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Preparation of Conjugated Linoleic Acid Enriched Derivatives by Conventional and Biphasic Isomerisation

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Abstract

Kyselka J., Thomes L., Remišová S., Dragoun M., Berčíková M., Filip V. (2016): **Preparation of conjugated linoleic acid enriched derivatives by conventional and biphasic isomerisation**. Czech J. Food Sci., 34: 511–521.

The preparation of conjugated linoleic acid (CLA)-enriched free fatty acids by industrial processes compared with our biphasic isomerisation experiments in a special designed reactor enabling the preparation of CLA esters was evaluated. Our experiments further revealed the main disadvantage of semi-synthetic alkali isomerisation to be the formation of conjugated *E,E*-octadecadienoic acid isomers (2.92–3.44%) and the bioavailability of free fatty acid products. Urea fractionation technology improved the quality of the reaction mixture, but at the same time the yield of rumenic acid was decreased on purification. Therefore, we decided to apply complexes of noble metals in order to isomerise linoleic acid ester derivatives. The known Wilkinson's hydrogenation catalyst, $RhCl(PPh_3)_3$, was found to be the most effective. We investigated the preparation of bioavailable CLA-enriched triacylglycerols. Special attention was paid to recycling of Wilkinson's catalyst.

Keywords: rumenic acid; mass spectrometry; Wilkinson's catalyst**;** ionic liquids; homogeneous catalysis

The growth of ruminal bacteria is inhibited by dietary intake of polyunsaturated fatty acids (PUFAs). Linoleic acid was demonstrated to affect bacterial cell division (Jenkins *et al*. 2008; Liavonchanka & Feussner 2008). Therefore, ruminal microflora had to develop a protective metabolic pathway, the biohydrogenation of double bonds in PUFAs. The anaerobic bacterium *Butyrivibrio fibrosolvens* is the most tolerant strain to growth inhibition caused by linoleic acid, because it has the highest activity of linoleic acid Δ^{12}-*cis*,Δ^{11}-*trans* isomerase (EC 5.2.1.5.), which carries out bioconversion of linoleic acid into (9*Z*,11*E*)-octadeca-9,11-dienoic acid (rumenic acid). Thus, the isomerase system has a physiological function (Kepler *et al*. 1971; Hunter *et al*. 1976; Devillard *et al*. 2006; Liavonchanka & Feussner 2008). It is the first step of the linoleic acid biotransformation cascade resulting in the formation of stearic acid. Key intermediates are rumenic and vaccenic acid (Kepler *et al*. 1971; Hunter *et al*. 1976; Devillard *et al*. 2006).

The functional importance of the biohydrogenation pathway is the regulation of PUFA dietary intake in order to depress the growth inhibition of mixed rumen microorganisms. Enzymatic activity of linoleic acid Δ^{12}-*cis*,Δ^{11}-*trans* isomerase is interesting when positional and geometric isomerisation of the pentadienyl system occurs without any activation by functional groups (Kepler *et al*. 1971; Hunter *et al*. 1976; Devillard *et al*. 2006; Jenkins *et al*. 2008; Liavonchanka & Feussner 2008). Analogical PUFA isomerases were described to be present in

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other bacteria (*Lactobacillus acidophilus*, *Propionibacterium acnes*, *Bifidobacterium* species) and red algae (*Ptilota filicina*). Despite the fact that the linoleic acid Δ^{12}-*cis*,Δ^{11}-*trans* isomerase and all other PUFA isomerases have slightly different organisation of the active centres, the biotransformation mechanism is believed to be similar (Liavonchanka *et al*. 2006). Active sites of isomerases consist of three domains forming the hydrophobic channel inside the enzyme, from which one has non-covalently bound flavin adenine dinucleotide (FAD). Enzymatic hydrogen abstraction from the C-11 *bis*-allylic position ($pK_a = 35$) and subsequent double bond allylic shift occur only with free PUFAs (Liavonchanka *et al*. 2006; Liavonchanka & Feussner 2008; Okuyama & Maskill 2014). First of all, triacylglycerol ester bonds have to be cleaved in order to release fatty acid substrates. They can associate with isomerase hydrophobic pocket. In other words, substrate specificity requirements involve the presence of free carboxyl group and *cis*-9,*cis*-12-diene system. A protic mechanism was suggested for the hydrogen abstraction and regiospecific addition, when the deuterium was incorporated into the substrate during the experiments performed at deuterium oxide (Kepler *et al*. 1971). However, the concerted reaction mechanism and radical isomerisation in the absence of oxygen were not completely excluded. PUFA isomerase reverts to the initial state after the product [(9*Z*,11*E*)-octadeca-9,11-dienoic acid or (10*E*,12*Z*)-octadeca-10,12-dienoic acid] dissociates the active site (Kepler *et al*. 1971; Liavonchanka *et al*. 2006; Liavonchanka & Feussner 2008).

