-tocopherol (≥96.0%, Sigma–Aldrich Chemie, Steinheim, Germany) or D-δ-tocopherol (≥90.0%, Sigma–Aldrich Chemie) in diethyl ether in presence of BF_3 (10%) as catalyst until the solution became yellow [25]. Reaction mixture was analyzed by GC-flame ionization detector (FID).

2.3 Antioxidants

The following phenolic acids were used (Fig. 1): *p*-hydroxybenzoic acid = 4-hydroxybenzoic acid (99%; Merck); caffeic acid = 3,4-dihydroxycinnamic acid (99%; Alfa Aesar, Karlsruhe, Germany); protocatechuic acid = 3,4-dihydroxybenzoic acid (≥97%; Sigma–Aldrich Chemie); gentisic acid = 2,5-dihydroxybenzoic acid (≥99%; Sigma–Aldrich Chemie); vanillic acid = 3-methoxy-4-hydroxybenzoic acid (98%; Merck); ferulic acid = 3-methoxy-4-hydroxycinnamic acid (98%; Merck).

2.4 Preparation of alkyl esters of phenolic acids

Used alcohols: methanol – 99.8%, Penta (Praha, Czech Republic); ethanol – 99.8%, Merck; propanol – 99.5%, Penta; butanol – 99.5%, Lachner s.r.o. (Neratovice, Czech Republic); hexanol – ≥ 98.0%, Sigma–Aldrich Chemie.

Alkyl esters of phenolic acids were prepared by esterification of phenolic acids by relevant alcohol, *p*-toluenesulfonic acid monohydrate was used as catalyst, according to Merkl

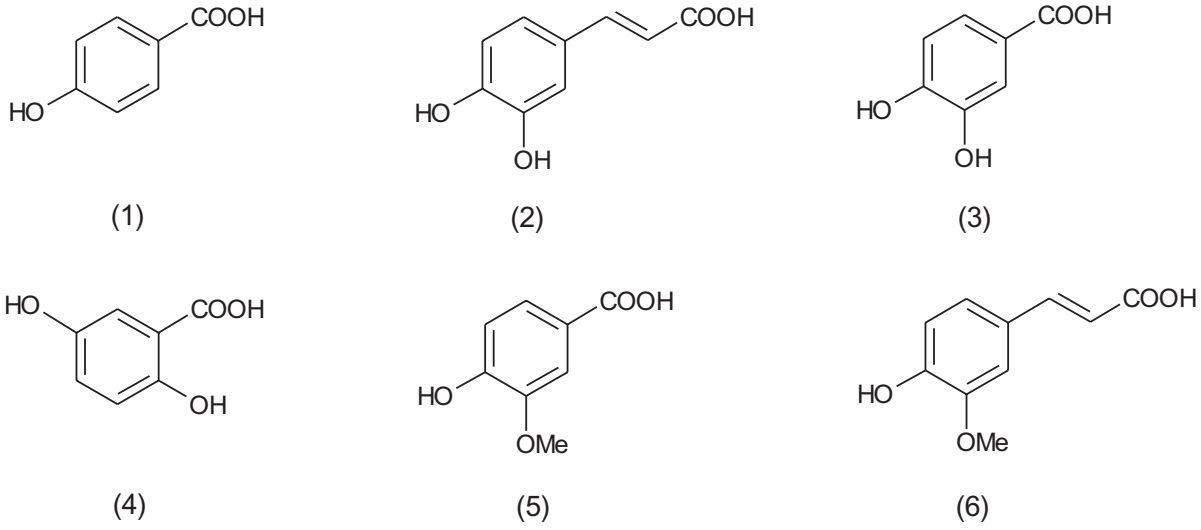

Figure 1. Chemical structure of *p*-hydroxybenzoic acid (1); caffeic acid (2); protocatechuic acid (3); gentisic acid (4); vanillic acid (5); ferulic acid (6).

et al. [10]. Prepared alkyl esters (≥98%) were methyl-, ethyl-, propyl-, butyl- a hexyl esters of phenolic acids (butyl ester and hexyl ester of vanillic acid were not prepared).

The content of studied antioxidants in all test media for determining their antioxidant activity was 3 mmol/kg.

2.5 Instrumental methods for determining of antioxidant activity

2.5.1 Oxidograph method (ML Oxidograph, Mikrolab Aarhus A/S, Hojbjerg, Denmark)

A sample of oil with an added antioxidant was measured at 110°C under oxygen atmosphere and the decrease in oxygen pressure was recorded. The induction period was determined as the time interval when oxygen pressure does not decrease – $IP_{Oxidograph}$ [16]. Induction period is mean value of three replicate analyses.

2.5.2 Rancimat method (Rancimat 743, Metrohm, Herisau, Switzerland)

A sample of oil with an added antioxidant was reacted at 120°C and air was bubbled through the sample during the reaction. Volatile secondary oxidative products were carried to the demineralized water and a conductivity of water was recorded. The induction period was determined as the time interval when the conductivity does not increase – $IP_{Rancimat}$ [26]. Induction period is mean value of three replicate analyses.

The protection factor of an antioxidant was calculated from Eq. (1):

$$PF = \left(1 - \frac{IP_{oil}}{IP_{oil+antioxidant}}\right) \times 100\,[\%] \quad (1)$$

where PF is the protection factor, IP_{oil} is induction period of oil without antioxidant; $IP_{oil+antioxidant}$ is induction period of sample of oil with antioxidant.

This equation is more suitable for determination of antioxidant effect than equation $F = IP_{oil+antioxidant}/IP_{oil}$ [9, 27] because antioxidant effect is in the range 0–100%, substances with prooxidant effect have negative values.

2.6 Fatty acid composition by GC-FID

The fatty acid profiles were analyzed using the modified methods ISO 5509:2000 and ISO 5508:1990, according to Zárubová et al. [28].

2.7 Tocopherol content by GC-FID

An oil sample (1.5 g) with addition of internal standard (5α-cholestane – 0.503 mg) and antioxidant (ascorbic acid – 0.3 g) was saponified by 70 mL of methanolic KOH solution (2 mol/L) for 60 min under reflux. Unsaponifiable fraction was extracted three times by diethyl ether (100 mL), washed three times by water (100 mL) and the solvent was evaporated with vacuum rotary evaporator (Buchi Laboratortechnik, Flawil, Switzerland). Two percent solution of unsaponifiable fraction in diethyl ether was prepared and then analyzed by GC-FID (Agilent Technologies 6890) with column Optima 17-TG (Macherey-Nagel, Dueren, Germany) – dimension 0.32 mm × 25 m, film thickness 0.1 μm. Helium was used as carrier gas with flow rate 1 mL/min. Sample (1 μL in split rate 1:25) was sprayed at 300°C. Analysis was isothermal (262°C). Detection was carried out at temperature 300°C (hydrogen flow was 40 mL/min, air flow was 450 mL/min, nitrogen flow as make up was 25 mL/min). Relative percentage was converted to weight percentage and expressed as mg/kg. Results are mean values of three replicate analyses.

2.8 Statistical analysis

All the values of induction periods and protection factors are presented as mean values ± SD, calculated from the results of three replicate analyses. Results were analyzed by applying a Student *t*-test with significance level $p = 0.05$. Microsoft Office Software (Excel, version 2003) was used to evaluate a correlation between the values of induction periods measured by the Oxidograph and Rancimat methods.

3 Results and discussion

Original sunflower oil was used as the test medium with higher tocopherol concentration. Tocopherol-stripped sunflower oil and FAME were used as test media with significantly lower or zero tocopherol concentration. Theoretically, methylation of pure tocopherols offered a further possibility how to remove tocopherol activity. Antioxidant activity of mono- and dihydroxyphenolic acids and their esters was determined in original sunflower oil and FAME in concentration 3 mmol/kg. The most effective antioxidants – methyl, ethyl, propyl, and butyl ester of caffeic acid were tested in original sunflower oil, tocopherol-stripped sunflower oil, and FAME for the determination of influence of different tocopherol concentration on final oxidative stability of samples.

3.1 Test media for determining the oxidative stability

Refined (original) sunflower oil served as the test medium with high concentration of linoleic acid. Naturally present tocopherols in the oil may affect the antioxidant activity of phenolic compounds tested (Table 1). Tocopherol concentration in original sunflower oil was lower in comparison with the data of Tasan and Demirci [29]. Tocopherol loss can be explained by careless conditions during deodorization (such as high temperature, heating time, pressure, and stripping steam dosage) [30].

Tocopherols can be removed from sunflower oil by adsorption on silica gel using the technique of column chromatography. Another way to remove tocopherols from the oil is methanolysis of sunflower oil and subsequent vacuum distillation of FAME prepared from sunflower oil (the tocopherols remain in the distillation residue). FAME prepared in such a manner have a similar fatty acid composition as original sunflower oil, but tocopherols are completely removed.

3.1.1 Tocopherol-stripped sunflower oil

Removing of tocopherols by adsorption chromatography led to oxidation of tocopherol-stripped sunflower oil. The decrease of linoleic and linolenic acids (Table 1) was evident from fatty acid composition. The total tocopherol content was considerably reduced from 149.0 to 8.7 mg/kg (Table 1). Oxidative stability of tocopherol-stripped sunflower oil ($IP_{Rancimat} = 2.51$ h; $IP_{Oxidograph} = 2.0$ h) decreased in comparison with that of the original sunflower oil ($IP_{Rancimat} = 2.94$ h; $IP_{Oxidograph} = 3.3$ h) due to a decrease in the level of naturally present tocopherols. This method of removing of tocopherols from oil is not suitable for two reasons: relatively high amounts of the residue remains in test medium and some oxidation changes of test medium can be assumed, in contrast with the argument of Waraho et al. [18].

3.1.2 FAME prepared from sunflower oil

Fatty acid composition of FAME was nearly equal to the original sunflower oil but the tocopherol content was found to be zero (Table 1). Oxidative stability of FAME ($IP_{Rancimat} = 0.82$ h; $IP_{Oxidograph} = 0.1$ h) decreased significantly in comparison with that of the original sunflower oil ($IP_{Rancimat} = 2.94$ h; $IP_{Oxidograph} = 3.3$ h) due to the complete removal of naturally present tocopherols from original sunflower oil. This method of removing of the tocopherols is optimal as it results in zero tocopherol concentration and insignificant oxidation changes.

3.2 Methylation of α-, γ-, and δ-tocopherol by diazomethane (CH_2N_2)

There is a hypothesis that a hydroxyl group in the tocopherol molecule could be protected by methylation [25] and thus the antioxidant effect of naturally present tocopherols might be eliminated.

It was found by GC-FID (Table 2) that δ-tocopherol reacts with CH_2N_2 more readily in comparison with γ- and α-tocopherol. This fact can be explained by the positive inductive effect of substituents in the tocopherol molecule (three methyl groups in the case of α-tocopherol and two methyl groups in the case of γ-tocopherol). Methyl groups (mainly in *ortho* position to the hydroxyl group) decrease the reactivity of the hydroxyl group. Nevertheless, because the degree of tocopherol methylation was found to be negligible, this method (tested for the purpose to eliminate the antioxidant activity of naturally present tocopherols in sunflower oil) failed.

Table 2. Conversion of tocopherol (TO) to tocopherol methyl ether ($TO–CH_3$) determined in the 1% reaction mixture after chemical reaction of tocopherols with diazomethane.

Tocopherol	TO (%)	$TO–CH_3$ (%)
α	99.90 ± 0.10	0.10 ± 10^{-3}
γ	99.80 ± 0.10	0.20 ± 10^{-3}
δ	99.00 ± 0.10	1.00 ± 10^{-3}

3.3 Comparison of two different methods used for determining the oxidative stability

Oxidograph and Rancimat methods are screening methods with shorter analysis time, the results relate with traditional parameter of lipid oxidation such as peroxide value at 20°C [31]. Introduction of these methods brought about a significant progress in prediction of induction periods during lipid autoxidation and in antioxidant research at the end of 20th century. Especially, the Rancimat method displaced the previously widely used and normalized Schaal oven test.

There are some differences between the Oxidograph and Rancimat methods. During the Oxidograph method the oil sample is measured at 110°C and oxygen atmosphere is formed over the sample (oxygen concentration is high and interface between the sample and the oxygen atmosphere is small), whereas during the Rancimat method the oil sample is measured at 120°C and bubbled by air (oxygen concentration is lower and interface between sample and air is large). The Rancimat temperature of 120°C was chosen according by Mateos et al. [32] in order to obtain similar values of induction periods (to shorten analysis time) as in the case of Oxidograph method.

Despite of the different principles and conditions of both instrumental methods, a linear correlation was evaluated between induction periods determined by both the Oxidograph and Rancimat method (Fig. 2) in the same lipid matrix. On the basis of this correlation it is suggested that the used antioxidants have very low or no volatility at Rancimat method conditions. The antioxidant volatility significantly decreases the induction period [33].

3.4 Antioxidant effect of phenolic acids and their alkyl esters

When measured in original sunflower oil containing the common levels of natural tocopherols (Fig. 3), caffeic acid

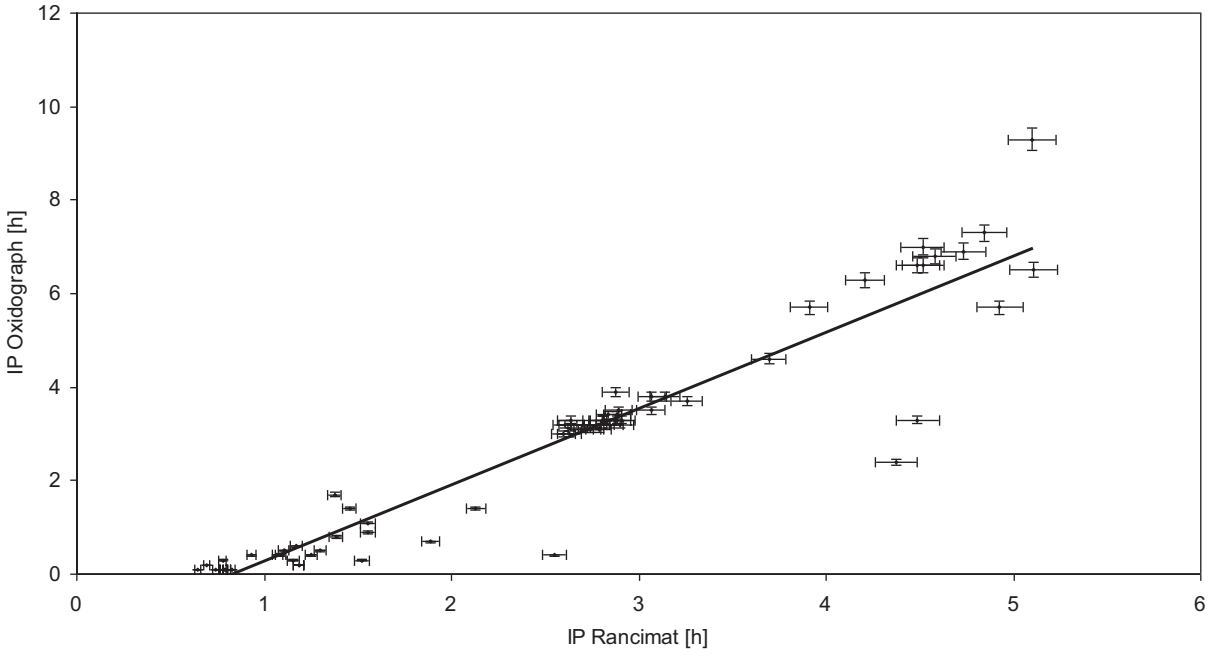

Figure 2. Linear correlation between the induction periods determined by Rancimat method ($IP_{Rancimat}$) and by Oxidograph method ($IP_{Oxidograph}$) $-$ $IP_{Oxidograph}$ (h) $= 1.6433\ IP_{Rancimat}$ (h) $- 1.4122$; $r = 0.890$ ($p<0.05$). Data used were obtained as results of all experiments. Regression analysis was carried out considering all the data ($n = 68$). Error bars express SD ($n = 3$).

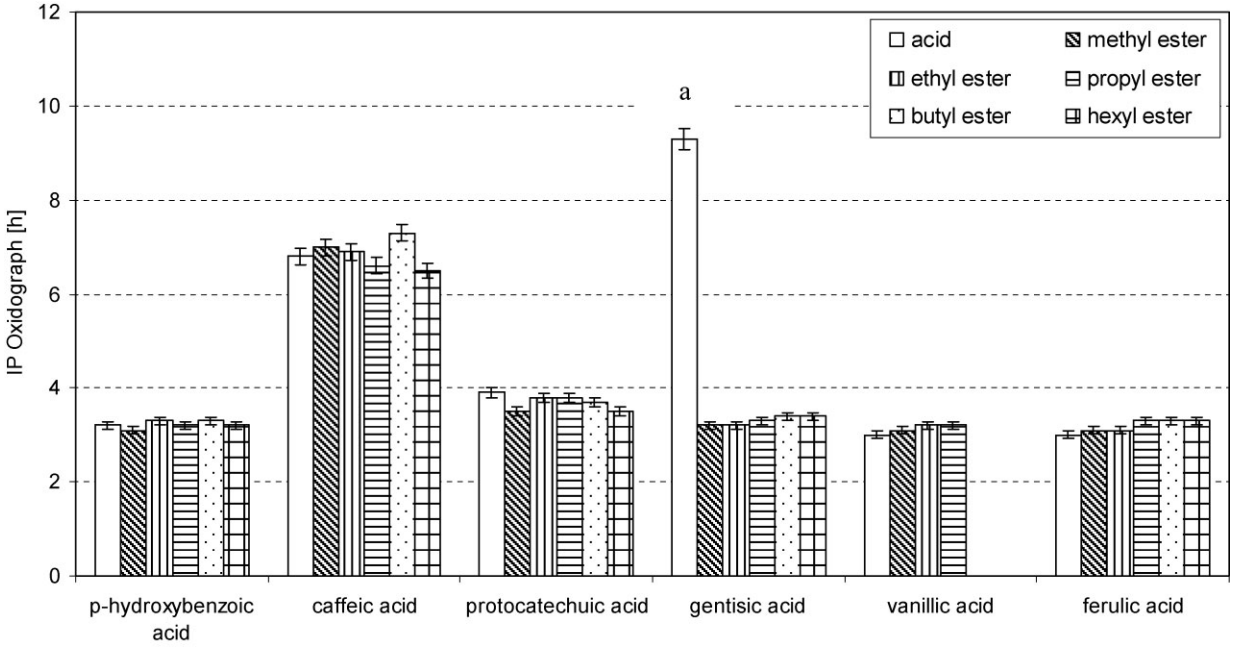

Figure 3. Oxidative stability of phenolic acids and their alkyl esters expressed as induction periods measured in original sunflower oil (total tocopherols 149.0 mg/kg) using the Oxidograph method (IP of original sunflower oil as a blank $= 3.3$ h). Error bars express SD ($n = 3$). [a]indicates significant difference ($p<0.05$).

and its alkyl esters exhibited significant antioxidant activity, while the antioxidant activity of protocatechuic acid and its alkyl esters was lower. In the case of both 3,4-dihydroxyphenolic acids (caffeic and protocatechuic acids), no statistically significant differences were found between antioxidative properties of acids and their alkyl esters. The antioxidant effect of gentisic acid alone (2,5-dihydroxyphenolic acid) was the greatest of all studied antioxidants. In contrast, alkyl esters of gentisic acids showed lower antioxidant activity. The explanation for this behavior might be that the strong antioxidant effect of gentisic acid is caused either by synergism with naturally present tocopherols in the original sunflower oil or by the formation of hydrogen bonds between the carboxylic group and hydroxyl group in position 2 in the molecule of gentisic acid.

When measured in tocopherol-free FAME prepared from original sunflower oil (Fig. 4), caffeic acid and its alkyl esters had the highest antioxidant activity of all studied antioxidants. Caffeic acid showed a similar antioxidant effect as its methyl, ethyl, and butyl esters, whereas propyl and hexyl esters of the caffeic acid had significantly lower effects. In emulsion systems a cut-off effect was reported. Antioxidant effect of phenolipids with different chain length is changed nonlinearly which is explained by different antioxidant location [12, 13]. In FAME (a nonpolar system) antioxidants with shorter chain length occur on oil–air interface (the critical point is butyl ester of caffeic acid), while antioxidants with longer chain length are dissolved in the oil phase. Generally, the antioxidant activity of alkyl esters of caffeic acid decreased with increasing length of their alkyl chain in conformity with the polar paradox hypothesis [5]. Protocatechuic acid and its alkyl esters exhibited a considerable but much lower antioxidant activity. The antioxidant activity of protocatechuic acid, as opposed to caffeic acid, was observed to be higher than that of its alkyl esters, the antioxidant activity of which increased with increasing length of their alkyl chain. Gentisic acid had a similar antioxidant effect as its alkyl esters. Based on the results obtained, it can be concluded that there is a significant synergistic effect between gentisic acid and naturally present tocopherols in the original sunflower oil, which is evident in Fig. 3, where the extraordinarily high antioxidant activity of gentisic acid can be seen.

Methyl, ethyl, propyl, and butyl esters of caffeic acid were chosen as the most effective antioxidants of all antioxidants studied. Their protection factors were determined using the Rancimat method in original sunflower oil (tocopherol content 149.0 mg/kg), in tocopherol-stripped sunflower oil (tocopherol content 8.7 mg/kg) and in FAME prepared from original sunflower oil (tocopherol-free medium). Protection factors of all alkyl esters of caffeic acid decreased with increasing concentration of tocopherols in the respective test medium (Fig. 5), which indicates that naturally present tocopherols in sunflower oil have an antagonistic effect on the antioxidative properties of alkyl esters of caffeic acid.

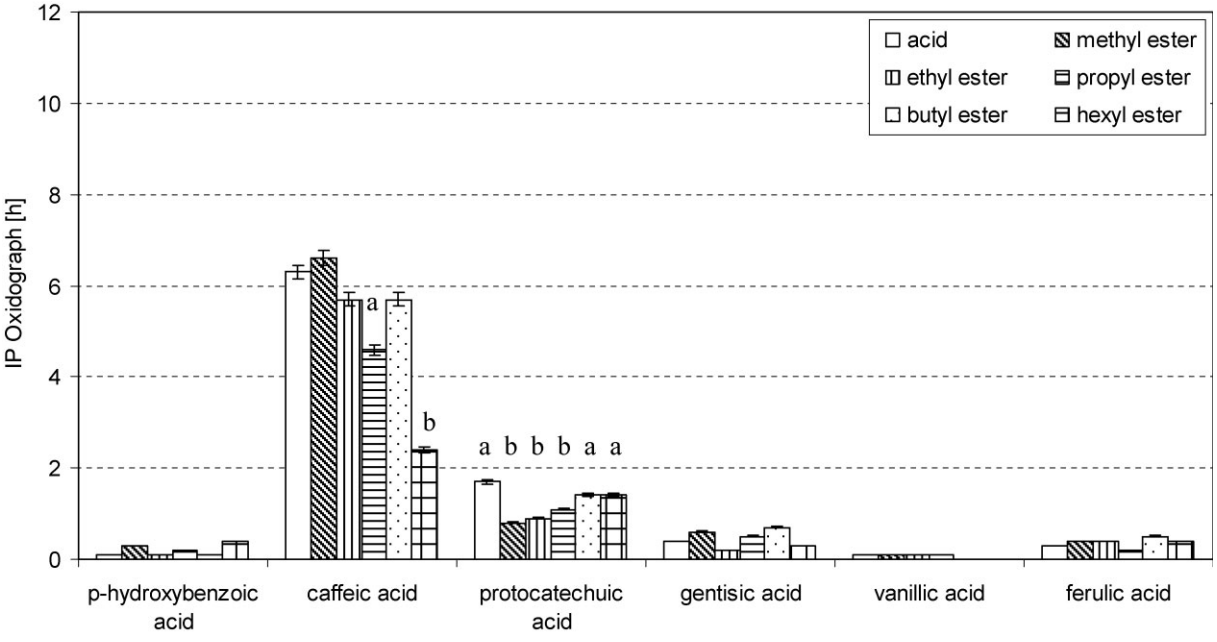

Figure 4. Oxidative stability of phenolic acids and their alkyl esters expressed as induction periods measured in FAME prepared from original sunflower oil (tocopherol-free test medium) using the Oxidograph method (IP of FAME as a blank = 0.1 h). Error bars express SD ($n = 3$). [a,b]different letters in the same group indicate significant differences ($p<0.05$).

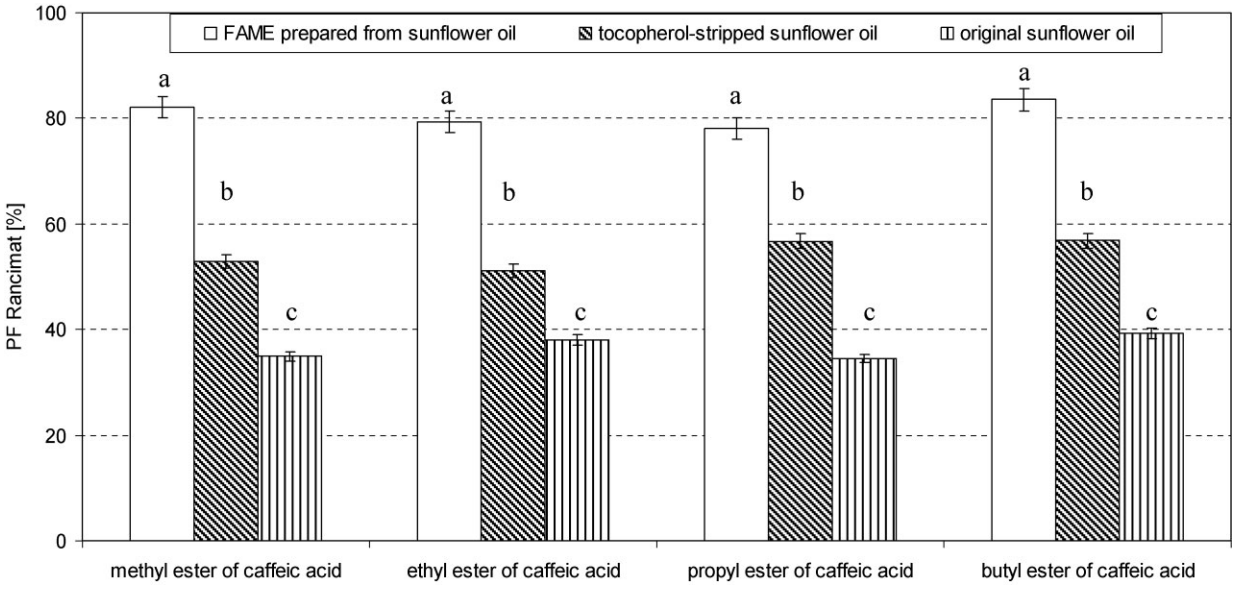

Figure 5. Protection factors of methyl, ethyl, propyl, and butyl esters of caffeic acid measured in FAME prepared from original sunflower oil (tocopherol-free test medium), in tocopherol-stripped sunflower oil (total tocopherols 8.7 mg/kg) and in original sunflower oil (total tocopherols 149.0 mg/kg) using the Rancimat method. Error bars express SD ($n = 3$). [a,b,c]different letters in the same group indicate significant differences ($p<0.05$).

4 Conclusions

Distilled FAME prepared from original sunflower oil may serve as an optimal tocopherol-free test medium for determining the antioxidant activity of various antioxidants. The chemical reaction of tocopherols with diazomethane tested for the purpose of eliminating their antioxidant activity failed due to the negligible degree of methylation of hydroxyl groups in the tocopherol molecules.

3,4-Dihydroxyphenolic acids (caffeic and protocatechuic acids) were evaluated to be more active antioxidants than monohydroxyphenolic acid (*p*-hydroxybenzoic acid), 2,5-dihydroxyphenolic acid (gentisic acid), and 3-methoxy-4-hydroxyphenolic acids (vanillic and ferulic acids).

Naturally present tocopherols in the original refined sunflower oil proved to have a synergistic effect on gentisic acid, whereas gentisic acid measured in tocopherol-free FAME had insignificant effect.

On the other hand, tocopherols appeared to have an antagonistic effect on alkyl esters of caffeic acid, because their protection factors decreased with increasing concentration of tocopherols in the respective test medium. Moreover, their antioxidant activity decreased with increasing length of alkyl chain in conformity with the polar paradox hypothesis.

This work was supported by funding from the Czech Ministry of Education, Youth and Sports (MSM 6046137305). Part of these results was presented on 7th Euro Fed Lipid Congress in Graz, Austria.

The authors have declared no conflict of interest.

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RESEARCH ARTICLE

European Journal of
Lipid Science and Technology
www.ejlst.com

Mechanism Controlling High-Temperature Degradation of Sunflower Oil Triacylglycerols in the Absence of Oxygen

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The aim of the present study is to describe the mechanism controlling heat-induced formation of sunflower oil triacylglycerol and fatty acid methyl ester oligomers. The unique combination of high-performance size-exclusion chromatography with hyphenated electrospray ionization mass spectrometry (MS), atmospheric pressure chemical ionization-MS, and high-temperature gas chromatography-MS techniques allows differentiating between radical coupling species and Diels–Alder cycloadducts. Targeted analysis of thermally degraded sunflower oils confirms the exact structures of various acyclic oligomers accompanied by less-abundant products of pericyclic transformations. A series of model experiments simulate the impact of dienophile nature on the course of Diels–Alder reactions. Thus, α-tocopherylquinone, δ-tocopherylquinone, and methyl-(*E*)-11-oxoundec-9-enoate are synthesized as naturally occurring dienophiles bearing electron-withdrawing groups. The geometry of poor dienophiles does not affect concerted cyclization, while the structure of electron deficient dienophiles can overcome low reactivity.

***Practical Application*: In the absence of oxygen, heat-induced degradation of polyunsaturated triacylglycerols proceed predominantly via a radical pathway, whereas concerted reactions represent minor mechanisms. Sunflower oil triacylglycerol molecules in the system without propagation stage can be effectively protected by natural and/or synthetic antioxidants. Application of chelates is also recommended. However, antioxidant-derived quinones, such as α-tocopherylquinone, can enter the Diels–Alder reaction even more easily than dienophiles without electron-withdrawing groups. Unsaturated core aldehydes possess the same reactivity. Examination of the mechanism controlling high-temperature degradation of triacylglycerols is especially important for processing engineers in edible oil refineries and food technologists. New perspective may help them to minimize undesirable changes in polyunsaturated species.**

1. Introduction

Numerous side reactions of polyunsaturated fatty acids (PUFAs) occur at high temperatures in the practical absence of air. Industrially relevant edible-oil-processing technologies involve deodorization (220–240 °C, 2–4 mbar), deodorization–deacidification (240–260 °C, 1–2 mbar), and vacuum frying applications.[1,2] The final refining stage almost completely removes undesirable volatile compounds (low M_w carbonyls, free fatty acids) and significantly reduces the level of triacylglycerol (TAG) hydroperoxides to yield high-quality vegetable oils, that are bland-tasting, odorless, and acceptable to consumers.[2,3] High temperatures also accelerate the formation of new polyunsaturated TAG artifacts, which definitely remain in edible oils.[3,4] Among the PUFA and TAG degradation products, oligomers, cyclic fatty acid monomers (CFAMs), and geometric and positional fatty acid isomers are quantitatively the most abundant.[2,5] The formation of artifacts is strongly dependent on the refining conditions, the level of crude oil oxidation, and the number of methylene interrupted double bonds of polyunsaturated TAG molecules.[2,3] Heat-induced alteration of TAG in the system without oxygen and thus a propagation stage for radical reactions is significantly different from the well-known autoxidation mechanism. Moreover, thermal degradation of TAG is far from monomechanistic.[5–7]

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DOI: 10.1002/ejlt.202000228

In the initial phase of high-temperature treatment, TAG hydroperoxides rapidly decompose to alkoxyl (BDE = DH_{298}(RO–OH) = 184 kJ mol^{-1}) and peroxyl (BDE = DH_{298}(ROO–H) = 377 kJ mol^{-1}) radicals, which cause homolytic splitting of bis-allylic and allylic C–H bond of PUFA. It is an essential step for heat-induced geometric and positional isomerization as well as other alterations.[5,8–10] Although hydroperoxides accelerate the isomerization, high temperatures employed during deodorization/deacidification more than fulfil the energy requirements for C–H bond dissociation of PUFA (BDE = DH_{298}(bis-allylic C–H) = 327–335 kJ mol^{-1}).[5,6,11] An addition–elimination mechanism has been proposed to describe the formation of PUFA geometrical isomers by radical species. By geometric isomerization of linoleic and linolenic acids, three and seven thermodynamically stable *trans* isomers can be formed.[2,12] The consumption of *trans* fatty acid monoenes can increase the risk of cardiovascular disease, while *trans* octadecadienoic acid isomers can promote inflammation by inhibiting eicosanoid metabolism.[13] Positional isomerization of PUFA is associated with the formation of a conjugated double bond system. It can proceed by a free radical chain mechanism, while an antarafacial [1,3]-sigmatropic rearrangement is less favored for steric reasons (Möbius topology). At temperatures above 200 °C, conjugated linoleic acid (CLA) isomers can undergo a suprafacial [1,5]-hydrogen shift.[5,12,14]

The last category of monomeric PUFA artifacts is represented by CFAMs with a wide variety of cyclopentyl, cyclopentenyl, cyclohexyl, and cyclohexenyl ring structures. Intermolecular cyclization of polyunsaturated fatty acyl moieties proceeds significantly at temperatures above 210 °C and is enhanced by the presence of unstable α-linolenic acid.[12,15] Based on the current state of knowledge, the intermolecular cyclization can involve free radical allylic intermediates as well as a concerted cyclization mechanism providing five- or six-membered ring structures. Cyclopentyl CFAM are considered to be more abundant.[12,15] Although isomerization and polymerization reactions of fatty acids predominate, the formation of CFAM is not a negligible (≤0.7 wt%) phenomenon since CFAM are highly absorbed in the intestine, but their potential toxicity is questionable.[2,5,15,16]

Nonpolar TAG polymers are in fact a heterogeneous group of TAG dimers, trimers, and oligomers with a typical molecular weight of TAG dimers ranging from 1700 to 1900 g mol^{-1}. Similar to CFAM and *trans* fatty acids, they do not cause sensory adverse effects. If accumulated, they accelerate the development of heat-induced alterations and undesirable deepening of color, increased viscosity, and reduced heat transfer.[12,17,18] On the other hand, their detrimental effects for humans are negligible since the rate of absorption of TAG polymers in the gastrointestinal tract is very slow.[7] The free radical chain mechanism and Diels–Alder reaction are mainly used to describe the formation of either acyclic or cyclic TAG polymers.[12,17] Intra- and intermolecular couplings of two allyl radicals provide prevailing/predominating acyclic dehydrodimers achieved by –C–C– linkage. In a medium without oxygen, the Diels–Alder reaction represents a parallel concerted mechanism involving a conjugated double bond system and a dienophile.[6,7,12] Heat-induced isomerization results in all-*trans* CLA (s-*cis* conformation), an essential reactant for consecutive oligomerization. The concerted cyclization yields cyclohexene derivatives according to normal electron demand in the Diels–Alder reaction.[5,19]

Table 1. Physicochemical properties of starting sunflower oil.

Fatty acid content [%]		Triacylglycerol profile [%]	
C16:0	6.04	ECN 42 (LLL)	20.44
C16:1	0.12	ECN 44 (LLO, PLL)	36.52
C18:0	3.32	ECN 46 (OLO, POL, SLL)	19.82
C18:1	31.62	ECN 48 (OOO, SOL)	4.29
C18:1 *trans*	0.07	ECN 50 (SOO, SOP, SLS)	0.43
C18:2	56.77	ECN 52 (SOS, SSP)	0.17
C18:2 *trans*	0.27	RBD sunflower seed oil characteristics	
C18:3	0.06	Saponification value (mg KOH g^{-1})	195.1
C20:0	0.25	Iodine value (g linoleic acid (I_2) per 100 g)	110.1
C22:0	0.73	Acid value (mg KOH g^{-1})	0.07
C24:0	0.29	Peroxide value (meq.act. O kg^{-1})	0.65

Polyunsaturated TAGs are affected considerably in the course of edible oil processing or deep-fat frying treatments. The heat-induced degradation of TAGs is far from monomechanistic and produces various reaction products. In the first part of this work, we investigated the predominant pathway for the formation of TAG oligomers and CFAM in the absence of oxygen. In the second part, the impact of dienophiles on the course of the Diels–Alder reaction has been studied. Our conclusions may help to reduce the formation of potentially detrimental compounds in commercially produced vegetable fats and oils.

2. Experimental Section

2.1. Reagents and Materials

Methyl heptadecenoate, methyl linoleate, 3-pyridylcarbinol, Wilkinson's catalyst, tin(II) chloride dihydrate, anhydrous ethanol, 1-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide (BMIM), α-tocopherol, iron(III) chloride hexahydrate, tetrahydrofuran (THF), 9-decenoic acid, acrolein dimethyl acetal, dichloromethane, second-generation Hoveyda–Grubbs catalyst, methyl oleate, methyl elaidate, methyl stearate, and palladium on activated charcoal (Pd/C, 5% Pd basis) were purchased from Sigma-Aldrich. Merck precoated silica gel F_{254} plates were used for thin-layer chromatography. Spots were detected by heating after spraying with 5% phosphomolybdic acid in EtOH. All other reagents and solvents were of analytical grade. Sample of sunflower oil was purchased from ADM, Czech Republic. Physicochemical properties of starting material are summarized in **Table 1**.

2.2. High-Temperature Degradation of Sunflower Oil TAGs in the Absence of Oxygen

Heat-induced degradation of sunflower oil TAG in the absence of oxygen was performed in the isomerization tube (height 254 mm, inner diameter 25 mm). A 50.0 g sample of sunflower oil was loaded into the apparatus through which a stream of argon

(Ar 4.8; purity 99.998 %) was passed upwards (1 dm^3 h^{-1}) by means of a glass capillary. The batch was subsequently heated in a thermostat (modified oven of a Chrom 4 gas chromatograph, LP Prague) to a temperature of 240, 250, or 260 °C, monitored with a digital thermometer (F200, Automatic Systems Laboratories, Croydon, UK). At given intervals, aliquots (0.5 g) were taken through Teflon septum for analysis of fatty acid methyl esters (FAMEs), and measurements of TAG monomers and polymers, and CFAMs, using glass syringe flushed with argon prior sampling. Detailed reaction scheme is provided in Figure S1, Supporting Information. Controlled TAG degradation was carried out over a period of up to 170 h.

2.3. Determination of Positional and Geometric Isomers of Unsaturated Fatty Acid Isomers and CFAM by GC-FID and GC-MS

Positional and geometric unsaturated FAME isomers were determined according to AOCS Official Method Ce 1f-96.[20] Methyl heptadecanoate was used as an internal standard. The analysis was performed on an Agilent 6890N gas chromatograph (Agilent Technologies, USA) coupled with a flame-ionization detector (FID) and SP 2560 capillary column (Supelco, Bellefonte) 0.25 mm × 100 m, and film thickness of 0.2 µm. The conditions of analysis were as follows: hexane solution of FAME (1%) was used for the injection (1 µL), split injection (1:50) at 220 °C; carrier gas flow (He) 1 mL min^{-1}; analysis at 175 °C for 120 min; FID detection at 250 °C, flow of H_2 40 mL min^{-1}, air flow 450 mL min^{-1} and make-up gas (N_2) flow, 45 mL min^{-1}. Determinations were performed in duplicate.

Qualitative and quantitative analyses of CFAMs in the form of methyl esters and 3-pyridylcarbinol ester were carried out by GC-FID and GC-MS. In brief, hydrogenation of FAMEs was carried out in a 50 mL round-bottom flask equipped with a magnetic stirrer, thermometer, reflux condenser, and connected to a hydrogenation manifold. The reactor was charged with FAME (0.5 g) and 15 mg of Pd/C (5% Pd basis) in 24 mL of heptane/isopropanol (2:1 v/v), and the system was flushed three times with hydrogen. The reaction mixture was hydrogenated in an excess of hydrogen at 70 °C for 24 h. Spent palladium catalyst was filtered off and the solvent was evaporated to dryness. At this point, urea fractionation afforded CFAMs as well as fatty acid oligomers in the form of methyl esters. Preparation of 3-pyridylcarbinol esters was done according to Christie and Han.[21] GC-MS (EI) was recorded on an Agilent 7820A GC system and 5975 Series MSD (Agilent Technologies, Santa Clara, CA, USA).

2.4. Qualitative and Quantitative Analyses of TAG Oligomers by HPSEC-ELSD, APCI-MS, and ESI-MS

Heat-induced formation of TAG dimers, trimers, and higher oligomers was determined by high-performance size-exclusion chromatography with evaporated light-scattering detector (HPSEC-ELSD) according to ISO method 16931:2009.[22] The amount of oligomeric TAGs was quantified using the external standard method. Fresh refined, bleached, and deodorized (RBD) sunflower oil was used as the external standard. Quantitative analysis was carried out using an Agilent 1100 Series HPLC chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a 5 µm PLgel column (7.5 mm × 300 mm, Agilent Technologies). The parameters of the analysis were as follows: THF solution of polymerized oils (3–5 mg mL^{-1}) was used for the injection (20 µL); THF mobile phase flow 0.6 mL min^{-1}; analysis at 25 °C for 15 min. The temperature of the ELSD was 50 °C. Each sample was run in triplicate. The error bars in scatter plots represent standard deviations.

Qualitative analysis of TAG oligomers was carried out at the University of Alberta using a 4000 QTRAP LC–MS/MS mass spectrometer (Applied Biosystems, Streetsville, ON, Canada) after previous separation of oligomer fractions by HPSEC-RID (Agilent Technologies) at UCT Prague. For direct atmospheric pressure chemical ionization (APCI-MS) in the positive ion mode, the conditions were as follows: ion source—heated nebulizer with high-purity nitrogen (99.995%) with values 15 (GS1), 0 (GS2), curtain gas 10 psi, nebulizer current 3 kV, temperature of source 400 °C, unit mass resolution for Q1 and Q3, and scan range 50–2800 m/z in 3 s. The fragmentation values as CE, DP, EP, and CCexP were 10–60 eV, 115, 10, and 10 V, respectively. The mass spectrometer was also equipped with an electrospray ion source (ESI-MS). For direct ESI-MS operating in the positive ion mode, the conditions were as follows: nitrogen gas was used as nebulizing (GS1) and collision gas (GS2). The values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were 4.5 kV, 400 °C, 15, 15, and 10 psi, respectively. The declustering potential was 115.0 V, the collision energy was 25.0 eV, and the collision exit potential was 10.0 V. The spectra were obtained over a mass range of m/z 50–2000. All MS measurements were processed using Analyst 1.5 program (Applied Biosystems).