An important dietary source of conjugated linoleic acid (0.29–1.13wt%) is the fat from ruminants occurring in milk, meat, and the products made of them (Velíšek 2002; Jenkins *et al*. 2008). It is generally accepted that the consumption of dairy fat in Europe can reach 30 g per day (Akoh & Lai 2005). Although conjugated linoleic acid (CLA) isomers are believed to have health benefits (anticarcinogenic action, adipogenesis inhibition), milk fat is a rich source of saturated and *trans*-fatty acids considered to be health risk factors, i.e. increased risk of cardiovascular diseases (Velíšek 2002; Akoh & Lai 2005; Henry 2007; Leray 2015). It has stimulated a deep interest in CLA-enriched human diet. Therefore, the first CLA dietary supplements were marketed in 1995 in the United States (Henry 2007). They are produced semi-synthetically by alkali isomerisation of sunflower or safflower seed oil. It is interesting to note that current dietary supplements and preventive nutrition products are ill-defined mixtures containing almost equal proportions of free rumenic acid and (10*E*,12*Z*)-octadeca-10,12-dienoic acid, with the presence of several minor isomers and other fatty acids (Akoh & Lai 2005; Henry 2007; Leray 2015).

Triacylglycerols are the most important lipids in human diet with very high efficiency of their digestion and absorption (Henry 2007). Several conjugated linoleic acid isomers can be present in triacylglycerol species whereas only rumenic acid is incorporated into phosphoglycerolipids (Ha *et al*. 1987; Henry 2007).

The aim of this study was the preparation of more easily bioavailable conjugated linoleic acid acylglycerols and its comparison with conventional semi-synthetic methods. Our conclusions may help to design a procedure for the preparation of acylglycerol-enriched CLA dietary supplements.

MATERIAL AND METHODS

Linoleic acid, isopropylidene glycerol, Wilkinson's catalyst, trilinolein, and ionic liquids were purchased from Sigma Aldrich Company (Steinheim, Germany). All other reagents and solvents were of analytical grade. Standard refined sunflower seed oil (content of free fatty acids 0.06wt%, peroxide value 2.67 ± 0.05 mmol ½ O_2/kg, phosphorus content 2.0 mg/kg), standard castor bean oil (content of free fatty acids 8.10wt%, iodine value 84 g I_2/100 g, content of ricinoleic acid 89.9%), which were purchased from the local producer Glencore Grain Czech s.r.o. (Ústí n/L., Czech Republic), were the input feedstock of the isomerisation experiments.

Commercial CLA supplements (MedPharma, NordPharma) were purchased from a local drugstore.

Preparation of CLA as free fatty acids by alkali isomerisation of sunflower seed oil and its urea fractionation. Alkali isomerisation of sunflower seed oil is the most common semi-synthetic method used to prepare CLA dietary supplements. Conjugation of linoleic acid double bonds was performed as previously described according to IUPAC method 2.206 1987 (Berdeaux *et al*. 1998) to simulate commercial production. In brief, 150 g of potassium hydroxide-ethylene glycol solution (20.5–20.6% w/w) was placed in the glass isomerisation tube (glass tube equipped with a capillary reaching to the bottom of the vessel, height 280 mm, inner diameter 65 mm). The current of argon (purity 99.998%, oxygen content < 3 ppm) passed

doi: 10.17221/362/2016-CJFS

through the reagent solution by means of the capillary to remove air and to agitate the alkaline solution gently at 150°C. After addition of 56 g of sunflower seed oil into isomerisation tubes, temperature was increased to 180°C. The resulting mixture was heated overnight at 180°C. CLA isomers were partitioned between *n*-hexane and acidified water phase. *n*-Hexane extracts were washed with water to neutral reaction and dried over anhydrous sodium sulphate. Fatty acid methyl esters (FAME) were obtained by Fischer esterification. An aliquot of alkali isomerised fatty acids (100 mg) was dissolved in hexane (4 ml), and a urea (1.5 g) mixture with 30 µl methanol was added. Adducted *trans*,*trans*-octadecadienoic acids were filtered off after standing overnight (Christie 1989).