2.5. Qualitative and Quantitative Analyses of FAME Oligomers by HTGC-FID, HTGC-MS, HPSEC-ELSD, APCI-MS, and ESI-MS

Quantitative analysis of FAME oligomers was carried out using HPSEC-ELSD as described in Section 2.4. The amount of oligomeric FAME was quantified using the external standard method. Methyl heptadecenoate was used as the external standard. Simultaneous high-temperature gas chromatography (HTGC) analysis was performed on an Agilent 6890n gas chromatograph (Agilent Technologies) coupled with a FID and an Optima-17-TG capillary column (Macherey–Nagel, Germany) 0.32 mm × 30 m, film thickness 0.1 µm. The conditions of the analysis were as follows: isooctane solution of FAME oligomers (1%) was used for the injection (1 µL), split injection (1:50) at 320 °C; flow of carrier gas (He) 1.5 mL min^{-1}; the temperature in the oven was programmed as follows: 80 °C (1 min); 80–250 °C (20 °C min^{-1}); 250–370 °C (5 °C min^{-1}); 370 °C (12 min); FID detection at 380 °C, flow of H_2 40 mL min^{-1}, air flow 450 mL min^{-1} and make-up gas (N_2) flow 45 mL min^{-1}. Determinations were performed in duplicate.

Qualitative analysis of FAME oligomers was carried out using a 4000 QTRAP LC–MS/MS mass spectrometer (Applied Biosystems). Mass spectra of the compounds were obtained in positive-ion mode using both the ESI and the APCI ion sources. In the positive-ion mode experiments, the compounds were typically detected as either proton $(M + H)^+$, sodium $(M + Na)^+$,

or ammonium $(M + NH_4)^+$ adducts. The spectra were obtained over a mass range of m/z 50–2000. All MS measurements were processed using the Analyst 1.5 program (Applied Biosystems). HTGC-MS (EI) was recorded on Agilent 7820A GC system and 5975 Series MSD (Agilent Technologies) under the same conditions used for HTGC-FID.

2.6. Qualitative and Quantitative Analyses of TAG Monomers by RP-HPLC-ELSD, APCI-MS, and ESI-MS

Degradation of individual TAG species was determined quantitatively by reversed phase HPLC with an ELSD according to the DGF Standard Method C-V 13a.[23] TAG/equivalent carbon number (ECN) 38 (*sn*-1,2-didodecanoyl-3-tetradecanoylglycerol; purity >98 %) was used as an internal standard. The separation of TAGs according to ECN was carried out using an Agilent 1100 Series HPLC chromatograph (Agilent Technologies) with a Separon SGX C18 5 µm pre-column (30 mm × 3.3 mm; Tessek) and Nucleosil 120-5-C18 column (250 mm × 4 mm, 5 µm; Watrex). The parameters of the analysis were as follows: acetone solution of TAG was used for the injection (50 µL); acetone/acetonitrile/methanol (4:2:1) mobile phase flow 1 mL min^{-1}; analysis at 40 °C for 55 min. The temperature of the ELSD was 90 °C. Each sample was run in triplicate. The error bars in scatter plots represent standard deviations. Qualitative analysis of TAG monomers was carried out using a 4000 QTRAP LC–MS/MS mass spectrometer (Applied Biosystems). Mass spectra of the compounds were obtained in positive-ion mode using both the ESI and the APCI ion sources. In the positive-ion mode experiments, the compounds were typically detected as either proton $(M + H)^+$, or ammonium $(M + NH_4)^+$ adducts. The spectra were obtained over a mass range of m/z 50–2000. All MS measurements were processed using the Analyst 1.5 program (Applied Biosystems). The content of tocopherols in selected samples was determined quantitatively according to ISO method 9936:2016.[24]

2.7. Synthesis of Diene Component: Conjugated Isomers of Methyl Linoleate (1)

Homogeneous isomerization of methyl linoleate was carried out as previously described using a jacketed glass reactor equipped with sintered glass at the bottom.[25] The stream of nitrogen passed upwards to agitate the reaction mixture thoroughly. The reactor was charged with methyl linoleate (13.58 mmol, 4.0 g), Wilkinson's catalyst (0.07 mmol, 63 mg), and $SnCl_2 \cdot 2H_2O$ (0.17 mmol, 38 mg) in 3 mL of anhydrous ethanol and 5 mL of ionic liquid (BMIM). The reaction mixture was isomerized at 60 °C for 90 min, after which time the solution was allowed to cool. After evaporation of ethanol, purification by column chromatography (silica, hexane/ethyl acetate, from 100:0 to 90:10) afforded 3.884 g (13.19 mmol) of CLA isomers.

2.7.1. Conjugated Isomers of Methyl Linoleate (1)

The mixture of 9*cis*,11*trans*-18:2 (46.1%), 10*trans*,12*cis*-18:2 (48.2%), all-*trans*-CLA isomers (1.7%), and other *cis*,*trans* and *trans*,*cis*-CLA isomers (4.0%) determined by GC-FID was obtained in 97.1% yield as a colorless liquid. R_f = 0.55 (hexane/diethyl ether/formic acid, 40:10:1); ^{1}H NMR (500 MHz, $CDCl_3$): mixture of geometric and positional isomers, some hydrogen atoms gave more than one signal δ 0.88 (3*H*, t, J = 5.2 Hz, CH_3), 1.25–1.37 (18*H*, m, CH_2), 2.00–2.15 (4*H*, m, 8-CH_2 and 13-CH_2), 2.30 (2*H*, t, J = 7.5 Hz, 2-CH_2), 3.66 (3*H*, s, COOCH_3), 5.26–5.34 (1*H*, m, 9-C*H* and 13-C*H*), 5.61–5.68 (1*H*, m, 12-C*H* or 10-C*H*), 5.94 (1*H*, t, J = 10.9 Hz, 10-C*H* or 12-C*H*), 6.28 (1*H*, t, J = 13.5 Hz, 11-C*H*); ^{13}C NMR (126 MHz, $CDCl_3$): mixture of geometric and positional isomers, the assignment of 9*cis*,11*trans*-18:2 carbon atoms determined as: δ 14.10 (C-18), 22.63 (C-17), 24.95 (C-3), 27.66 (C-8), 28.92 (C-15), 29.05 (C-4), 29.10–29.75 (C-5/C-6/C-7), 29.43 (C-14), 31.75 (C-16), 32.90 (C-13), 34.10 (C-2), 51.42 (COOCH_3), 125.57 (C-11), 128.71 (C-10), 129.91 (C-9), 134.77 (C-12), 174.30 (C-1); carbon atoms of 10*trans*,12*cis*-18:2 determined as: δ 27.64 (C-14), 32.86 (C-9), 125.66 (C-11), 128.57 (C-12), 130.15 (C-13), 134.58 (C-10); high-resolution APCI-MS m/z 294.2509 $[M + H]^+$.

2.8. Synthesis of Dienophiles with an Electron-Withdrawing Groups: *para*-α-Tocopherylquinone (2a), *para*-δ-Tocopherylquinone (2b), and Methyl-(*E*)-11-oxoundec-9-enoate (3)

Synthesis of tocopherylquinones was carried out as described previously.[26] In brief, to a stirred solution of α-/δ-tocopherol (5.69 mmol) in diethyl ether (22 mL) was added dropwise $FeCl_3 \cdot 6H_2O$ (16.6 mmol, 4.5 g) in 34 mL of aqueous methanol (1:1 v/v) until the reaction mixture turned to a final orange color. After the mixture was stirred for 60 min at 25 °C, it was taken up in 50 mL of diethyl ether and washed with a saturated solution of NaCl (3 mL × 50 mL). The solvent was evaporated under reduced pressure to achieve a lipophilic residue, which was purified by flash chromatography on a silica gel (70–230 mesh) column to yield the fraction of α-tocopherylquinone or δ-tocopherylquinone (eluted with hexane/ethyl acetate, from 100:0 to 50:50 v/v).

2.8.1. para-α-Tocopherylquinone (2a)

The product was obtained in 92% yield as a yellow oil. R_f = 0.64 (hexane/ethyl acetate, 1:1); UV (ethanol) λ_{max} 262 nm (19 500 L mol^{-1} cm^{-1}); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81–0.82 (12*H*, m; 13a, 17a, 21a, and 22-CH_3), 1.19 (3*H*, s, 9a-CH_3), 1.03–1.48 (23*H*, m, complex), 1.96 (6*H*, s, 2a, and 3a-CH_3), 1.99 (3*H*, s, 5a-CH_3), 2.49–2.52 (2*H*, m, 7-CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 11.91 (C-5a), 12.24 (C-2a), 12.32 (C-3a), 19.64 (C-13a), 19.72 (C-17a), 21.31 (C-7), 21.40 (C-11), 22.59 (C-21a), 22.69 (C-22), 24.46 (C-19), 24.76 (C-15), 26.56 (C-9a), 27.93 (C-21), 32.73 (C-17), 37.25 (C-12), 37.38 (C-16), 37.65 (C-18), 39.33 (C-20), 40.16 (C-8), 42.26 (C-10), 72.51 (C-9), 140.13 (C-5), 140.36 (C-3), 140.46 (C-2), 144.46 (C-6), 187.15 (C-1), 187.57 (C-4); HRESI-MS m/z 445.3686 $[M - H]^+$.

2.8.2. para-δ-Tocopherylquinone (2b)

The product was obtained in 40.8% yield as a red oil. R_f = 0.65 (hexane/ethyl acetate, 1:1); UV (ethanol) λ_{max} 276.5 nm; ^{1}H NMR

(500 MHz, $CDCl_3$) δ 0.82–0.85 (12*H*, m; 13a, 17a, 21a, and 22-CH_3), 1.19 (3*H*, s, 9a-CH_3), 0.99–1.55 (23*H*, m, complex), 2.09 (3*H*, s, 3a-CH_3), 2.49 (2*H*, m, 7-CH_2), 6.55 (1*H*, m, C*H*), 6.65 (1*H*, m, C*H*); ^{13}C NMR (126 MHz, $CDCl_3$) δ 19.62 (C-13a), 19.76 (C-17a), 22.63 (C-21a), 22.74 (C-22), 24.47 (C-19), 24.80 (C-15), 27.98 (C-21), 32.73 (C-17), 37.30 (C-12), 39.38 (C-20), 40.12 (C-8), 42.40 (C-10), 72.58 (C-9), 128.01 (C-3), 133.59 (C-5), 145.73 (C-2), 148.17 (C-6), 185.50 (C-1), 186.46 (C-4); HRESI-MS m/z 419.35304 $[M + H]^+$.

For the synthesis of methyl-(*E*)-11-oxoundec-9-enoate, cross-metathesis was employed according to Globisch et al.[27] In brief, to a stirred solution of methyl dec-9-enoate (3.26 mmol, 0.6 g) and acrolein dimethyl acetal (9.79 mmol, 1.0 g) in dichloromethane (25 mL) purged with nitrogen was added the second-generation Hoveyda–Grubbs catalyst (41.3 µmol, 25.9 mg). After the mixture had been stirred for 5 h at room temperature, fresh catalyst (41.3 µmol, 25.9 mg) was added and the reaction continued for another 20 h. After evaporation to dryness, purification by column chromatography (silica, diethyl ether/pentane (50:50) with 0.1% triethylamine) yielded dimethyl acetal of methyl-(*E*)-11-oxoundec-9-enoate. The reaction intermediate was deprotected in the presence of hydrochloric acid (0.01 mol L^{-1}). The product was taken up in 20 mL of CH_2Cl_2 and washed with a saturated solution of NaCl (2 mL × 10 mL). The solvent was evaporated under reduced pressure and the residue was worked up by silica gel chromatography (eluted with hexane/ethyl acetate, from 95:5 to 50:50 v/v) to afford methyl-(*E*)-11-oxoundec-9-enoate.

2.8.3. Methyl-(E)-11-oxoundec-9-enoate (3)

The product was obtained in 36% yield as a colorless oil. R_f = 0.53 (hexane/ethyl acetate, 1:1); UV (hexane) λ_{max} 233 nm; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.29–1.51 (8*H*, m, CH_2), 2.23 (2*H*, t, *J* = 7.4 Hz, 2-CH_2), 3.57 (3*H*, s, COOCH_3), 6.03 (1*H*, m, C*H*), 6.96 (1*H*, dt, *J* = 7.9, 15.5 Hz, C*H*), 9.53 (1*H*, d, *J* = 7.9 Hz, 11-C*H*); ^{13}C NMR (126 MHz, $CDCl_3$) δ 24.60, 27.56, 28.67, 28.72, 31.98, 32.43, 33.75, 51.19 (COOCH_3), 132.72 (C-9), 158.83 (C-10), 174.04 (C-1), 193.02 (C-11); HRESI-MS m/z 235.1318 $[M + Na]^+$.

2.9. Examination of the Diels–Alder Reaction Mechanism between the Diene Component and Various Dienophiles

Model experiments simulating Diels–Alder reactions between conjugated FAMEs and selected dienophiles were performed in a special reactor (RTP-15, IOCB Prague) at 260 °C for 10 h as shown in Figure S2, Supporting Information. To the conjugated isomers of methyl linoleate (0.68 mmol, 200 mg) and methyl stearate (0.68 mmol, 203 mg) as internal standard in ACE conical reaction vials equipped with magnetic "V" stirrers was added 0.68 mmol of selected dienophile (methyl oleate, methyl elaidate, α-tocopherylquinone, or methyl-(*E*)-11-oxoundec-9-enoate). All reactions were performed strictly under an argon atmosphere to avoid autoxidation. At the given intervals, aliquots (10 mg) were taken through a Teflon septa by Hamilton syringe flushed with argon for the analyses of FAMEs, APCI-MS, and ESI-MS experiments. ESI-MS and APCI-MS spectra of Diels–Alder products were measured with LC-MS LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) and LC-MS TSQ Quantum Access MAX triple quadrupole mass spectrometer (Thermo Fisher Scientific) in Central Laboratories of UCT Prague. Electron impact mass spectrometry (EI-MS) spectra were also recorded.

3. Results and Discussion

3.1. Examination of Sunflower Oil TAG Degradation and the Formation of TAG Oligomers by RP-HPLC-ELSD/APCI-MS, HPSEC-ELSD, and HPSEC-RID Coupled with ESI-MS

Despite the number of articles related to TAG oligomers, a detailed structural analysis and the mechanism of their formation have not been investigated extensively. Our study focuses on sunflower oil TAGs with an examination of the mechanism of heat-induced degradation. High-temperature experiments were simulated in the isomerization tube at 240, 250, and 260 °C and oxygen was fully excluded by a stream of argon. Specific formation pathways of TAG oligomers and their structural complexity were analyzed by a unique combination of preparative HPSEC-RID coupled with ESI-MS.

Physicochemical properties of starting material are summarized in Table 1. Refined sunflower oil had a peroxide value of 0.65 meq.act.O kg^{-1} and an acid value of 0.07 mg KOH g^{-1}, indicating that the sunflower oil was of good RBD quality. The profile of sunflower oil TAGs was determined by RP-HPLC-ELSD. TAG monomers with ECN values from 42 to 52 accounted for 81.67% with trilinoleoylglycerol (LLL), LLO, OLO, OOO, PLL, POL, SLL, and SOL being the most abundant. APCI-MS/MS analysis of the most important TAG, OLO (M_w = 883.42 g mol^{-1}), and LLO (M_w = 881.40 g mol^{-1}) was used as a diagnostically useful tool for the determination of fatty acyls esterified in the *sn*-2 position. APCI-MS/MS spectra were found to contain abundant parent $[M + H]^+$ and daughter $[M+H-R_iCOOH]^+$ ions. Predominating diacylglycerol-like fragment ions at m/z 600.0 and 602.0 corresponded to the neutral loss of oleic acid. Thus, linoleic acid, which was attached preferentially to the *sn*-2 position, was only poorly displaceable.

The fate of polyunsaturated TAG monomers at 260 °C is shown in **Figure 1**. Within 35 h, sunflower oil samples were thermally stable, after which time the content of TAGs dropped sharply. At 35 h, the function had an inflection point and the degradation of TAG reached the so-called induction period. The number of double bonds clearly defined thermal stability of individual TAG species as follows: ECN48 (OOO, SOL; k_1 = 0.007 h^{-1}) > ECN46 (OLO, POL, SLL; k_2 = 0.009 h^{-1}) > ECN44 (LLO, PLL; k_3 = 0.013 h^{-1}) > ECN42 (LLL; k_4 = 0.021 h^{-1}). Three bis-allylic positions in the molecule of LLL tripled the rate of TAG degradation. The formation of dimers and trimers from TAG is already known to occur in oils and fats, while the direct coupling of two dimers was observed in vegetable oils for the first time. This was a novel finding (**Figure 2**). It was confirmed that higher temperatures favored oligomerization. Moreover, degradation of polyunsaturated TAGs at 260, 250, and 240 °C exhibited clear induction periods of 35, 50, and 75 h, respectively.

A sunflower oil sample heated for 165 h at 240 °C was selected for the preparative HPSEC fractionation of pure TAG dimers (dTAG). A direct infusion ESI-MS technique was used to generate ammoniated TAG dimer ions $[M + NH_4]^+$ by the addition of

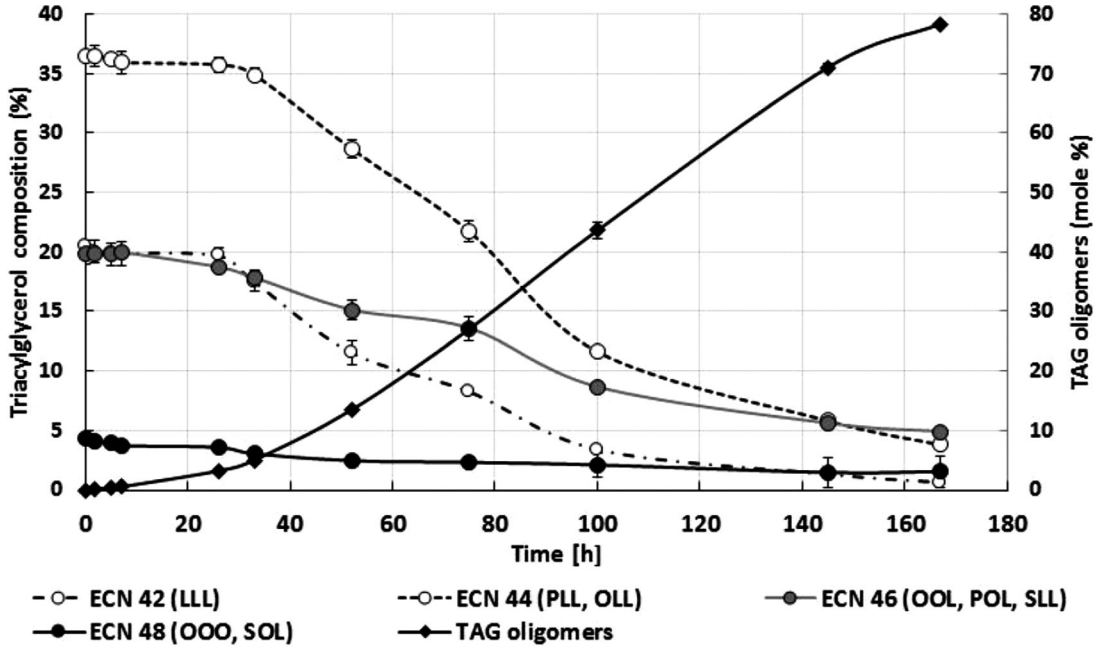

Figure 1. Heat-induced degradation of sunflower oil TAG species at 260 °C.

excess ammonium acetate, as shown in **Figure 3**. Targeted analyses of dTAG adduct ions confirmed the high diversity of isolated species in the region of m/z values from 1700 to 1900. The wide variation of $[M + NH_4]^+$ ions could be explained by diverse incoming TAG reactants as well as multiple mechanisms involved in TAG dimerization. Moreover, TAG dimers could be ionized differently based on ECN values under the conditions of ESI-MS experiments. The most intense adduct ion of the m/z value of 1781.0 was suggested to be a dimer of TAGs ($C_{114}H_{204}O_{12}N$) LOL 54:5 (ECN 44) and LOO 54:4 (ECN 46) formed by a radical coupling reaction. If we take into account the number of possible radical species formed and the number of *sn* positions, the mass of 1781.0 represented dozens of acyclic dehydrodimers achieved by –C–C– linkage. However, the mass of 1781.0 could also be assigned to cyclohexene derivatives of two LOL 54:5 formed by a concerted cyclization according to normal electron demand Diels–Alder reaction. Ammoniated parent ions in the region of the m/z values 1728.0 ($C_{110}H_{198}O_{12}N$) and 1754.0 ($C_{112}H_{200}O_{12}N$) corresponded to TAG dimers with one or two molecules of ester bound palmitic acid, whereas peaks above 1800.0 m/z contributed to dTAG with arachidic, behenic, and lignoceric fatty acyls. MS/MS spectra of selected $[M + NH_4]^+$ ions, including the mechanism of fragmentation, are shown in Figure S3, Supporting Information.

The results presented in Figure 3 also demonstrated that collision-induced dissociation provided diacylglycerol-like fragment ions $[M+NH_4\text{-}NH_3\text{-}R_iCOOH]^+$ derived from TAG dimers after the neutral loss of free fatty acids from *sn*-1 and *sn*-3 positions. The most intense dTAG diacylglycerol-like product ion of m/z 1482.7 was assigned to the neutral loss of oleic acid and ammonia from the TAG dimer $C_{114}H_{204}O_{12}N$ adduct. Further collision-induced dissociation of DAG-like fragment ions yielded several peaks at m/z 1121.4, 1146.5, 1195.4, and 1220.5, which corresponded either to the neutral loss of ketene R_2–CH=C=O or R_2COOH (R_2COOH = linoleic acid) with 74 m/z as a part of the glycerol backbone. The same fragmentation pattern was observed for TAG monomers.[28]

3.2. Examination of the Predominant Formation Pathway for TAG Oligomers in the Absence of Oxygen

An intermolecular and intramolecular Diels–Alder reaction used to be the guiding concept in understanding various aspects of heat-induced oligomerizations of TAG. It was the object of our study to confirm or reject pericyclic pathways. However, in the previous chapter, we demonstrated that direct resolution of TAG dimers was not entirely sufficient while searching for concerted cyclization. The exact structure of sunflower oil FAME oligomers was, therefore, shown to definitively explain thermal degradation from a mechanistic point of view.

The transformation of PUFAs, which is illustrated in **Figure 4**, involved induction periods similar to polyunsaturated TAG species (Figure 2). Geometric and positional isomerization of fatty acyls was an essential transformation proceeding via the radical pathway. At the beginning, the lifetime of radicals was very short due to the high content of tocopherols (≈550 mg kg^{-1}). However, during the induction period, antioxidants were fully consumed and the lifetime of radicals was prolonged; a dynamic equilibrium was, thus, established between various fatty acid isomers. Geometric and positional isomers reached a clear maximum after 50, 100, and 165 h at 260, 250, and 240 °C, respectively. The sum of conjugated fatty acid isomers, including all-*trans* CLA (s-*cis* conformation) derivatives, did not exceed 2.36–3.21 wt%. Contrary to previous reversible steps, fatty acid oligomerization was essentially nonreversible at high temperatures. As shown in Figure 4, FAME oligomers were formed to a significant extent (8.25–56.67 wt%), reaching a maximum after 165 h.

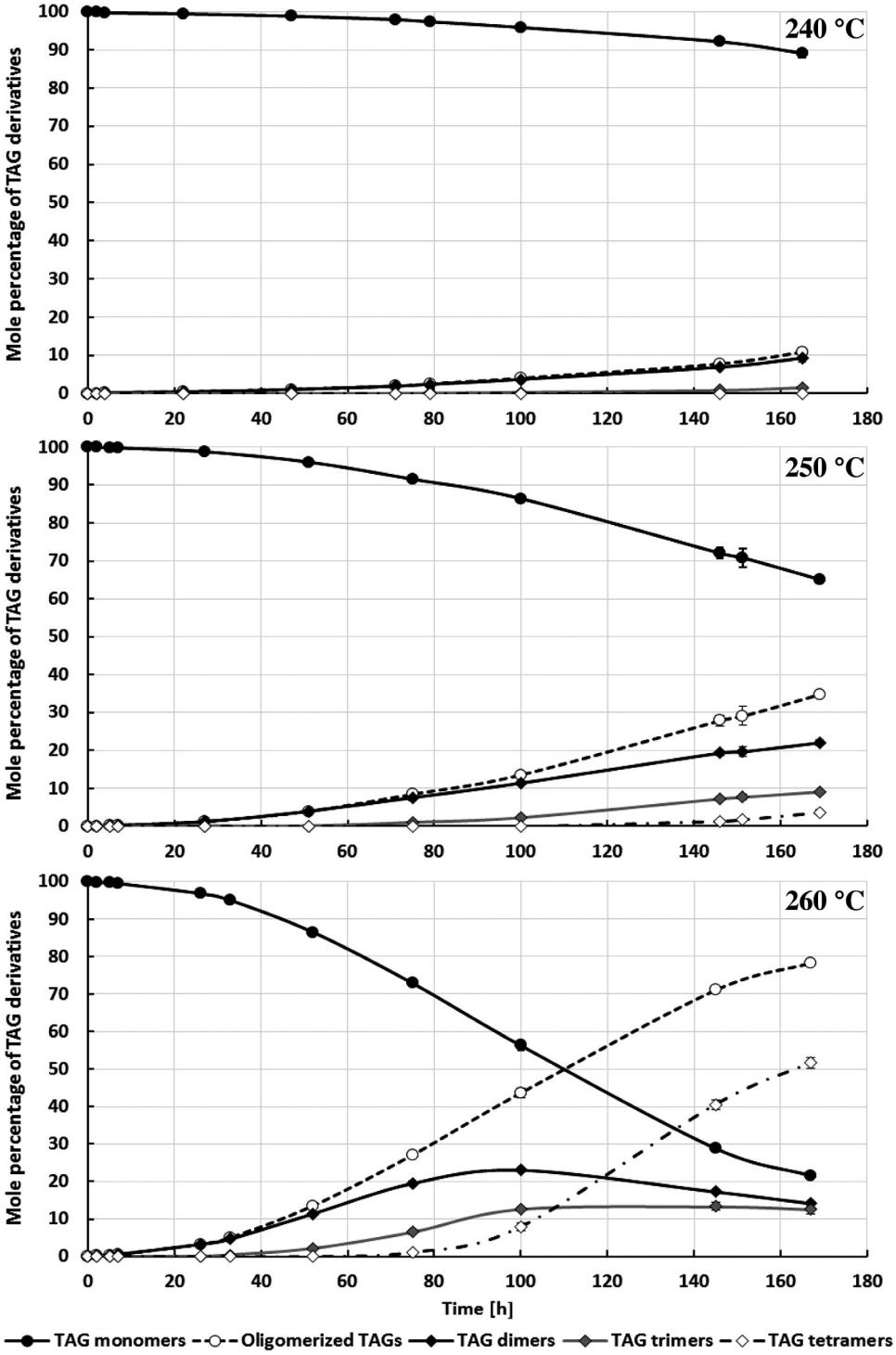

Figure 2. Impact of a prolonged heating of sunflower oil on the formation of TAG oligomers.

Complete structural information about the origin of FAME oligomers was provided by transesterification, preparative HPSEC-RID techniques coupled with hyphenated ESI-MS, APCI-MS, and HTGC-MS. ESI-MS results (**Figure 5**, upper image) demonstrated the presence of protonated parental ions with m/z values of 588.0 and 882.3, corresponding to FAME dimers and trimers accompanied by less-abundant FAME tetramers (m/z 1176.6) and pentamers (m/z 1480.2). The most intense adduct ions were proposed as acyclic oligomers formed by coupling reactions of radical species. Compound structures presented in Figure 5 (upper image) could be considered as illustrative, since the distribution and stereospecificity of products

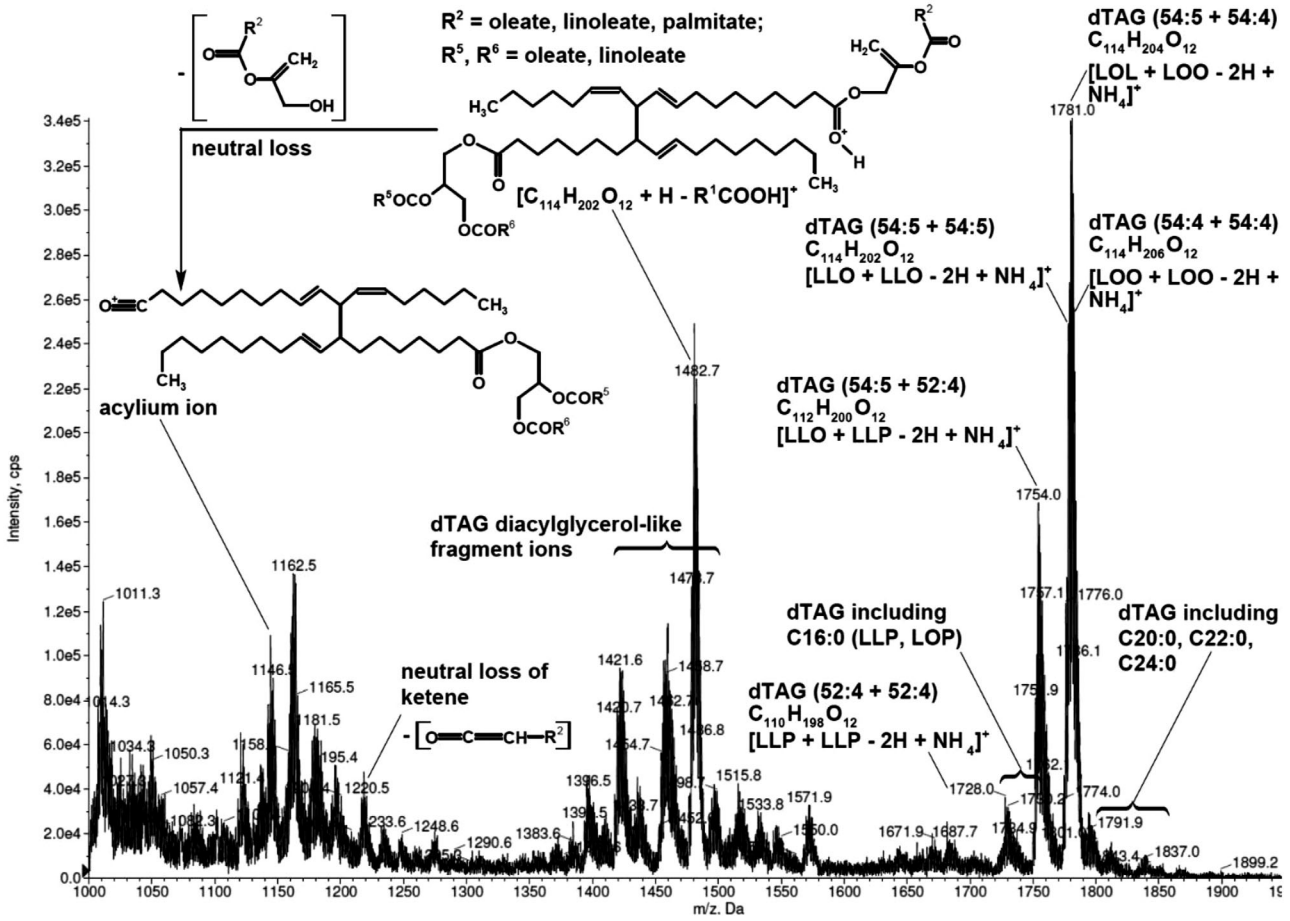

Figure 3. ESI-MS spectrum of TAG dimers $[M + NH_4]^+$ isolated by HPSEC and their collision-induced dissociation fragmentation pattern.

formed was statistically random. APCI-MS results shown in Figure S4, Supporting Information, demonstrated the presence of adduct ions $[M + Na]^+$ with m/z values of 611.8, 906.1, and 1195.5 corresponding to sodiated parental ions of FAME dimers, trimers, and tetramers. Targeted HTGC-MS analysis was applied while searching for Diels–Alder cycloadducts. Here, we present detailed EI-MS spectra of cycloadducts formed by the Diels–Alder reaction of methyl octadeca-10,12-dienoate with methyl octadec-9-enoate. The mass spectrum of the selected compound (Figure 5, lower image) exhibited an intense molecular ion at m/z 590 accompanied by direct loss of a methoxy group (m/z 559) via α-cleavage. Fragments at m/z 519, 505, 491, and 477 originated from the loss of 5, 6, 7, and 8 carbon atoms, respectively, from the aliphatic chain, while the base peak (m/z 433) was derived from cleavage of the C11 fragment. The EI-MS spectrum of 3-pyridylcarbinol derivatives of FAME dimers showed a similar fragmentation pattern shifted to m/z 667 ($M^{+\bullet}$- pyridine), 596 ($M^{+\bullet}$- pyridine—C4 fragment), 553 ($M^{+\bullet}$- pyridine—C8 fragment), and 510 ($M^{+\bullet}$- pyridine—C11 fragment).

CFAMs were the last category of linoleic acid artifacts that definitely remained in thermally degraded sunflower oil. Combined hydrogenation, urea fractionation, and adequate derivatization techniques successfully afforded enriched CFAM in the form of 3-pyridylcarbinol esters. Resolution of their structure and identification of specific ring positions was carried out using GC-MS. Among CFAM, the picolinyl ester of 7-(2-hexyl-1-cyclopentyl)heptanoic acid predominated, as shown by the EI-MS spectra in Figure S5, Supporting Information, (upper image) with a unit gap between m/z 220 and 288. Sunflower oil samples were subjected to prolonged heat treatments at 240, 250, and 260 °C for 165 h, resulting in the formation of 0.19, 0.40, and 0.60 wt% of CFAM, respectively. Thus, each temperature increment of 10 °C caused a twofold increase in the formation of total cyclopentyl and cyclohexyl fatty acid alteration products (Figure S5, Supporting Information, lower image).

3.3. The Impact of Poor Dienophile Geometry on the Course of the Diels–Alder Reaction

The first series of model experiments simulated Diels–Alder reactions between conjugated FAMEs and dienophiles without special activating groups. Methyl esters of oleic and elaidic acids were selected deliberately as poor dienophiles because their geometries were significantly different. All experiments were performed strictly under an argon atmosphere to avoid autoxidation. Moreover, reactions proceeded in the absence of residual catalysts

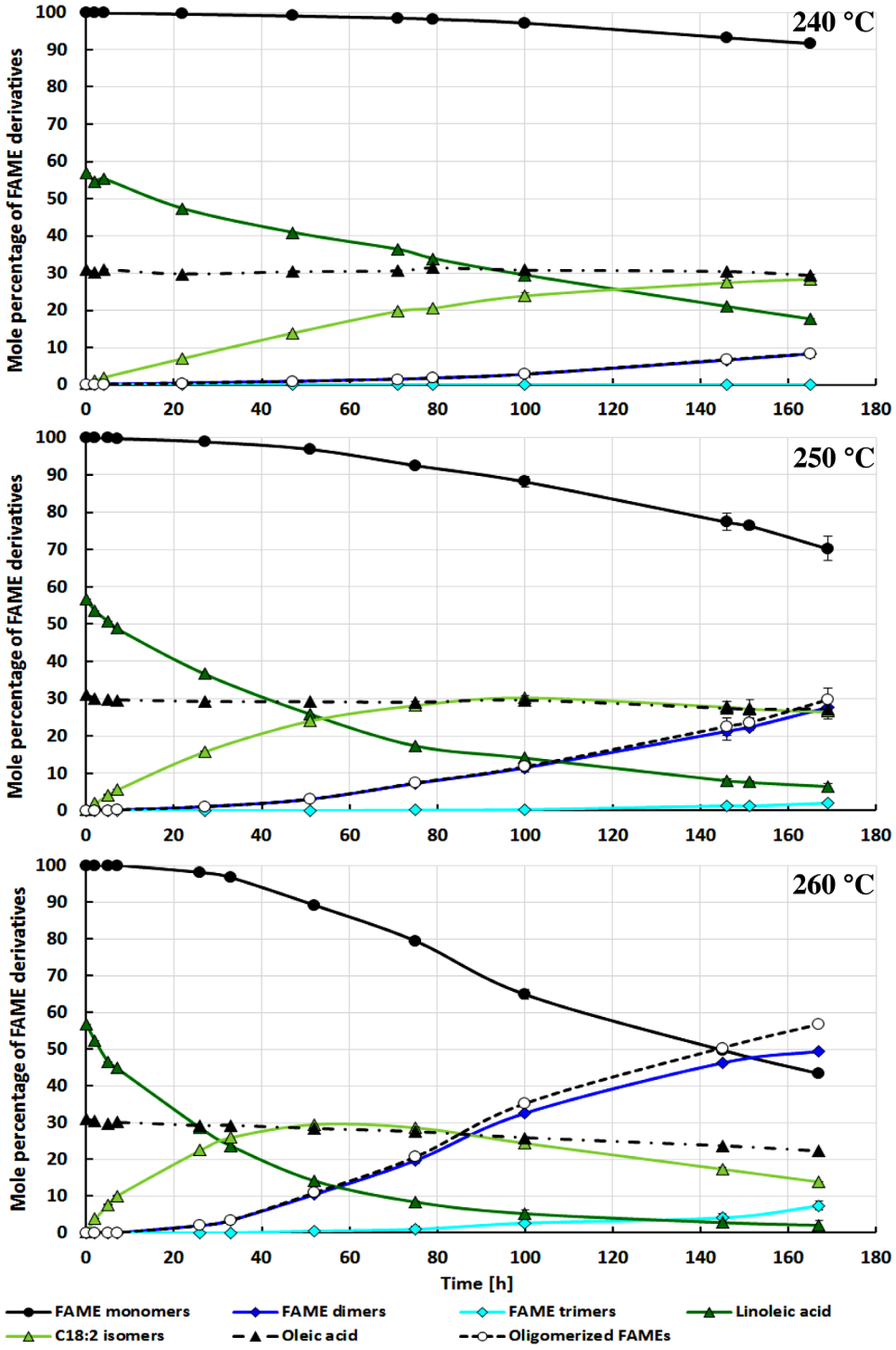

Figure 4. Impact of a prolonged heating of sunflower oil on the formation of FAME oligomers after transesterification of heated sunflower oils.

such as free fatty acids and Lewis acids to be strictly thermally activated.

In our study, we employed homogeneous isomerization of methyl linoleate by Wilkinson's catalyst in the presence of an ionic liquid ($[BMIM]^+[(CF_3SO_2)_2N]^-$) and $SnCl_2 \cdot 2H_2O$ at 60 °C for 90 min. This was an attractive, fast, and green approach for the preparation of conjugated isomers of methyl linoleate, with a final isolation yield of 97.1%. The exact structural elucidation of synthesized product was based on the comparison of retention characteristics and 1D-NMR spectra with literature.[25,29,30] ^{13}C APT spectra showed intense –CH= and allylic CH_2 signals at δ 27.66 (C-8), 129.91 (C-9), 128.71 (C-10), 125.57 (C-11), 134.77

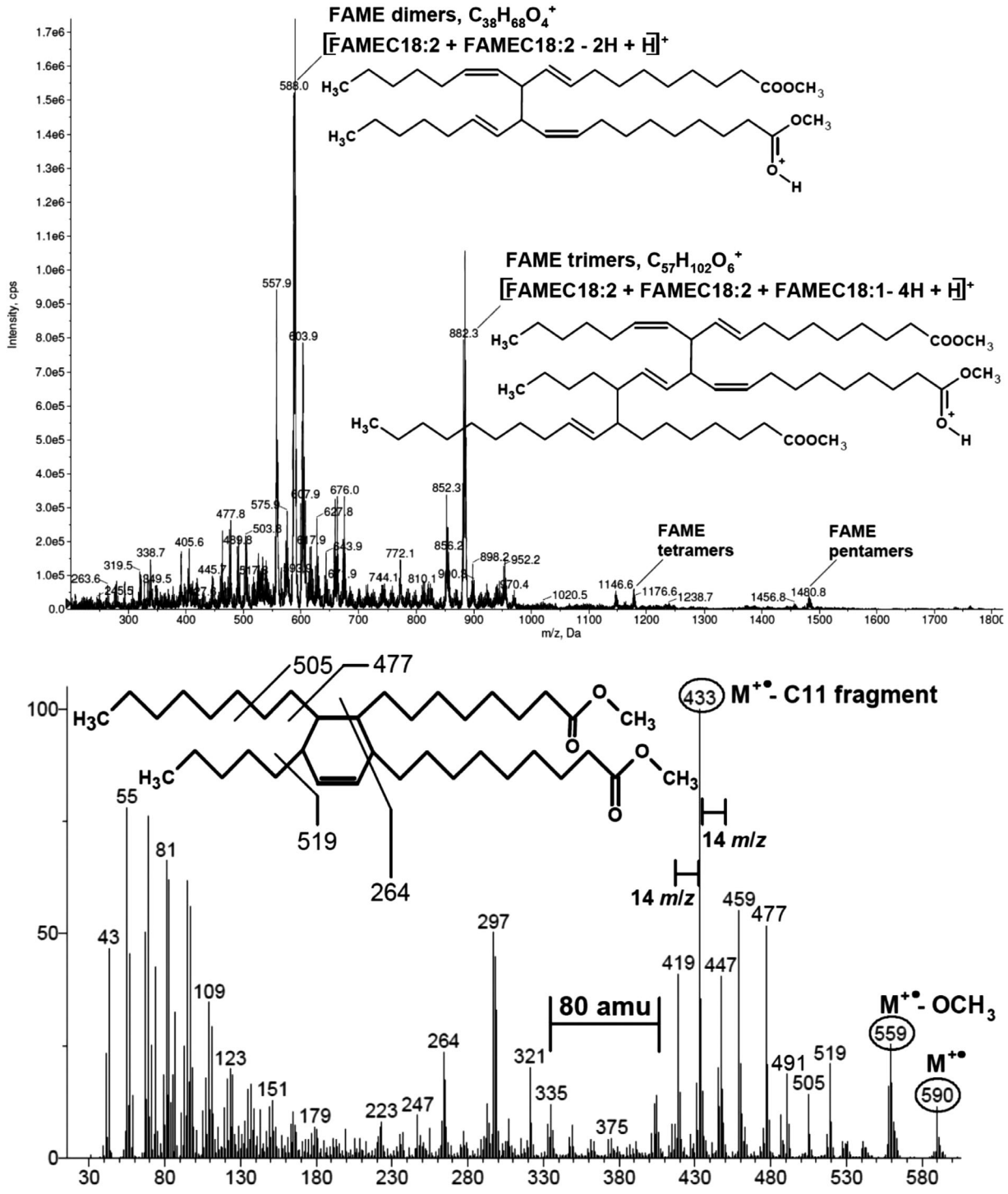

Figure 5. ESI-MS spectrum of protonated FAME oligomers isolated by HPSEC (upper image). Detailed EI-MS spectrum of the Diels–Alder cycloadducts obtained by HTGC-MS (lower image).

(C-12), and 32.90 (C-13) typical for 9*cis*,11*trans*-18:2, while APT carbon signals at δ 32.86 (C-9), 134.58 (C-10), 125.66 (C-11), 128.57 (C-12), 130.15 (C-13), and 27.64 (C-14) were assigned to the 10*trans*,12*cis*-18:2 CLA isomer. Furthermore, the ^{1}H NMR and APCI-MS results were in accordance with literature data.[30]

Quantification of individual geometrical and positional isomers was carried out by GC-FID analysis using an SP 2560 capillary column. The mixture of reaction products contained 9*cis*,11*trans*-18:2 (46.1%), 10*trans*,12*cis*-18:2 (48.2%), all-*trans*-CLA isomers (1.7%), and other *cis*,*trans* or *trans*,*cis*-CLA isomers (4.0%).