Preparation of rumenic acid by castor bean oil dehydration. The semi-synthetic preparation of (9*Z*,11*E*)-octadeca-9,11-dienoic acid (rumenic acid) was based on dehydration of castor bean oil. The first step involved the conversion of secondary alcohol groups to methanesulphonates. Methanesulphonyl chloride (0.23 g, 2.01 mmol) was used to obtain mesylates from castor bean oil (5.00 g, 5.36 mmol) in the presence of triethylamine (1.45 g, 14.33 mmol) on an ice water bath (Yang *et al*. 2002; Villeneuve *et al*. 2005). Methanesulphonated castor bean oil was extracted with *n*-hexane from a hydrochloric acid solution. Extracts were washed with water to neutral reaction and dried over anhydrous sodium sulphate. Alkali-catalysed dehydration of ricinoleate methanesulphonates was performed in an ethylene glycol alkaline solution (15% w/w). The current of argon agitated the solution at 100°C in the same tube as described above. Purification of reaction mixture and preparation of fatty acid methyl esters were done in the same manner as in the case of alkali isomerisation (IUPAC method 2.206 1987; Berdeaux *et al*. 1998).

Preparation of CLA as fatty acids and FAMEs by biphasic isomerisation experiments with Wilkinson's catalyst in ethanol/ionic liquid system. Biphasic isomerisation experiments were performed in a reactor (glass tube, height 220 mm, inner diameter 18 mm) fitted with sintered glass (S4) in the lower part and gas exhaust in the upper part, placed in a thermostat and held at 60°C. The temperature was monitored with a digital thermometer F200 (Automatic Systems Laboratories, Croydon, UK). The stream of nitrogen which passed through the sintered glass upwards was regulated and allowed strong agitation of the reaction mixture. To 5 ml of the ionic liquid (1-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide) [BMIM]·[NTf_2] were added 63 mg of Wilkinson's catalyst (0.07 mmol, $RhCl(PPh_3)_3$) and 38 mg of $SnCl_2 \cdot 2H_2O$ (0.17 mmol). After stirring for 10 min in a glass reactor, linoleic acid or linoleic acid methyl ester (13.6 mmol) and 3 ml of absolute ethanol were added and the biphasic reaction was initiated (Larock *et al*. 2001; Consorti *et al*. 2009). At the given intervals, aliquots were taken for analysis. CLA derivatives were separated from impurities by passing them through solid-phase extraction cartridges (silica gel Supelclean™ LC-Si SPE tubes, 6 ml, 1 g). Chromatographed samples were used for ICP-MS (ICP/MS XSERIES II) analysis to determine the Rh content in central laboratories of the University of Chemistry and Technology in Prague. First-order rate constants of the time dependent isomerisation of linoleic acid derivatives were calculated.