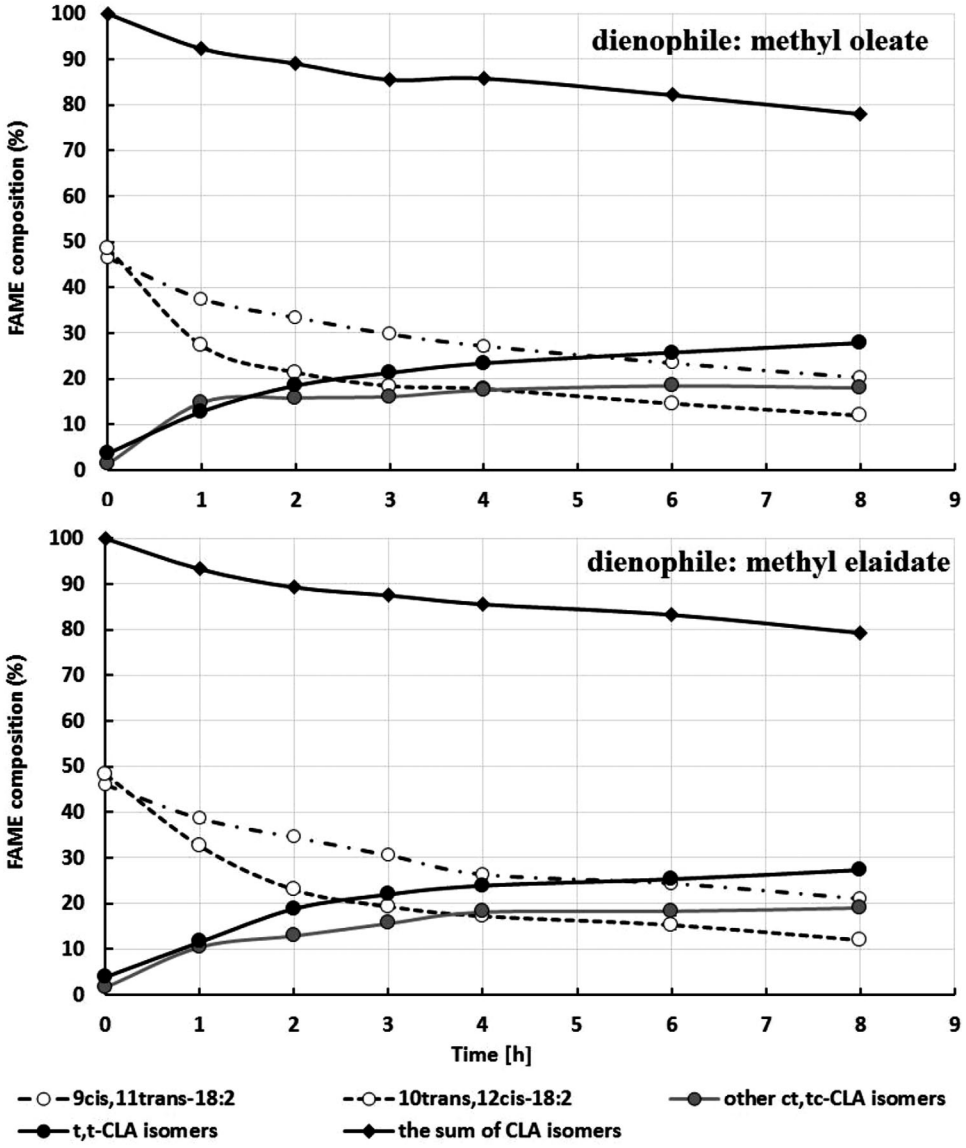

Figure 6. The impact of poor dienophile geometry on the course of the Diels–Alder reaction.

Pericyclic construction of six membered rings required the formation of *trans,trans*-CLA isomers with a cisoid conformation. As shown in **Figure 6**, key derivatives were formed to a significant extent (27.3–27.8 wt%), reaching a maximum after 10 h of model experiments. However, [1,5]-sigmatropic rearrangements were identified as the prevailing pericyclic reaction at 260 °C in the absence of catalyst, as illustrated by the decrease in 9*cis*,11*trans*- and 10*trans*,12*cis*-CLA isomers. For thermal [1,5]-sigmatropic migrations of hydrogen, the conjugated fatty acid derivatives must also be able to adopt the cisoid conformation.[31] Destaillats and Angers suggested that *cis*-C_n,*trans*-C_{n+2} and *trans*-C_n,*cis*-C_{n+2} CLA isomers with a s-*cis* conformation were suitable reactants for the pericyclic [1,5]-sigmatropic mechanism, while *trans,trans*-CLA isomers represented dead-end products.[29] In our study, predominant 9*cis*,11*trans*- and 10*trans*,12*cis*-CLA isomers also underwent extensive suprafacial [1,5]-hydrogen migrations, but the profile of observed reaction products was rather complicated. We have found that different rotamers of *cis*-C_n,*trans*-C_{n+2} and *trans*-C_n,*cis*-C_{n+2} CLA isomers could provide more than one geometric isomer. If we take into account thermal [1,5]-sigmatropic migrations of hydrogen along the molecule of the 9*cis*,11*trans*-CLA isomer, this could lead to the formation of major 8*t*,10*c*-C18:2 accompanied by 8*c*,10*c*-C18:2 due to steric reasons. These products took place in further and/or reversible [1,5]-hydrogen shifts. However, a steep increase in *trans,trans*-CLA isomers could not be explained by a [1,5]-sigmatropic mechanism with suprafacial (allowed) geometry. Reactions nevertheless occurred (Figure 6) and thus a stepwise radical mechanism was preferred rather than involving antarafacial (forbidden) pathways.

With respect to high temperature, duration, and the composition of reactants, model oligomerizations were performed in favor of concerted [4+2]-cycloadditions. *trans,trans*-CLA isomers with a cisoid conformation were believed to react 100 times faster compared to *cis*-C_n,*trans*-C_{n+2} and *trans*-C_n,*cis*-C_{n+2} CLA

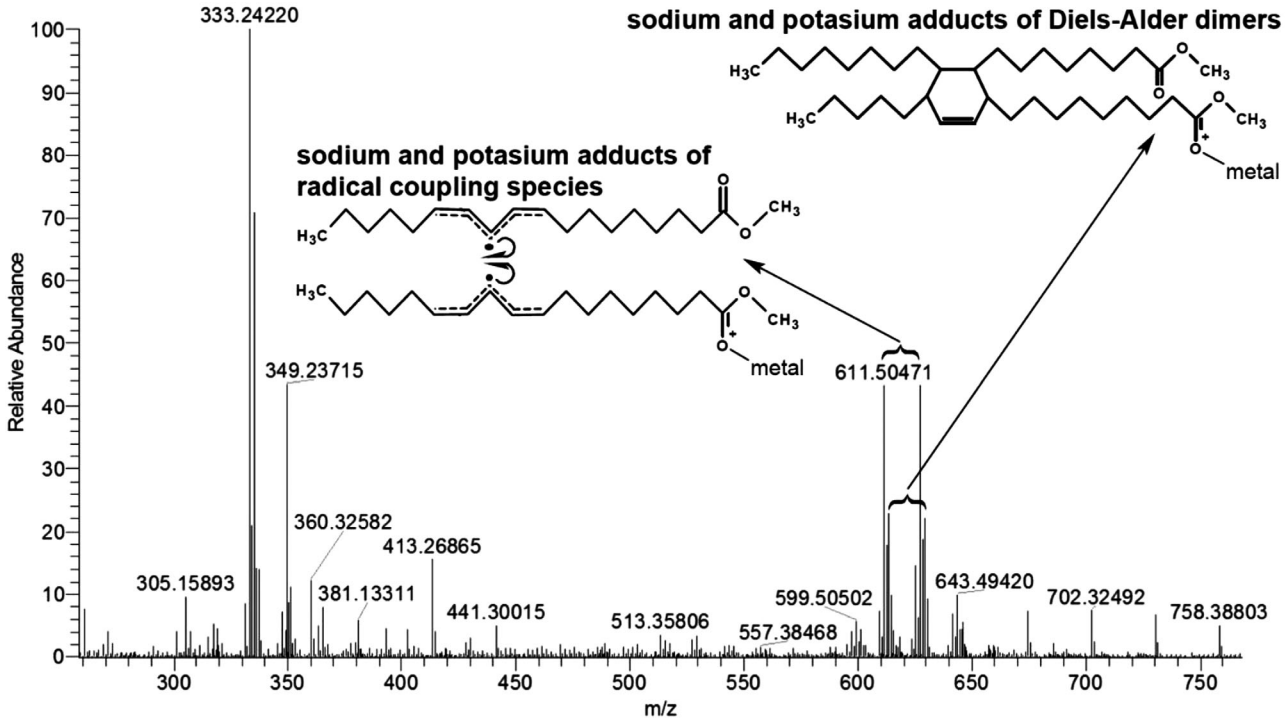

Figure 7. ESI-MS spectrum of model system with methyl elaidate and FAME oligomers in the form of sodium/potassium adducts.

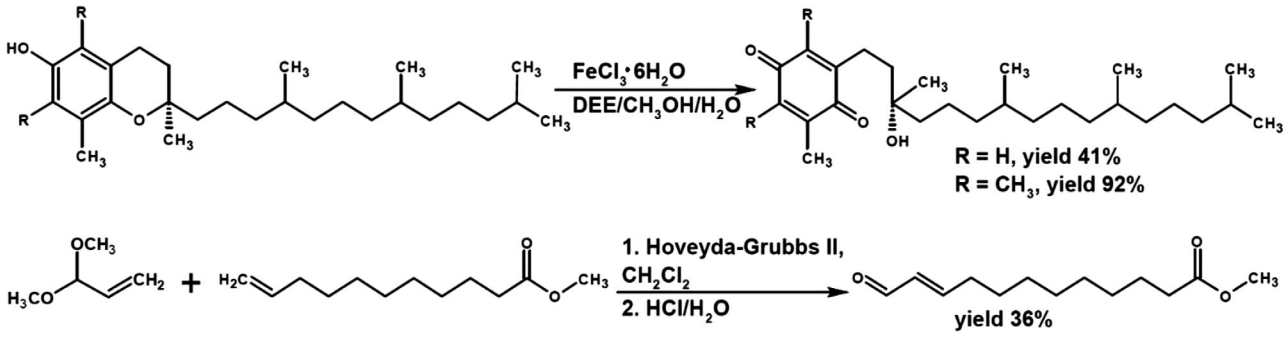

Figure 8. Organic synthesis of naturally occurring dienophiles bearing EWG.

isomers, which had to adopt the conformation by rotation.[31] In fact, higher molecular weight compounds and other artifact were formed to a substantial extent (20.7–21.9%) as shown in Figure 6, but concerted Diels–Alder pathway products were less abundant. **Figure 7** illustrates the ESI-MS spectrum of FAME oligomers. The most intense Na^+/K^+ adduct ions of the *m/z* 611.50482 ($[C_{38}H_{68}O_4Na]^+$) and 627.49953 ($[C_{38}H_{68}O_4K]^+$) were suggested to be dimers of methyl elaidate and conjugated methyl linoleate, formed by radical coupling reactions. Adduct ions of 613.52125 ($[C_{38}H_{70}O_4Na]^+$) and 629.51541 ($[C_{38}H_{70}O_4K]^+$) represented dozens of pericyclic products achieved by the Diels–Alder reaction. Concerted [4+2]-cycloaddition of methyl octadec-9-enoates was under geometric control, since stereospecific syn addition of methyl elaidate was slightly favored. In the case of methyl oleate, Diels–Alder cycloadducts comprised 24%, while syn addition of methyl elaidate reached 31%.

3.4. The Impact of Dienophile-Activating Groups on the Course of the Diels–Alder Reaction

It was the aim of the second model series to investigate the reactivity of naturally occurring dienophiles towards conjugated linoleate. In edible fats and oils, tocopherylquinones, volatile aldehydes, and core aldehydes were particularly common dienophiles-bearing COR and CHO electron-withdrawing groups (EWG). *para-α*-Tocopherylquinone (*α*-TQ) (2a), *para-δ*-tocopherylquinone (*δ*-TQ) (2b), and methyl-(*E*)-11-oxoundec-9-enoate (3) were synthesized in good-to-quantitative yields (36–92%), as shown in **Figure 8**. The presence of the by-product, *ortho-δ*-tocopherylquinone, was excluded by combined 1D-NMR analysis and HRESI-MS techniques. Preparation of the representative unsaturated core aldehyde was based on cross-metathesis of methyl dec-9-enoate and acrolein dimethyl

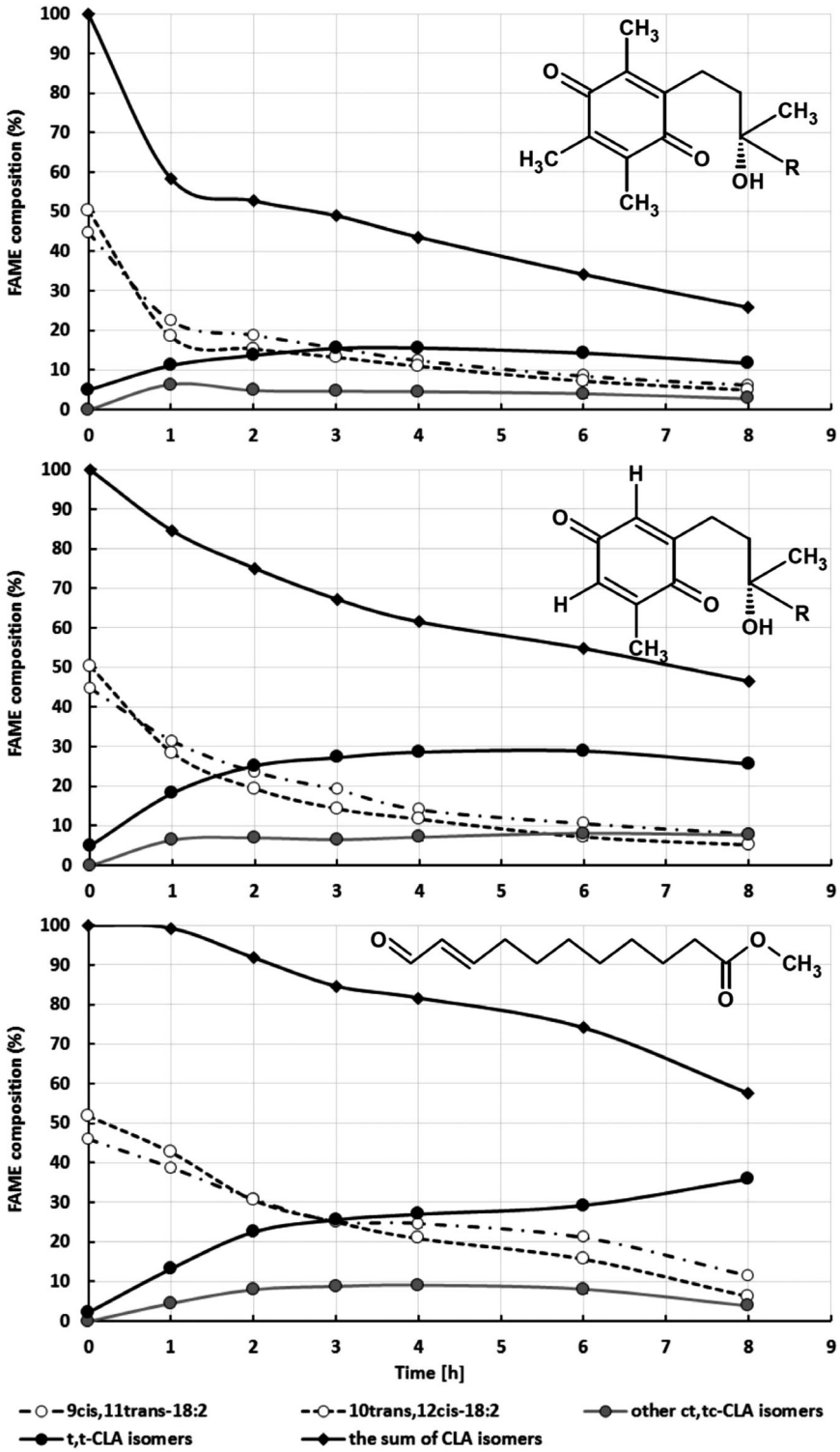

Figure 9. The impact of dienophile EWG group on the course of the Diels–Alder reaction.

acetal, catalyzed by a second-generation Hoveyda–Grubbs catalyst.

The impact of dienophile EWG on the course of the Diels–Alder reaction is summarized in **Figure 9**. All experiments exhibited a sharp decline in CLA derivatives providing products of concerted cyclization. The best dienophile was surprisingly the most substituted α-TQ. In the presence of α-TQ, the sum of CLA isomers decreased by 74.3%, and after 8 h of the model experiment, positive APCI-MS results exhibited protonated [4+2]-cycloadducts of conjugated methyl linoleates and α-TQ of the m/z

741.65810 and their dehydration products of the m/z 723.62695 accompanied by dimers (m/z 893.71689) of α-TQ. Reactions of δ-TQ and methyl-(*E*)-11-oxoundec-9-enoate were rather complicated because a competitive oligomerizetion of δ-TQ was highly involved as shown in Figure S6, Supporting Information, while core aldehyde (enophile) underwent ene (Alder-ene) reactions. Nevertheless, protonated [4+2]-cycloadducts of conjugated methyl linoleates with δ-TQ and methyl-(*E*)-11-oxoundec-9-enoate were clearly identified at m/z values of 714.61370 and 507.36915, respectively.

4. Conclusions

Heat-induced degradation of sunflower oil TAGs at 240–260 °C exhibited clear induction periods, after which time original tocopherols were fully consumed and thus the formation of artifacts was dramatically accelerated. In the practical absence of oxygen, geometric and positional isomerization preceded both TAG oligomerization and PUFA cyclization. The exact structures of sunflower oil TAG and FAME oligomers were successfully elucidated by preparative HPSEC with hyphenated ESI-MS, APCI-MS, and HTGC-MS techniques. The most intense ammoniated parent ions $[M + NH_4]^+$ in the region of the m/z values 1728.0 ($C_{110}H_{198}O_{12}N$) and 1781.0 ($C_{114}H_{204}O_{12}N$) were assigned to acyclic dehydrodimers of TAG achieved by –C–C– linkage. Direct infusion ESI-MS and APCI-MS techniques demonstrated the presence of protonated parent ions (m/z values of 588.0 and 882.3) and sodium adducts (m/z values of 611.8 and 906.1) of FAME dimers and trimers. Although coupling reactions of radical species prevailed, concerted cyclization was not fully excluded. Finally, targeted HTGC-MS results confirmed the exact structure of Diels–Alder cycloadducts formed by the reaction of methyl octadeca-10,12-dienoate with methyl octadec-9-enoate.

Oligomerization of TAGs was widely described by the Diels–Alder reaction and thus concerted cyclization represented the guiding theory. However, in the absence of oxygen and antioxidants, radical pathways represented the real mechanism behind heat-induced formation of TAG and FAME oligomers. The first series of model experiments shed light on the reactivity of conjugated methyl linoleate with poor dienophiles. Concerted [4+2]-cycloaddition of methyl octadec-9-enoates was under geometric control, since stereospecific syn addition of methyl elaidate was slightly favored. Moreover, synthesized CLA isomers (9*cis*,11*trans*-18:2, 10*trans*,12*cis*-18:2), which adopted the cisoid conformation, underwent extensive suprafacial (allowed) [1,5]-hydrogen shifts, while the formation of *trans*,*trans*-CLA isomers was a consecutive radical reaction. Antarafacial (forbidden) pathways were fully excluded and thus a stepwise addition–elimination mechanism was proposed as the correct one. Electron-deficient dienophiles helped to a significant extent to overcome the low reactivity of conjugate dienes.

Abbreviations

α-TQ, *para*-α-tocopherylquinone; δ-TQ, *para*-δ-tocopherylquinone; APCI, atmospheric pressure chemical ionization; BMIM, 1-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide; CFAM, cyclic fatty acid monomers; CLA, conjugated linoleic acid; ECN, equivalent carbon number; EI-MS, electron impact mass spectrometry; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; FAME, fatty acid methyl ester; GC-FID, gas chromatography coupled with flame-ionization detector; GC-MS, gas chromatography coupled with mass spectrometry; HPSEC, high-performance size-exclusion chromatography; HTGC, high-temperature gas chromatography; PUFA, polyunsaturated fatty acids; RBD, refined, bleached, and deodorized; TAG, triacylglycerol; TLC, thin-layer chromatography

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors are indebted to Erik Pešek. The authors sincerely thank Ivana Bartošová for NMR analyses. This work was supported by Ministry of Agriculture of the Czech Republic, National Agency of Agricultural Research QK1910302.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.K.: conceptualization, data curation, investigation, methodology, supervision, visualization, and writing original draft. K.C.: investigation and methodology. D.L.-L.: investigation. J.C.: investigation and methodology. T.V.: data curation and investigation. K.A.: data curation, investigation, writing original draft, review, and editing. I.H., M.B., M.M., and V.F.: writing original draft, review, and editing.

Data Availability Statement

Data are available on request from the authors.