Mass spectrometry of Diels-Alder cycloadducts of conjugated linoleic acid methyl esters with 4-phenyl-1,2,4-triazoline-3,5-dione, maleic anhydride, and 1,4-benzoquinone. The determination of double bond positions in alkali-isomerised conjugated linoleic acid methyl esters required specific chemical modification of the conjugated diene system. Three powerful dienophiles (4-phenyl-1,2,4-triazoline-3,5-dione, maleic anhydride, and 1,4-benzoquinone) were selected for derivatisation prior to analysis by mass spectrometry. Derivatisations were performed in the following manner. In brief, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 3 mg, 17.0 µmol) was mixed with CLA methyl esters (1 mg, 3.4 µmol) in 3 ml of dichloromethane for 10 s at room temperature (Young & Vouros 1987; Dobson 1998). The maleic acid anhydride and 1,4-benzoquinone adducts were prepared by solvent-free thermal cycloaddition (150°C, 1 h under nitrogen) of CLA methyl esters (3 mg, 10.2 µmol) with 10.2 µmol of maleic acid anhydride (Biermann *et al*. 2007) or 1,4-benzoquinone. Diels-Alder cycloadducts of CLA methyl esters with several dienophiles were separated from impurities by passing them through solid-phase extraction cartridges (silica gel Supelclean™ LC-Si SPE tubes, 6 ml, 1 g). Analyses of Diels-Alder adducts were performed on a Gas Chromatograph 7890A GC System connected with a mass spectrometer (5975 C VLMSD; both Agilent Technologies, Santa Clara, USA). Compounds were separated on a 30 m HP-5MS column (Agilent Technologies, USA) using a temperature gradient of 80°C increased to 320°C at 15°C/min, held for 20 minutes.

Determination of positional and geometrical isomers of unsaturated fatty acids by capillary

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gas-liquid chromatography (CGLC). Positional and geometrical unsaturated fatty acid isomers were determined according to AOCS Official Methods Ce 1f-96. Methyl heptadecanoate was used as an internal standard. The analysis was performed on Agilent 6890N Gas Chromatograph (Agilent Technologies, USA) and SP™ 2560 capillary column (Supelco, Bellefonte) 0.25 mm × 100 m, film thickness 0.2 μm was used. The conditions of the 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/minute. Determinations were performed minimally in triplicate.

Preparation of CLA-enriched triacylglycerols from 1-monolinolein by biphasic isomerisation experiments with Wilkinson's catalyst in ethanol/ionic liquid system. To a stirred solution of linoleic acid (50 mmol), 4-dimethylaminopyridine (DMAP, 6.25 mmol), and isopropylidene glycerol (57 mmol) in anhydrous CH_2Cl_2 (200 ml) on an ice water bath was added dropwise a solution of N,N'-dicyclohexylcarbodiimide (DCC, 62.5 mmol) in 30 ml of CH_2Cl_2. The reaction mixture was allowed to warm up to laboratory temperature during 16 h of stirring under the stream of argon. Precipitated dicyclohexylurea was filtered off with the Witt filtration apparatus. The CH_2Cl_2 solution, washed with hydrochloric acid (0.5 mol/dm^3) and water, was dried over anhydrous sodium sulphate (Neises & Steglich 1978; Köhler & Grosch 1999). Linoleoyl isopropylidene glycerol was purified by column chromatography on silica gel. 1-Monolinolein was deprotected by refluxing in the mixture of water (300 ml) and hydrochloric acid (32 ml, 0.5 mol/dm^3) for 10 minutes. Monoacylglycerol was extracted with diethyl ether/tetrahydrofuran (200 ml, 40 : 10, v/v) from saturated $NaHCO_3$ solution and saturated NaCl solution. The extract was dried over anhydrous sodium sulphate and 1-monolinolein was purified by column chromatography on silica gel (Neises & Steglich 1978; Köhler & Grosch 1999). Biphasic isomerisation experiments were performed in a reactor provided with sintered glass (S4) in the lower part and gas exhaust in the upper part, placed in a thermostat and held at 60°C. The stream of nitrogen which passed through the sintered glass upwards was regulated and allowed strong agitation of the reaction mixture. To 5 ml of the ionic liquid (1-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide) were added 63 mg of Wilkinson's catalyst (0.07 mmol, $RhCl(PPh_3)_3$) and 38 mg $SnCl_2 \cdot 2H_2O$ (0.17 mmol). After stirring for 10 min in a glass reactor, 1-monolinolein (13.6 mmol) and 3 ml of absolute ethanol were added and the biphasic reaction was initiated (Larock *et al*. 2001; Consorti *et al*. 2009). At the given intervals, aliquots were taken for analysis. Pure triacylglycerol was synthetised according to the known procedure by using palmitoyl chloride in the presence of pyridine (Bentley & Mccrae 1970). CLA-TAG purity was determined by the HT-GC method. Commercial trilinolein was isomerised in the same manner as described above.