Keywords

conjugated linoleic acid, core aldehydes, Diels–Alder reaction, tocopherylquinones, triacylglycerol oligomers

Published online:

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Antimicrobial and Antioxidant Properties of Phenolic Acids Alkyl Esters

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Abstract

Merkl R., Hrádková I., Filip V., Šmidrkal J. (2010): **Antimicrobial and antioxidant properties of phenolic acids alkyl esters**. Czech J. Food Sci., **28**: 275–279.

Some phenolic acids alkyl esters (methyl, ethyl, propyl, butyl and hexyl) and determine their antioxidant and antimicrobial activities were prepared. The antimicrobial activity against the tested microorganisms *Escherichia coli* DMF 7503, *Bacillus cereus* DMF 2001, *Listeria monocytogenes* DMF 5776, *Fusarium culmorum* DMF 0103, and *Saccharomyces cerevisiae* DMF 1017 was investigated and expressed by minimum inhibitory concentration (MIC) in the range of 1.2–20mM. The inhibitory activity of phenolic acids butyl esters was found to be higher than that of methyl esters (MIC below 1.25mM). The antioxidant activity of the selected phenolic acids alkyl esters was investigated by Rancimat method. The esters of 3,4-dihydroxyphenolic acids (protocatechuic and caffeic acids) exhibited higher antioxidant activities in comparison with the respective phenolic acids. The highest antioxidant activity was found in the case of caffeic alkyl esters.

Keywords: phenolic acid; antioxidant properties; antimicrobial properties

The selected phenolic compounds are plant secondary metabolites naturally present in almost all plant materials, including food products of plant origin and other substances such as propolis. Many biological effects of these compounds, such as anti-inflammatory, antiviral, antibacterial, antiatherogenic, and anticarcinogenic properties have already been reported. These compounds are considered to be an integral part of human food (Psomiadou & Tsimidou 2002). Phenolic acid derivatives are often isolated and applied as a blend of plant species extracts. An exception is phenethyl ester of caffeic acid which has been identified as one of the major components of honeybee propolis. Biological activities are well known in the group of alkyl esters for *p*-hydroxybenzoic acids (parabens) (Grunberger *et al.* 1988). They are used widely as antimicrobial preservatives in pharmaceuticals, cosmetics, foods, and beverages and their potential toxicity and pharmacological activities have been evaluated. Each phenolic derivative has a low acute toxicity, which increases with the increasing length of the alkyl chain. Butyl ester is approximately three times more toxic than methyl ester. It appears that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid can be safely applied in food and drug preservatives, which have been recommended by Matthews *et al.* (1956). The maximum daily intake of parabens is 0.42 mg/kg, as reported by Soni *et al.* (2005). Carcinogenicity and estrogenicity of phenolic derivatives, however, have been little studied (Soto *et al.* 1991). The

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046137305.

correlation between the molecular structure and antioxidant activity of phenolic substances has been described by Cuvelier *et al.* (1992). Monophenols were found by them to be less effective than polyphenols. Moreover, they found that the second hydroxyl group at either *ortho* or *para* position increases the antioxidant activity, and the activity of monophenols increases considerably with one or two methoxylic substituents.

In this work, the alkyl esters studied were synthesised from pure acids to reduce the polarity of the final substances, which increases the solubility in oil and also facilitates the access to the lipophilic cell wall of microorganism.

MATERIALS AND METHODS

Chemicals. Sigma-Aldrich Chemie GmbH (Steinheim, Germany): protocatechuic acid (3,4-dihydroxybenzoic acid), ≥ 97%; gentisic acid (2,5-dihydroxybenzoic acid), ≥ 99%; *p*-hydroxybenzoic acid (4-hydroxybenzoic acid), 99%; Merck (Hohenbrunn, Germany): vanillic acid (4-hydroxy-3-methoxybenzoic acid), ≥ 98%; ferulic acid (4-hydroxy-3-methoxycinnamic acid), ≥ 98%; *p*-toluenesulfonic acid, ≥ 98%; ethanol, 96%; Alfa Aesar (Karslruhe, Germany): caffeic acid (3,4-dihydroxycinnamic acid), 99%; Penta (Strakonice, Czech Republic): propanol, ≥ 99.5%, methanol ≥ 99.8%; Lachema (Strakonice, Czech Republic): butanol, ≥ 99.5%; Fluka (Steinheim, Germany): hexanol, ≥ 98%

Phenolic acid alkyl esters. Phenolic acid alkyl esters were obtained by the reaction of phenolic acid (commercial source) with the respective alcohols. The acid was firstly dissolved in alcohol and the catalyst (*p*-toluenesulfonic acid in the ratio of 0.3–1:1 w/w of phenolic acid) was then added. The reaction was carried out continuously under reflux (2–6 h; 65–95°C) (Etzenhouser *et al.* 2001). The isolation of the phenolic acid derivatives was performed by the method described by Silva *et al.* (2000) with a little modification. After cooling, the solvent was evaporated. The mixture was dissolved in ethyl acetate and neutralised with 8.4% w/w Na_2CO_3 and subsequently washed with 1% w/w NaCl solution. The organic phase was separated and dried overnight over Na_2SO_4. The products were purified by flash-chromatography (silica gel, hexane/ethyl acetate 7:3) or by crystallisation in benzene with a small addition of the appropriate alcohol. All compounds were obtained with a high yield and their purity was confirmed by TLC-FID. The purity of the compounds was greater than 98%. Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 and cellulose plates (Merck, Hohenbrunn, Germany). The mobile phase was chloroform/methanol (9:1). The spots were visualised under UV (254 nm) (Nagaoka *et al.* 2002).

Antioxidant activity. The antioxidant activities of the phenolic acid derivatives (36mM) prepared were determined using the Rancimat apparatus (Rancimat 743, Metrohm, Ltd., Herisau, Switzerland). The principle of this method is to bubble air through heated sunflower oil (Table 1) and monitor continuously the conductivity of demineralised water containing volatile secondary oxidative products. The analysis was performed at the temperature of 120°C with air flow of 20 l/h. The protection factor of antioxidants was calculated using Rancimat software according to the equation:

$$PF\ (\%) = IP\ (oil + antioxidant)/IP\ (oil) \times 100$$

where:

PF – protection factor

IP – duration of the induction period (Hrádková *et al.* 2009)

Antimicrobial activity. Antimicrobial activity of phenolic derivatives against Gram-positive and Gram-negative bacteria, yeast, and fungi was measured by means of the minimum inhibitory concentration (MIC). The MIC was defined as the lowest concentration of phenolic acid alkyl esters which would inhibit the visible growth of the microorganism after the respective incubation (Table 2) (Andrews 2001).

The antibacterial effect of phenolic acid derivatives was followed in microtitration plates. The

Table 1. Characterisation of sunflower oil

AV (mg KOH/g)			0.16	
PV (meq. act. O/kg)			3.28	
IV (g I_2/100 g)			122.06	
Tocopherol content (mg/kg)			120.00	
Composition of fatty acids (% w/w)	C14:0	0.07	C18:3	0.04
	C16:0	6.12	C20:0	0.30
	C18:0	4.05	C20:1	0.02
	C18:1	26.62	C22:0	0.82
	C18:2	61.71	C24:0	0.25

Table 2. Conditions of incubation

Organism	Incubation conditions
Escherichia coli DMF 7503	37°C in air for 20 h
Bacillus cereus DMF 2001	30°C in air for 20 h
Listeria monocytogenes DMF 5776	37°C in air for 20 h
Saccharomyces cerevisiae DMF 1017	25°C in air for 48 h
Fusarium culmorum DMF 0103	20–23°C in air for 72–120 h

culture grew at the corresponding temperature under aerobic conditions for 20–120 hours. The bacterial cultures used in the experiment were prepared freshly in nutrient broth, while yeast and fungi were prepared in malt extract. The initial density of bacterial strains was approximately 10^7 CFU/ml (CFU = colony-forming unit). In the case of *Fusarium* species, their spores were diluted to 10^4 CFU/ml. In each well of the microtitration plates, a volume of 50 µl suspensions of the microorganisms was mixed with 200 µl nutrient broth containing phenolic acid alkyl esters. The amount of antimicrobial agent was diluted with 30% ethanol. The final concentration of ethanol in the test broth was not above 2.5% because at this level the concentration has no influence on the growth of microorganism. The controls inoculated without antimicrobial agent were processed simultaneously (Bartošová *et al.* 2004).

Statistical analysis. All experiments were performed in three replications. The results were expressed as mean values ± SD (the corresponding error bars were displayed in the graphical plots). All statistical tests were performed at a confidence level of 95% (P = 0.05).

RESULTS AND DISCUSSION

The MIC values of the phenolic acids alkyl esters prepared are shown for each microorganism strains tested in Table 3. The MIC values of phenolic acids and alkyl esters determined for yeast (E) and fungi (D) were significantly different.

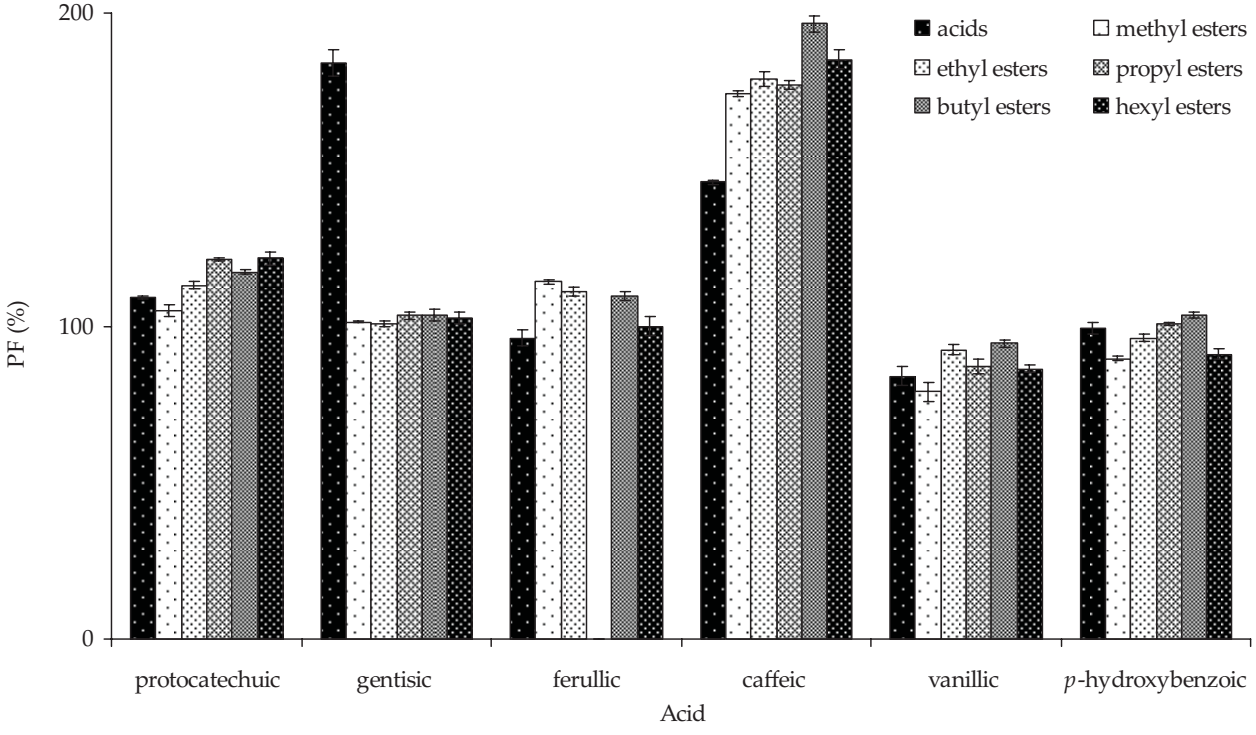
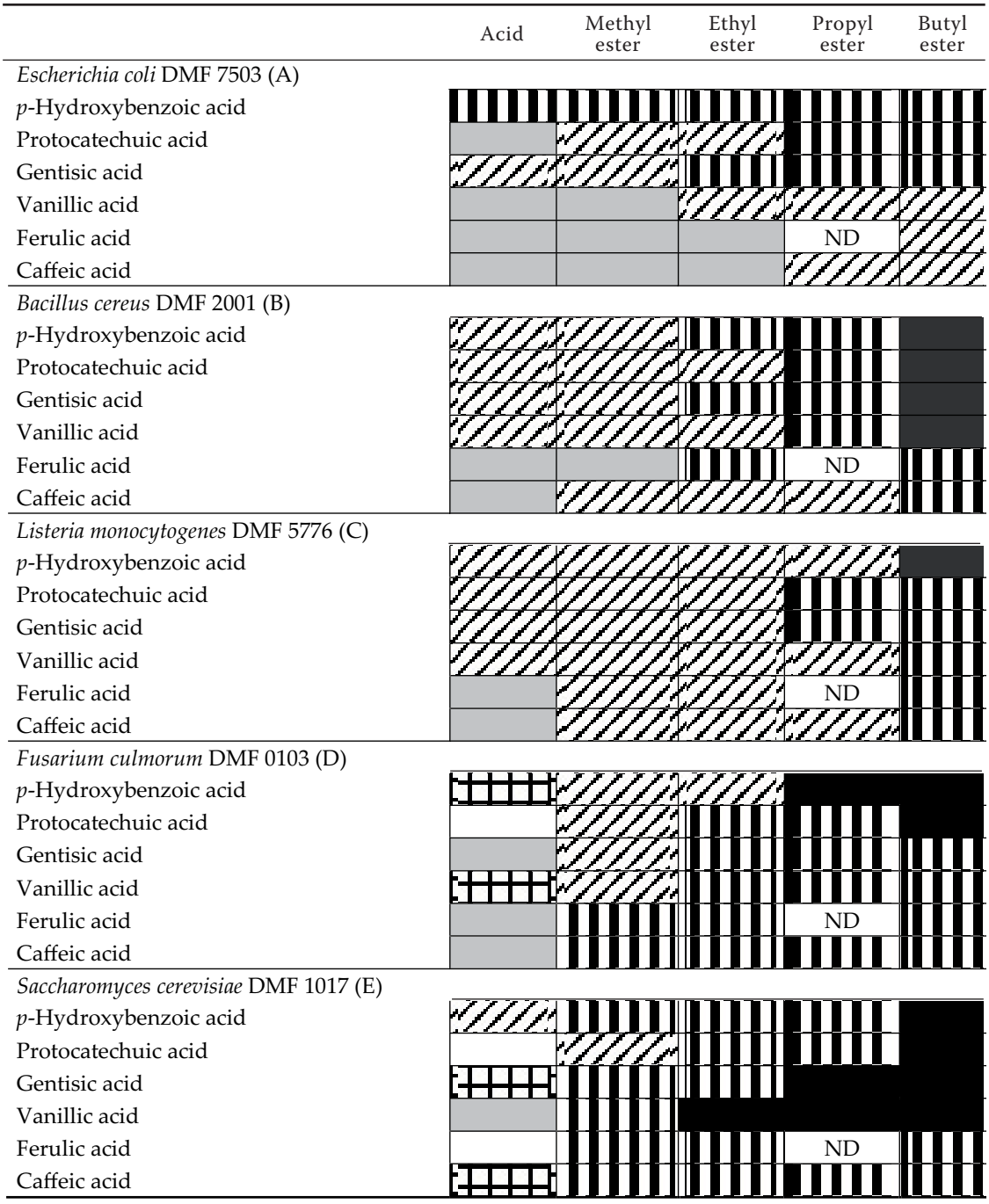


Figure 1. Protection factor (PF) of sunflower oil with phenolic acid and their alkyl esters (36mM), Rancimat method

Table 3. The minimum inhibitory concentrations (MIC) in mM of phenolic acid and their alkyl esters against tested microorganisms (A) *Escherichia coli* DMF 7503, (B) *Bacillus cereus* DMF 2001, (C) *Listeria monocytogenes* DMF 5776, (D) *Fusarium culmorum* DMF 0103, and (E) *Saccharomyces cerevisiae* DMF 1017



Possible explanation may reside in the higher proportions of lipids and phospholipids contained in the cell walls of the former. It can be seen most evidently in the case *Fusarium*. Significant differences between MIC values for Gram-negative (*Escherichia*) and Gram-positive (*Bacillus* and *Listeria*) bacteria were observed. Moreover, the sensitivity of Gram-positive bacteria was higher even in the case of phenolic acids and their methyl or ethyl esters.

Table 3 is arranged in the order from the lightest shade (the highest amount of the tested substance)

to the darkest one (the lowest amount of tested substance). Propyl ester of ferulic acid was not obtained in suitable purity, therefore its antioxidant activity was not determined.

Antioxidant properties of the substances, expressed as protective factors (PF), are shown in Figure 1. It is obvious that some compounds show pro-oxidation activity (ferullic acid, all derivatives of vanillic acid and some derivatives of *p*-hydroxybenzoic acid). Gentisic acid exhibited approximately doubled PF, whereas its ester forms exhibited considerably low PF as a result of losing their antioxidant properties. Masuda *et al.* (2008) described the antioxidant mechanisms of polyphenols (caffeic acid) as quinone form of dihydroxylbenzene that is much more easily oxidisable than the biological material.

CONCLUSION

Generally, the antimicrobial effect of phenolic acids derivatives increases with the increasing length of the alkyl chain. Butyl esters of phenolic acids effectively inhibit the growth of *Bacillus cereus* DMF 2001 and *Saccharomyces cerevisiae* DMF 1017.

Caffeic acid, its esters, and gentisic acid (only) show significant PFs (higher than 150%). Protocatechuic acid and its esters also possess antioxidant activity but their protection factor does not exceed 120%.

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Received for publication April 16, 2010
Accepted after corrections June 10, 2010

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Lipid Oxidation in Margarine Emulsions

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Abstract: Influence of different storage atmosphere (argon and oxygen atmosphere) and influence of monoacylglycerol´s emulsifier (with the carbon chain containing 10, 12, 14, 16, 18 carbon atoms and commercial emulsifier D and a model mixture of monoacylglycerols with the carbon chains containing 10, 12, 14 carbon atoms) on lipid oxidation in margarine emulsions were observed. The rate of lipid oxidation in emulsion with oxygen atmosphere depends on oxygen diffusion through the emulsion layer, while lipid oxidation in emulsion with inert atmosphere is influenced by initial oxygen concentration in water and fat phase. Lipid oxidation in emulsion also depends on acyl combination and the acyl length in emulsifier. Emulsions with monostearoylglycerol oxidized minimally while emulsions with a mixture of monoacylglycerols oxidized maximally.

Keywords: emulsifier; emulsion; lipid oxidation; margarine; monoacylglycerol

INTRODUCTION

Lipid oxidation in emulsion systems is a very complex feature which is usually influenced by many factors, for example the technological history of fat blend, the presence of oxygen in the package, the initial concentration of oxygen in the water phase and in the fat blend, the storage conditions of emulsions, the presence of prooxidants, antioxidants and the structure of interface [1, 2].

Technological fat blend history means previous oxidation of fats and oils of the blend before the emulsification. There is an effort to keep the minimal oxygen content in the fat blend which is close to zero after physical refining or deodorization.

During the storage of margarine emulsions the oxygen diffuses from the atmosphere to the fat blend and together, if the oxygen is presented in water phase, from the water phase to the fat blend.

Monoacylglycerol's emulsifier forms interface of emulsion. Type of emulsifier and its concentration also influence the rate of lipid oxidation [3, 4].

EXPERIMENTAL

Margarine composition. Fat phase: fat blend: 69.6%. Emulsifier –- monoacylglycerol (MAG): the concentration of MAGs in emulsifier was 98.4 to 99.9%. The concentration of MAGs in emulsions was 0.4%.

Synthesized MAG – Monocaprinoylglycerol (MCG) = 1-decanoylglycerol
- Monolauroylglycerol (MLG) = 1-dodecanoylglycerol
- Monomyristoylglycerol (MMG) = 1-tetradecanoylglycerol
- Monopalmitoylglycerol (MPG) = 1-hexadecanoylglycerol
- Monostearoylglycerol (MSG) = 1-octadecanoylglycerol
- A model mixture of monocaprinoylglycerol, monolauroylglycerol, monomyristoylglycerol (in the ratio of 1:8:1).

Commercial emulsifier D (the mixture of monopalmitoylglycerol, monostearoylglycerol and monooleoylglycerol), the concentration of MAGs was 94%.

Water phase: distilled water (29.9%), NaCl (0.1%), lactic acid (0.02%).

Storage conditions. The initial oxygen concentration was defined in emulsion. First type of emulsions: 250 ml of emulsions were stored under the oxygen atmosphere (oxygen volume was 450 ml, oxygen purity was >84% v/v) in glass jars (700 ml) with Twist-Off lids. Second type of emulsions:

250 ml of emulsions were stored under the argon atmosphere (argon volume was 10 ml, argon purity was < 99.9% v/v) in glass jars (260 ml) with Twist-Off lids. The precondition: Twist-Off lids would not allow gas exchange between outside and inside atmosphere of the glass jar. The storage temperature was 15°C.

Analytical methods used in this research.

– Peroxide value (PV) – IUPAC 2.501 (1987)
– Conjugated diens content (CD) – IUPAC 2.206 (1987)
– Anisidine value (AV) – IUPAC 2.504 (1987)
– Acid value = free fatty acid content (FA) – IUPAC 2.201 (1987)
– Oxidative stability of isolated fat blend from emulsion under the conditions of Schaal oven (the storage time at 60°C which is necessary to increase in peroxide value of fat blend to 10 milliequivalents of active oxygen per kg).

Emulsion sample. Figure 1(a) shows how the emulsions under the oxygen atmosphere were stored. Samples were taken from the surface (A), the core (B) and the bottom (C) for peroxide value determination and from the surface (A) and the bottom (C) for other analytical determinations. The rate of lipid oxidation in the different layers was limited due to oxygen diffusion through the emulsion.

Figure 1(b) shows how the emulsions under the argon atmosphere were stored. Samples were taken from the core of emulsion. Lipid oxidation in this system depends only on the initial oxygen concentration in the water and the fat blend.

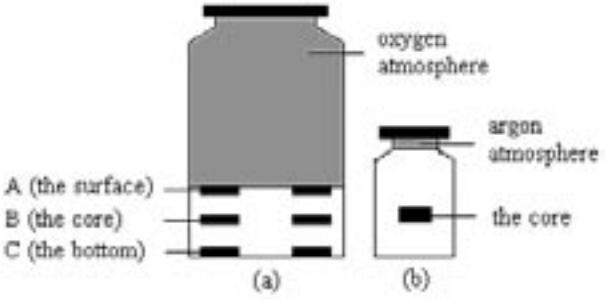

Figure 1. Samples of emulsion stored under oxygen (a) and argon atmosphere (b)

RESULTS AND DISCUSSION

The oxygen diffusion through emulsion layer and comparison of different storage atmosphere

During storage period the oxygen diffuses from the oxygen atmosphere to the emulsion surface and then through the emulsion layer. The oxygen dissolves in fat phase and reacts with fatty acids to hydroperoxides (Figure 2). Samples stored under the oxygen atmosphere were oxidized faster on the surface (A) than on the bottom (C). The rate of lipid oxidation depends on the rate of oxygen diffusion through the layer of the emulsion in the propagation phase of the reaction (between 10 and 15 weeks). Samples stored under the argon atmosphere oxidized only slightly. The range of oxidation changes depends on initial concentration of oxygen in both phases of emulsion.

The same results were obtained in the determination of conjugated diens content.

The anisidine value (Figure 3) and the free fatty acid content did not change during the whole storage period at 15°C. It means that the secondary oxidative products did not form from hydroperoxides under storage temperature (15°C).

Determination of oxidative stability of isolated fat blend

The determination of oxidative stability of isolated fat blend represents another approach to testing the changes in lipid oxidation in emulsion during storage period at 15°C (Figure 4).

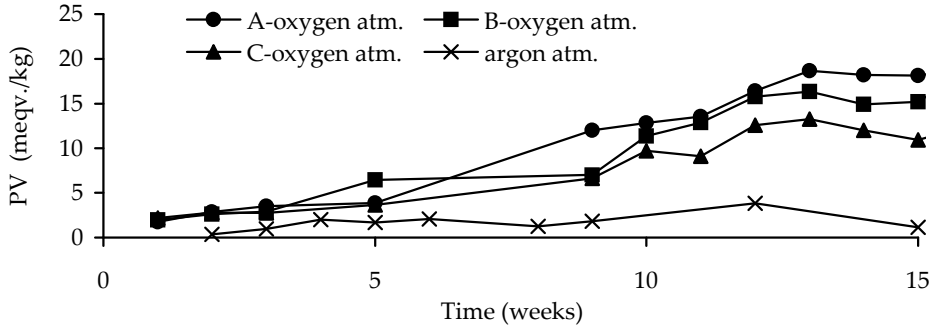

Figure 2. The dependence of peroxide value (emulsifier D) on the time at 15°C

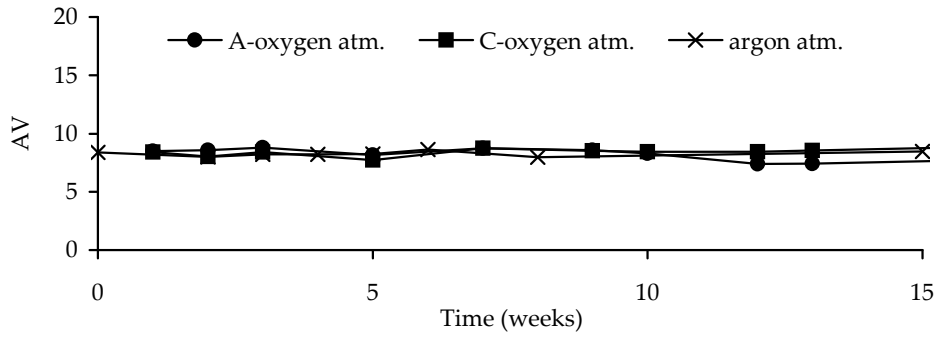

Figure 3. The dependence of anisidine value (emulsifier D) on the time at 15°C

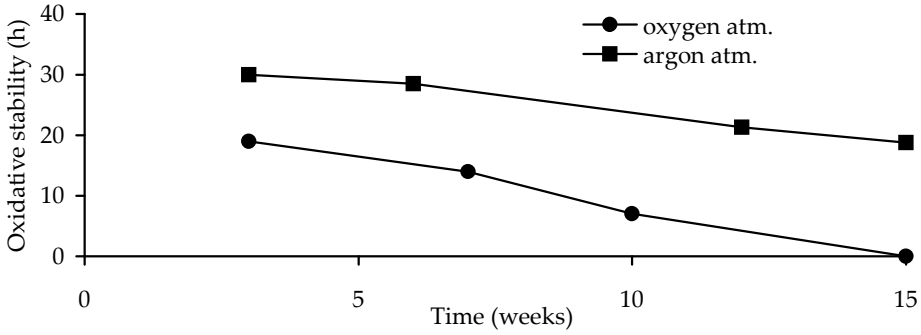

Figure 4. Oxidative stability of isolated fat blend of emulsion (MMG) under conditions of Schaal oven during storage period at 15°C

Oxidative stability of isolated fat blend of emulsion stored under oxygen atmosphere decreased to the zero during 15 weeks while oxidative stability of fat blend of emulsion stored under argon atmosphere decreased from 100% to 60%. We can suppose that it depends on the initial oxygen content in emulsion and the technological history of fat blend.

Influence of emulsion interface on lipid oxidation (Figure 5)

Emulsions containing emulsifier D or a model mixture of monoacylglycerols were oxidized to the greatest extent. The interface was heterogeneous and the oxygen diffusion was increased through this interface because three different acyl chains were presented on the interface in both cases.

The emulsion with monostearoylglycerol had the lowest oxidation level of all emulsions. The interface of this emulsion was very compact. The carbon chain consisting of 18 carbon atoms was the longest carbon chain of all monoacylglycerol's emulsifiers. The oxygen diffusion decreased through this interface and the lipid oxidation was minimal. The interactions are possible between monostearoyglycerol and fat blend crystals too.

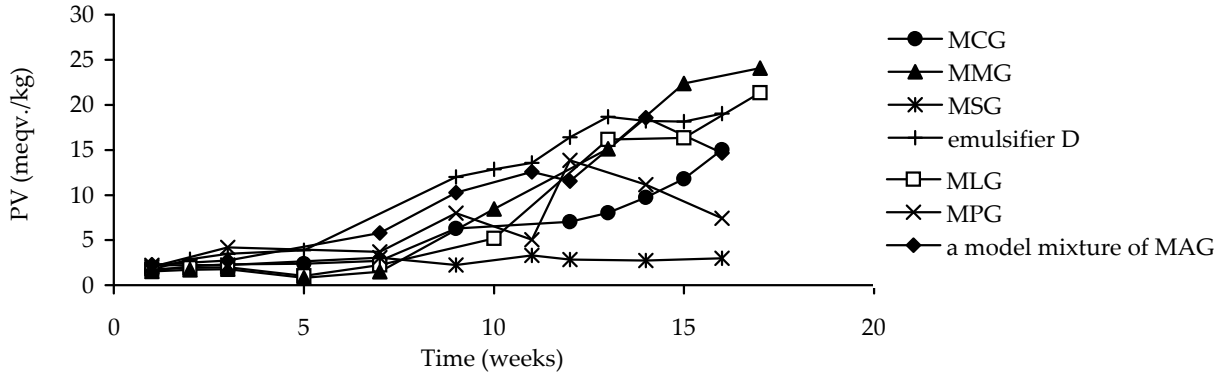

Figure 5. The dependence of peroxide value (on the surface) of emulsion with oxygen atmosphere on time at 15°C. Influence of the emulsifier type

CONCLUSIONS

The rate of lipid oxidation in the emulsions stored under oxygen atmosphere depends on the oxygen diffusion through the emulsion layer. The range of oxidation changes in the emulsions stored under inert atmosphere depends on initial oxygen concentration in both phases of emulsion.

Lipid oxidation in emulsion depends on acyl combination and the acyl length in emulsifier. Emulsion containing only monostearoylglycerol oxidized minimally while emulsion where the interface was created by a mixture of monoacylglycerols (such as emulsifier D or a model mixture of monocaprinoylglycerol, monolauroylglycerol and monomyristoylglycerol) oxidized to the maximum.

The content of secondary oxidative products did not change during whole storage period at 15°C.

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Lipid Oxidation in Dispersive Systems with Monoacylglycerols

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Abstract: Model fat blends with a monoacylglycerol emulsifier with different acyl chain (C10, C12, C14, C16, C18, C18:1, C20, C22) were prepared and stored under oxygen atmosphere 8 weeks at temperature 20°C. Influence of monoacylglycerol on oxidation and oxidation stability of the model fat blends was studied. The model fat blends were prepared by mixing of fully hydrogenated structured fats that contained only palmitic and stearic acid (fully hydrogenated zero-erucic rapeseed oil and fully hydrogenated palmstearin) and half-refined soybean oil. Lipid oxidation was measured by determination of the peroxide value. Volatile oxidation products were detected by the solid phase microextraction in connection with gas chromatography-mass detector (SPME/GC-MS). The oxidative stability was measured by the Rancimat method. Lipid oxidation in model system with 1-octadecenoylglycerol (MAG18:1) was the most extended. On the other hand minimal lipid oxidation was found out in the presence of 1-tetradecanoylglycerol (MAG14) and 1-hexadecanoylglycerol (MAG16).

Keywords: lipid oxidation; fat blend; emulsifier; monoacylglycerol

INTRODUCTION

Oxidation is the most important reaction that leads to increase of oils rancidity, viscosity and volatility because the low-molecular weight off-flavor compounds are formed. The off-flavor compounds do oil less acceptable or unacceptable to consumers or for industrial use as a food ingredient. Oxidation of oil also destroys essential fatty acids and forms toxic compounds and oxidised polymers (Ashavaryu *et al.* 2000; Choe & Min 2006).

Lipid oxidation in liquid oils and O/W emulsions is described very well. However, the lipid oxidation in dispersive systems as W/O emulsions and fat crystals in liquid oil is not described enough (Frankel 2001).

It has been found out that monoacylglycerols of saturated fatty acids retarded lipid oxidation in margarine emulsion. The emulsifier in emulsion can be accumulated at three various interfaces – emulsion/air, water/oil and liquid lipid/solid lipid of the fat blend (crystals triacylglycerols). The question is: Is the lipid oxidation influenced on monoacylglycerol emulsifier in fat blends, where the emulsifier can be accumulated at two interfaces – fat blend/air and liquid lipid/solid lipid (crystal network of the structured fat) (Garti *et al.* 1998; Pokorná *et al.* 2004).

The aim of this study was to find out the influence of the monoacylglycerol with different acyl chain on the rate of lipid oxidation of liquid phase in dispersive system.

MATERIALS AND METHODS

Fat blend composition

Structured fat: 19.7% w/w fully hydrogenated zero-erucic rapeseed oil (FH ZERO) or fully hydrogenated palmstearin (FH PST) (Table 1 shows fatty acid composition of structured fat).
Liquid oil: 79.7% w/w half-refined soybean oil.
Emulsifier: 0.6% w/w monoacylglycerols (MAGs), 1-decanoylglycerol (MAG10), 1-dodecanoylglycerol (MAG12), 1-tetradecanoylglycerol (MAG14),

Table 1. Fatty acid composition of structured fat (g FA/100 g ΣFA)

Fatty acid (FA)	FH ZERO	FH PST
C16:0	5.53	54.91
C18:0	91.04	42.43
C18:1	0.12	0.09
C18:2	0.00	0.24.
Others	3.31	2.33

1-hexadecanoylglycerol (MAG16), 1-octadecanoylglycerol (MAG18), 1-octadecenoylglycerol (MAG18:1), 1-eicosanoylglycerol (MAG20), 1-docosanoylglycerol (MAG C22) were synthesised (Martin 1953), purity > 95%.

Storage conditions

250 ml of fat blends were stored under the oxygen atmosphere (oxygen volume was 450 ml, oxygen atmosphere was > 84% v/v) in glass jars (700 ml) with Twist-Off lids. The storage temperature was 20°C.

Analytical methods used in this research

- Peroxide value (PV) – ČSN ISO 3960:1994.
- Solid phase microextraction in connection with gas chromatography-mass detector (SPME/GC-MS) – detection of volatile oxidation products (adsorption at 20°C), GC conditions: column HP5 (max. temperature 325°C, 0.25 mm × 30 m, thickness of stationary phase 0.25 μm, carrier gas He 0.9 ml/min), SPME fibre: Divinylbenzen/Carboxen/Polydimethylsiloxan (DVB/CAR/PDMS) 50/30 μm, length of fibre 1 cm, pH 2–11, max. temperature 270°C.
- Oxidation stability was estimated on the Rancimat instrument (conditions: air flow 20 l/h, temperature 120°C) and evaluated as time of induction period (h).
- Methods used for data processing – rectangle method (calculation of areas under the curves) and Student's *t* test at significance level α = 0.05 (Eckschlager *et al.* 1980).

Fat blends samples

Fat blend was separated to three layers: the surface, the core and the bottom. Samples were taken from the surface, the core and the bottom for the peroxide value determination. Samples for Rancimat method were taken from the core. Lids of the glass jars were equipped with septum to take directly sample of volatiles above the fat blend for SPME/GC-MS.

RESULTS AND DISCUSSION

Primary products of lipid oxidation

Oxygen diffuses from the oxygen atmosphere to the fat blend, dissolves in the fat blend and reacts with fatty acids to hydroperoxides. The amount of hydroperoxides decreases from the surface to the bottom. Figures 1 and 2 show the areas under the curves, which correspond with total content of hydroperoxides in 1 kg sample for 8 weeks. Fat blend (with FH ZERO as well as FH PST) with MAG18:1 had the highest content of hydroperoxides and was statistically significant different from the others fat blends. The lowest content was detected in fat blends with FH ZERO with MAG10, MAG14, MAG16 and MAG22 (Figure 1) and in fat blends with FH PST with MAG14 and MAG16 (Figure 2).

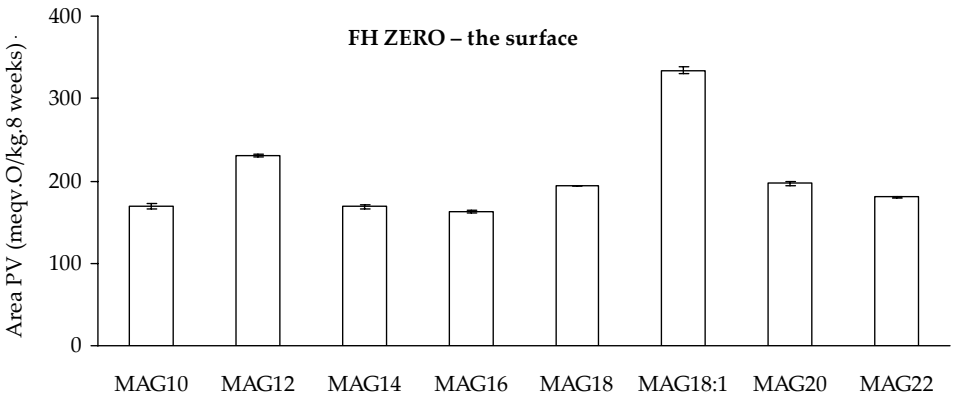

Figure 1. Areas under the curves of the peroxide value of fat blends with FH ZERO

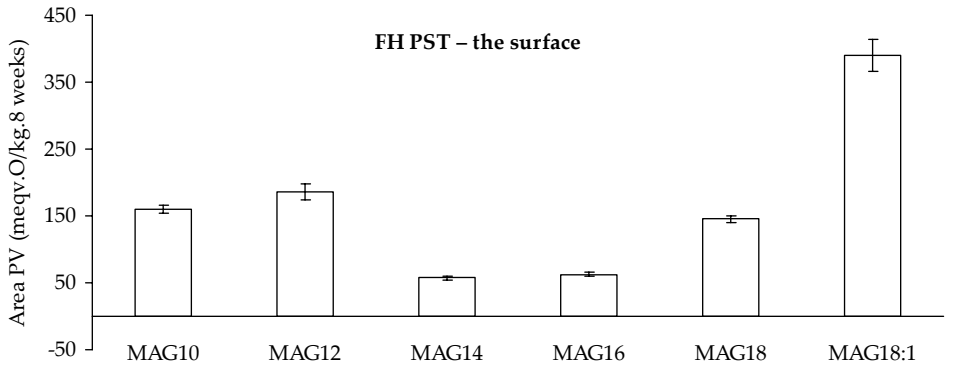

Figure 2. Areas under the curve of the peroxide value of fat blends with FH PST

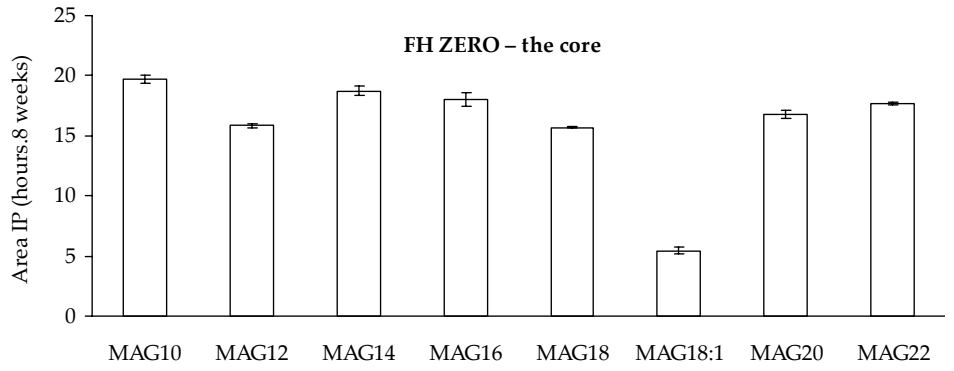

Figure 3. Areas under the curve of the oxidative stability of fat blends with FH ZERO

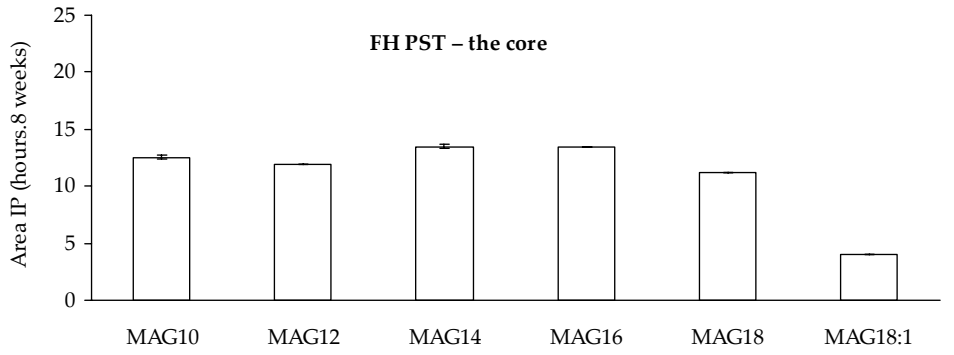

Figure 4. Areas under the curve of the oxidative stability of fat blends with FH PST

Differences between fat blends with these emulsifiers were not statistically significant.

Secondary products of lipid oxidation

Volatile oxidation products (hexanal, 2,4-heptadienal and nonanal) were detected by the method SPME/GC-MS. The maximal amount of all volatile products was detected in the second week of storage. Lower concentration was determined in fat blends with MAG12 and MAG18. Higher concentration was found out in fat blend with MAG18:1.

Oxidative stability by the Rancimat method

Oxidative stability is represented by the time (induction period – IP or induction time) in which an oil sample resists to oxidation at specific temperature. The sample was heated at 120°C and conductivity of volatile products of oxidation in the solution was measured. The oxidative stability decreases during the storage. The minimal oxidation stability had the fat blend with MAG18:1 and the maximal oxidation stability was extended in the fat blend with MAG16 (Figures 3 and 4). Statistically significant difference of areas under the curves was found out in the fat blend with

MAG18:1, which was significantly different from the fat blends with the others MAGs.

Lipid oxidation in model system with MAG18:1 was the most extended. On the other hand minimal lipid oxidation was found out in the presence of MAG14 and MAG16 at the both structured fats. Adsorption of monoacylglycerols on the crystal network with the tightest arrangement of emulsifier molecules can be expected. The presence of a *cis*-double bond in oleic acid of monoacylglycerol caused less tight adsorption of the emulsifier on the interface. Hence oxygen diffusion through the interface depends on the type of acyl chain of the monoacylglycerol emulsifier.

Acknowledgements: This work was supported by funding from the Ministry of Education, Youth and Sports of the Czech Republic (Project. No. MSM 6046137305).

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Lipid Oxidation of Fat Blends Modified by Monoacylglycerol

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Abstract

Spěváčková V., Hrádková I., Šmidrkal J., Filip V. (2012): **Lipid oxidation of fat blends modified by monoacylglycerol**. Czech J. Food Sci., **30**: 527–533.