RESULTS AND DISCUSSION

Preparation of CLA as free fatty acid products by alkali isomerisation of sunflower seed oil and castor bean oil dehydration. Preparations of CLA in the form of free fatty acids were performed in our laboratory either by alkali isomerisation of sunflower seed oil or by dehydration of methanesulphonated castor bean oil in order to simulate commercial batch production and to compare it with biphasic isomerisation experiments in the next section of this article.

Laboratory alkali isomerisation resulted in an equimolar mixture of rumenic acid (26.84%) and (10*E*,12*Z*)-octadeca-10,12-dienoic acid (28.18%) together with minor geometrical and positional isomers summarised in Figure 1. The reaction yield was 49.9%. It has been shown that laboratory made CLA and marketed preventive nutrition products have almost similar composition (Figure 1). The main disadvantage of semi-synthetic alkali isomerisation was the formation of undesired conjugated *E*,*E* isomers (2.92–3.44%; Figure 1). It was surprising to note that neither did commercial producers use any other purification methods such as urea fractionation. Therefore, the urea fractionation technology of fatty acid mixture was applied to increase the content of CLA in mother liquor and to decrease *E*,*E*-octadecadienoic acids by clathration (Christie 1989). The isomerised fractions of rumenic acid and (10*E*,12*Z*)-octadeca-10,12-dienoic acid were increased to 77.05%, while the content of total *trans*-fatty acids was decreased to 2.07%. However, rumenic acid was found to be better fixed in clathrates due to an observable yield loss on purification (Figure 1). Elimination of methanesulphonated castor bean oil

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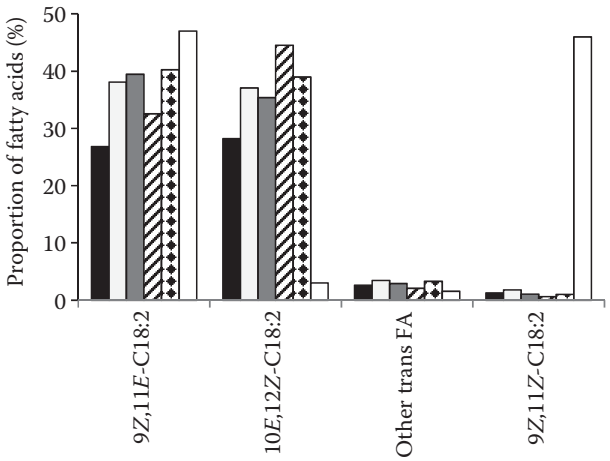

Figure 1. The proportion of isomerised fatty acids after alkali-isomerisation, alkali-elimination, biphasic isomerisation experiments, and the content of conjugated linoleic acid (CLA) in commercial supplements

was another route to the commercial nutrients (Yang *et al*. 2002; Villeneuve *et al*. 2005). The yield of the two-step process was not more than 55% due to the polymerisation side reactions of ricinoleate moiety (Yang *et al*. 2002; Villeneuve *et al*. 2005). However, dehydration of castor bean oil was unique transformation resulting in an equimolar mixture of rumenic acid (46.97%) and (9*Z*,11*Z*)-octadeca-9,11-dienoic acid isomer (45.99%; Figure 1).

Preparation of CLA as fatty acid derivatives by isomerisation experiments with Wilkinson's catalyst in ethanol/ionic liquid system. Homogeneous isomerisation of olefins is a fundamental catalytic transformation. Activation of double bonds is realised through the complexation of unsaturated hydrocarbons by transition metals. There are three possible mechanisms involved: (a) migration of the double bond by β-elimination; (b) allylic C-H activation (e.g. $Fe(CO)_5$, chromium carbonyls, arene-$Cr(CO)_3$); (c) isomerisation via metallocarbenes during metathesis (Grubbs catalysts) (Frankel 1970; Singer *et al*. 1977; Larock *et al*. 2001; Astruc 2007; Con-