Model dispersions of fat blends (FBs) with monoacylglycerols (MAG) of saturated fatty acids with different lengths of the acyl chain (MAG10–MAG18) and 1-octadecenoylglycerol and without MAG (as blank) were prepared. We find out the influence of the addition of monoacylglycerol on oxidation of the fat dispersion. Trihexadecanoylglycerol (tripalmitoylglycerol – TAG48) was used as the dispersive phase and soybean oil was used as the dispersive medium. Primary (conjugated diens) and volatile secondary (by SPME in connection with GC-MS) lipid oxidation products and oil stability index (OSI) were measured during autoxidation of the fat blends in storage conditions. MAGs with a shorter (or the same) acyl chain length (MAG10–MAG16) than the acyl chain length of the structured fat (TAG48) arrange tightly on the interface oil/crystals of structured fat, thus prevent lipid oxidation.

Keywords: conjugated diens; secondary oxidation products; oil stability index – OSI; structured fat; trihexadecanoylglycerol; tripalmitoylglycerol

Oxidation of lipids is one of the major reactions resulting in the decrease of food quality and acceptability, because it reduces the nutritional value and generates rancidity, causing undesirable flavours. Primary oxidation products are hydroperoxides which have no effect on flavour quality of foods. Hydroperoxides are unstable being decomposed to secondary oxidation products, a complex mixture of volatile (aldehydes, methyl ketones, hydrocarbons) and non-volatile compounds under storage temperatures. The extent of oxidation, formation of oxidation products, and oxidative stability are primarily dependent on the degree of unsaturation of the fatty acids present in triacylglycerols and on the structural differences beween the various triacylglycerols. Double bonds and methylene groups near to double bonds in unsaturated fatty acids are the active sites for free radicals formation and oxidation reaction is subsequently started (Shahidi 1998; Choe & Min 2006; Laguerre *et al.* 2007).

Lipid oxidation in oils and in emulsions of oil-in-water (O/W) type has been well described in many publications (Velasco & Dobarganes 2002; Anwar *et al.* 2003; Osborn & Akoh 2004; Beltran *et al.* 2005; Haiyan *et al.* 2007). Nevertheless the information on the oxidation of lipids in emulsions of water-in-oil (W/O) type or dispersive systems like fat blends is not sufficient (Frankel 2001; Pokorná *et al.* 2004).

Oxidation and oxidative stability of lipids in model fat blends–fat dispersions were studied in this work. The simple fat dispersion consists of fat crystals and/or their clusters which create three-dimensional network as the dispersive phase and

Supported by the Ministry of Education, Youth and Sports of the Czech Republic, Project No. MSM 6046137305, and by Specific University Research, Project No. MSM 21/2011.

liquid oil as the dispersive medium (Narine & Marangoni 1999). High melting fat crystals such as fully hydrogenated fats can exhibit gel-like behaviour in fat blends. Three-dimensional fat crystal network then exhibits the properties of organogel (Higaki *et al.* 2004). Liquid triacylglycerols closed in three-dimensional network are oxidised. The network itself can make a specific barrier against oxygen diffusion and adsorbed emulsifiers can intensify this barrier (Spěváčková *et al.* 2009). It has been found out that monoacylglycerols retarded lipid oxidation in margarine emulsion, a system of three phases (two limitation miscibility liquids and solid phase) (Pokorná *et al.* 2004). In margarine emulsions the emulsifier is adsorbed not only on the interfaces emulsion/air and water/oil but also on the interface liquid lipid/solid lipid of the fat blend (Garti *et al.* 1998). In the shortenings the emulsifier can be accumulated on the interfaces fat blend/air and liquid lipid/solid lipid. Emulsifiers such as monoacylglycerol can improve their physicochemical properties in two main ways. The first function of an emulsifier is to enable the formation of two distinct phases (formation of a stable pseudo-homogenous state) and the second one is to modify the behaviour of continuous oil phase (Martini & Herrera 2008).

This issue has been studied in a simple system of fat dispersions with fully hydrogenated zero-erucic rapeseed oil (containing more then 90 % (w/w) of stearic acid) as a structured fat. Positive barrier effect of 1-hexadecanoylglycerol was determined in these fat blends (Spěváčková *et al.* 2009). The aim of this paper was to study the influence of monoacylglycerol emulsifiers (with naturally occurring fatty acids) on lipid oxidation of model fat blends (FBs) with trihexadecanoylglycerol (TAG48).

MATERIAL AND METHODS

Chemicals. Glycerol (purity ≥ 99.5%; Sigma-Aldrich, Darmstadt, Germany), hexadecanoyl chloride (palmitoyl chloride, purity ≥ 98%; Sigma-Aldrich, Darmstadt, Germany), pyridine (purity ≥ 99.8%; Fluka, Germany), chloroform (purity ≥ 99.5%; Lach-Ner, Brno, Czech Republic), 3-dimethylamino-1-propylamine (purity ≥ 98%; Fluka, Germany), hydrochloric acid (purity ≥ 35%; Lach-Ner, Brno, Czech Republic), calcium chloride (purity ≥ 96 %; Lach-Ner, Brno, Czech Republic), *n*-hexane (purity ≥ 99 %, Penta, Prague, Czech Republic).

Synthesis of trihexadecanoylglycerol (tripalmitoylglycerol – TAG48). TAG48 was synthesised by the reaction of glycerol with hexadecanoyl chloride by the modified method (Sidhu & Daubert 1947; Täufel *et al.* 1960). The reaction was stopped after 12 h by the addition of 3-dimethylaminopropylamin. The reaction mixture was rinsed with 5% (w/w) aqueous solution of hydrochloric acid and subsequently with distilled water to neutral pH, and then the organic fraction was dried over calcium chloride. The chloroform was evaporated and the product was purified by crystallisation in *n*-hexane (two-times). The purity of TAG48 was higher than 98 % by HTGC/FID analysis while the contents of FFA, MAG and DAG were negligible.

FBs composition. 19.7 % (w/w) structured fat – TAG48; 79.7% w/w liquid oil – half-refined soybean oil (Setuza a.s., Ústí n. L., Czech Republic) (characterisation and fatty acids composition in Table 1); 0.6% w/w monoacylglycerol emulsifier. The emulsifiers 1-decanoylglycerol (MAG10), 1-dodecanoylglycerol (MAG12), 1-tetradecanoylglycerol (MAG14), 1-hexadecanoylglycerol (MAG16), 1-octadecanoylglycerol (MAG18), 1-octadecenoylglycerol (MAG18:1) were synthesised (Martin 1953). Purity of all MAGs was higher than 95%.

FB without emulsifier (WE) was prepared as a blank sample (20% w/w structured fat and 80% w/w liquid oil).

FBs preparation and storage conditions. FBs were prepared in duplicate vessels in argon atmosphere

Table 1. Characterisation and fatty acids composition [% w/w] of soybean oil

AV (mg KOH/g)	0.12
PV (meq. act. O/kg)	1.03
IV (g I_2/100 g)	130.4
CD (% w/w)	0.350
IP Rancimat (h)	4.06
IP Oxidograph (h)	3.60
C16:0	10.9
C18:0	4.5
C18:1	23.4
C18:2	53.0
C18:3	7.1
Others	1.1

AV – anisidine value; PV – peroxide value; IV – iodine value; CD – conjugated diens content; IP – induction period

(Piska *et al.* 2006). The mixture of structured fat, liquid oil and emulsifier was heated to 80°C. The liquid mixture was emulsified during 5 min with an emulsifying stirrer (1000 min^{-1}). FBs were stored over a long term in the darkness under an oxygen atmosphere (oxygen atmosphere was > 84% (v/v) – sensor CellOx 325, WTW; Weilheim, Germany) at temperature 20 ± 1°C.

Determination of conjugated diens content. Conjugated diens (CD) content was determined by the standard IUPAC 2.206 method (IUPAC 1987) every two weeks.

Measurement of oxidative stability. The oxidative stability of FBs was determined by the instruments Rancimat 743 (Metrohm Ltd, Herisau, Switzerland) and Oxidograph^{TM} (Mikrolab Aarhus A/S, Højbjerg, Denmark) every two weeks.

Rancimat procedure: 2.5 g of FB in the reaction vessel were bubbled through with air flow of 20 l/h at a temperature of 120 ± 1.6°C (Oil Stability Index method – OSI; AOCS Official Method Cd12b-92 1997).

Oxidograph procedure: 5 g of FB in the reaction vessel under oxygen atmosphere (filling of vessel by oxygen: flow of O_2 was 2–4 l/min for 20 s) were heated to 110°C (Velasco *et al.* 2004; Nogala-Kalucka et al. 2005).

Analyses of volatile compounds. The volatile compounds were extracted by the headspace-solid phase extraction (HS-SPME) and determined by gas chromatograph Agilent Technologies 7890 (Agilent Technologies, Santa Clara, USA) with mass detector Agilent Technologies 5975C (Agilent Technologies, Sant Clara, USA) (GC/MS). GC/MS with HP-5MS column (max. temperature 325°C, 0.25 mm × 30 m, thickness of stationary phase 0.25 µm; J&W Scientific, St. Louis, USA) was used.

HS-SPME procedure: SPME manual holder (Supelco, Bellefonte, USA) with 50/30 µm DVB/CAR/PDMS 1 cm fiber (Supelco, Bellefonte, USA). The fiber was conditioned for 60 min at 270°C as recommended by the manufacturer. 5.6 g of FB were capped with PTFE/Silicone septa in 15 ml vial (Supelco, Bellefonte, USA). The fiber was exposed to the sample headspace for 20 min at 20°C.

HS-SPME/GC/MS: The fiber was desorbed for 7 min in the splitless injection port at 240°C. The column temperature program was 40°C for 7 min and was followed by anincreased to 200°C at 5 K per minute. The flow rate of helium carrier gas was 0.9 ml/minute. The temperatures of the ion source and quadrupole were 230 and 150°C, respectively. The mass spectrometer operated in the electron impact (EI) ionisation mode at 70 eV and mass spectral data were acquired in the range 25–400 amu. The scan and selected ion monitoring (SIM) were used as the data acquisition mode, the chosen ions were 44 and 56 for hexanal and 44 and 58 for pentanal. The identification of the compounds was carried out by comparing their spectra with those of the NIST Mass Spectra library.

Calibration curves were prepared for two major volatile products – pentanal (valeraldehyde, purity ≥ 97%) and hexanal (purity ≥ 98%; both Fluka, Taufkirchen, Germany). These aldehydes were analysed in oil without volatile compounds. The calibration data were obtained under the same analysis conditions as were those for the samples and they are listed in Table 2.

Calculation of the area under the curve (AUC). The area under the curve for the individual MAGs was calculated from: the dependence of the CD content (expressed in % w/w) on storage time (expressed in weeks) – AUC_{CD}, the dependence of the oxidative stability (expressed in h) on storage time ($AUC_{Rancimat}$, $AUC_{Oxidograph}$), the dependence of the pentanal/hexanal concentration (expressed in µmol/kg) on storage time ($AUC_{pentanal}$, $AUC_{hexanal}$) by trapezoid method. The AUC correlated with the effects of MAGs on the formation of CD, pentanal, hexanal, and oxidation stability during storage.

Statistical analysis. The results were expressed as mean ± standard deviation (the corresponding error bars were displayed in the graphical plots). Statistical data analysis was performed by using Student's *t*-test at a confidence level of 95% ($P = 0.05$).

Table 2. The calibration data of standard volatile compounds by HS-SPME/GC/MS

Parameter	Hexanal	Pentanal
Average of retention time t_R (min)	9.123	5.169
Calibration curve	$y = 205.1x$	$y = 2838.8x$
Correlation coefficient (R^2)	0.9760	0.9626
Range of concentration of standard solution (µmol/kg)	1.14–1022.74	1.16–51.32

RESULTS AND DISCUSSION

The influence was studied of monoacylglycerol emulsifier type on the extent of oxidation and oxidative stability in model fat blends. Oxidative changes of model fat blends were investigated and the selected oxidation products were determined over 16-week period.

Primary oxidation products

Primary oxidation products are hydroperoxides. The polyunsaturated fatty acids have pentadiene system of double bonds. During oxidation, the conjugated system of double bonds is formed due to the rearrangement of double bonds (energy of he system is lowered). Thus, conjugated diens content correlates with the peroxide value (Shahidi 1998; Spěváčková *et al.* 2009).

The conjugated diens content increases with time (Figure 1). The areas under the curves of the dependence of CD content on time are presented in Table 3. Fat blends with MAG18, MAG18:1 and fat blend without emulsifier had the highest contents of conjugated diens and were statistically significantly different from the other fat blends.

Oxidative stability

Oxidative stability is an important factor for the prediction of fat/oil quality. It is represented as the resistance to oxidation under defined conditions and is expressed as the induction period (IP). The induction period was determined by two different methods: in the first one, Oil Stability Index method was measured by Rancimat, and in the second one, the decrease of oxygen pressure in the headspace was monitored by Oxidograph (Velasco *et al.* 2004; Nogala-Kalucka *et al.* 2005).

Table 3. The area under the curve of dependence of CD content on time (AUC_{CD})

	AUC_{CD}		AUC_{CD}
MAG10	6.812 ± 0.037	MAG16	6.892 ± 0.079
MAG12	6.268 ± 0.074	MAG18	8.449 ± 0.057
MAG14	6.524 ± 0.005	MAG18:1	9.634 ± 0.236
WE	9.458 ± 0.238		

Oxidative stability of FBs decreased during storage (Figure 2). The results obtained from Rancimat and Oxidograph are related, the IP determined by Rancimat instrument is higher than IP determined by Oxidograph instrument. The minimum oxidative stability was determined in fat blends with MAG18, MAG18:1 and in the fat blend without emulsifier by both methods (Table 4). These three FBs were statistically significantly different from the other FBs. Oxidative stability of FBs with MAG10, MAG12, MAG14, and MAG16 was generally higher. The differences in oxidative stability were not significant in this group.

Volatile secondary oxidation products

Volatile oxidation products were detected and the major products were quantified by HS-SPME/

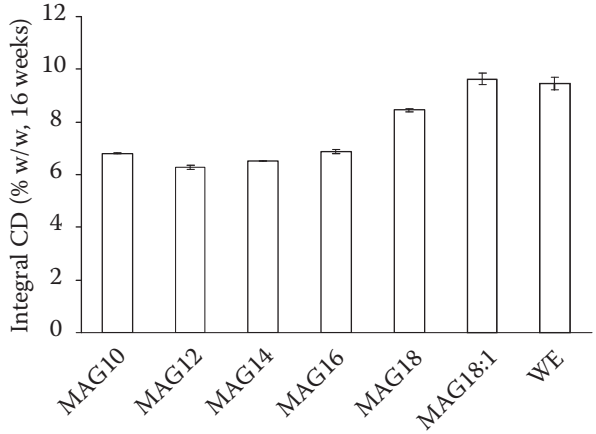

Figure 1. Conjugated diens content of FBs with MAGs during storage time

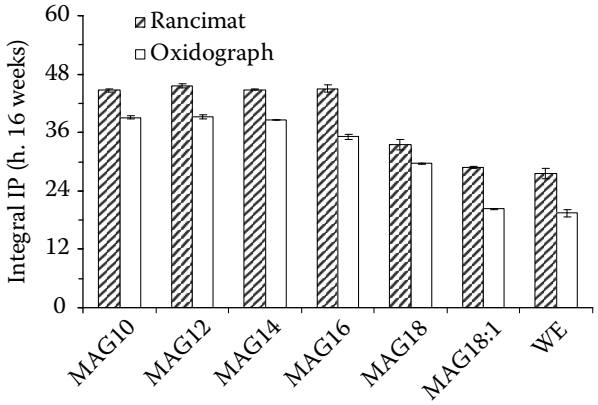

Figure 2. Oxidative stability of FBs with MAGs determined by Rancimat (A) and Oxidograph instrument (B) during storage time

Table 4. The area under the curve of dependence of $IP_{Rancimat}$ and $IP_{Oxidograph}$ on time ($AUC_{Rancimat}$, $AUC_{Oxidograph}$)

	$AUC_{Rancimat}$	$AUC_{Oxidograph}$
MAG10	44.71 ± 0.33	39.10 ± 0.26
MAG12	45.59 ± 0.46	39.20 ± 0.36
MAG14	44.83 ± 0.06	38.60 ± 0.05
MAG16	45.02 ± 0.77	35.10 ± 0.62
MAG18	33.49 ± 1.11	29.60 ± 0.09
MAG18:1	28.85 ± 0.16	20.30 ± 0.13
WE	27.57 ± 0.98	19.40 ± 0.79

Table 5. The area under the curve of dependence of pentanal and hexanal concentration on time ($AUC_{pentanal}$, $AUC_{hexanal}$)

SPME	$AUC_{pentanal}$	$AUC_{hexanal}$
MAG10	182.73 ± 10.96	2233.96 ± 63.81
MAG12	121.51 ± 7.29	888.05 ± 26.64
MAG14	166.86 ± 8.34	1389.08 ± 64.75
MAG16	182.88 ± 14.63	1226.56 ± 79.81
MAG18	213.98 ± 12.84	1781.49 ± 83.47
MAG18:1	129.61 ± 6.48	1784.03 ± 103.27
WE	186.66 ± 13.07	3919.39 ± 75.39

GC/MS. The major volatile compounds, as we had expected, were hexanal and pentanal because the liquid oil in the fat blends was soybean oil with a high content of linoleic acid (Table 1). Hexanal is formed from (9*Z*, 11*E*)-13-hydroperoxyoctadeca-9, 11-dienoic acid and pentanal is formed from (9*Z*, 12*Z*)-14-hydroperoxyoctadeca-9, 12-dienoic acid. The formation of pentanal from ω-6 polyunsaturated fatty acids may be also explained by the hexanal decomposition (Frankel 1993). Pentanal and hexanal were quantified using the calibration curve. These two compounds were detected at the beginning of storage. The content of hexanal stighly increased during storage (Figure 3). The areas under the curves of the dependence of pentanal and hexanal concentration on time in fat blends are shown in Table 5. The lowest concentration of both aldehydes was determined in fat blends with MAG12, MAG14, and MAG16. FB with MAG10 gave rise to a higher content of hexanal. On the other hand, the highest concentration of aldehydes (especially hexanal) was detected in the fat blend without emulsifier (blank – WE). This fat blend was statistically significantly different from the other fat blends (Tables 5 and 6).

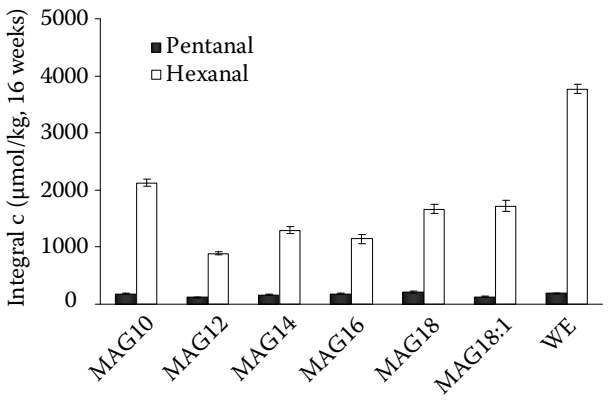

Figure 3. Hexanal concentration of FBs with MAGs determined by HS-SPME/GC/MS during storage time

CONCLUSION

The aim of this study was to find out the influence of MAG addition on the oxidation changes of model fat blends. A homological group was used of MAGs with saturated fatty acids (from MAG10 to

Table 6. Volatile secondary oxidation products of model fat blends by HS-SPME/GC/MS

FB with emulsifier	Volatile compounds				
MAG10	pentanal	hexanal	(*Z*)-2-heptenal	3,5-octadien-2-one	
MAG12	pentanal	hexanal	3,5-octadien-2-one		
MAG14	pentanal	hexanal	(*Z*)-2-heptenal	3,5-octadien-2-one	
MAG16	pentanal	hexanal	3,5-octadien-2-one		
MAG18	pentanal	hexanal	(*Z*)-2-heptenal	2,4-heptadienal	3,5-octadien-2-one
MAG18:1	pentanal	hexanal	(*Z*)-2-heptenal	2,4-heptadienal	3,5-octadien-2-one
WE	pentanal	hexanal	(*Z*)-2-heptenal	2,4-heptadienal	2,4-octadiene
	2-octenal	3,5-octadien-2-one	2,4-undecadien-1-ol	2-nonenal	(*E*,*E*)-2,4-decadienal

MAG18) and MAG18:1 (as a member of a homological group of MAG with unsaturated fatty acids).

From the point of view of primary oxidation products, volatile aldehydes (representing secondary oxidation products) and oxidative stability it is possible to split up the obtained results in three groups. The first one, FB without MAG (blank – WE) oxidises to the greatest extent in all monitored parameters. The second group, FBs with 1-octadecenoylglycerol (MAG18:1), 1-octadecanoylglycerol (MAG18), is a typical case of longer acyl chain length of MAG than the acyl chain length of the structured fat used. Primary oxidation products are formed to the greatest extent and their decomposition to secondary oxidation products is minimal in this group of FBs. Oxidative stability of this group of FBs is insufficient. The third group, FBs with MAG10, MAG12, MAG14, and MAG16 is a typical case of a shorter or the same acyl chain length of MAG as compared to the acyl chain length of the structured fat used. FBs of this group oxidise to the minimal extent in all monitored parameters.

There is a hypothesis that monoacylglycerols as surface active agents adsorb on the interfaces fat blend/air and oil/crystals of structured fat. MAGs with a shorter (MAG10–MAG14) or the same acyl chain length (MAG16) arrange on the interface oil/crystals of structured fat tightly and therefore the oxygen diffusion through the fat blend layer is retarded. MAGs with 18 carbon atoms in the acyl chain or with 18 carbon atoms and *cis* double bond in the acyl chain arrange on the interface oil/crystals of structured fat not so tightly and therefore the oxygen diffusion through the fat blend layer is accelerated.

In a previous paper (Spěváčková *et al.* 2009), where fat blends with different structured fats were studied, similar results were found out.

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Received for publication November 15, 2011
Accepted after corrections February 21, 2012

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