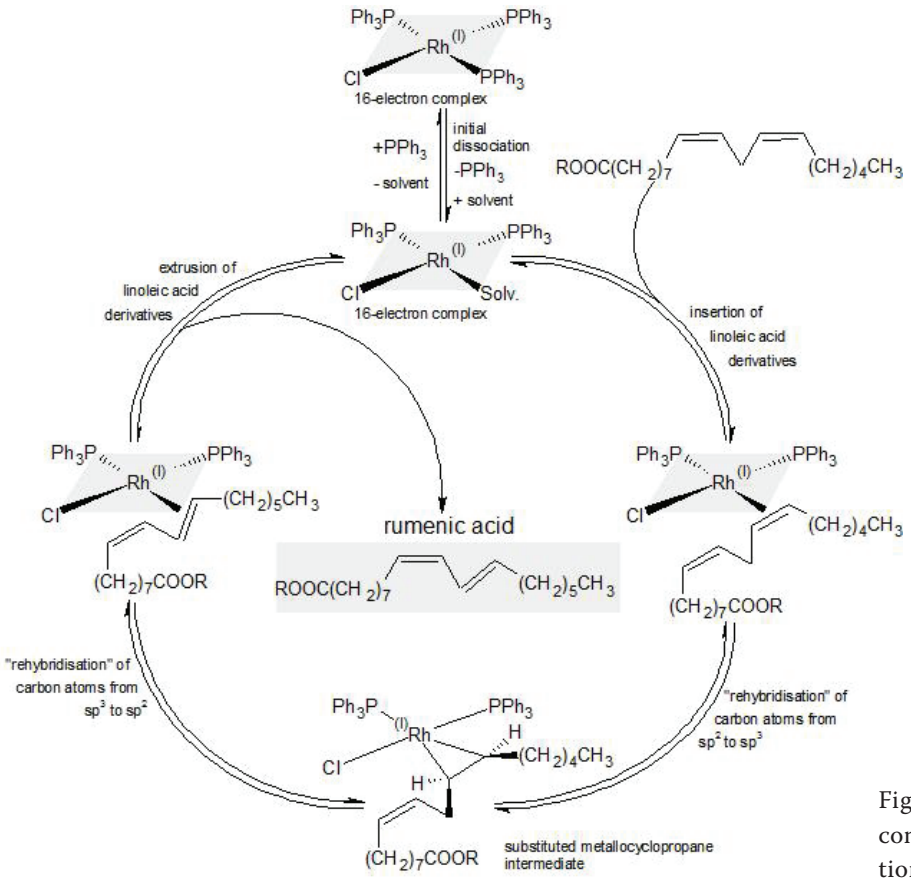

Figure 2 .The mechanism of the conjugated linoleic acid isomerisation by Wilkinson's catalyst

doi: 10.17221/362/2016-CJFS

sorti *et al*. 2009; Housecroft & Sharpe 2014). Mechanisms (b) and (c) were out of the question for our purposes due to the application of toxic metal carbonyls, undesired side reactions (hydrogenation, dehydrogenation), prolonged reaction times, and high amounts of the spent catalyst (Frankel 1970; Larock *et al*. 2001; Consorti *et al*. 2009). Therefore, we have decided to apply complexes of noble metals to isomerise linoleic acid derivatives. A known hydrogenation catalyst, $RhCl(PPh_3)_3$ (Wilkinson's catalyst), was found to be the most effective for our isomerisation experiments in various systems.

Wilkinson's catalyst has a square planar structure and the number of valence electrons is 16 with the oxidation state of rhodium +I (Figure 2) (Astruc 2007; Housecroft & Sharpe 2014). The reaction sequence was initiated by the dissociation of phosphine L ligand, which is assumed to be fast. The type of chemical bonding between an unsaturated fatty acid derivative and rhodium in $RhCl(PPh_3)_3$ was described by the Dewar-Chatt-Duncanson model, which explained the σ-donation of fatty acid double bonds to rhodium and the noble metal donated the electrons back by so called π-backbonding (Astruc 2007; Housecroft & Sharpe 2014). The key was the balance between the rhodium double bond of the linoleic acid complex and the metallocyclopropane transition structure according to the suggested reaction scheme (Figure 2) (Astruc 2007; Housecroft & Sharpe 2014). The hydridisation of alkene carbons changed from sp^2 to sp^3 with increasing π-backbonding, which caused the double bond shift (Figure 2). The formation of *trans*-unsaturated geometrical and positional isomer was preferred when the isomerisation was under thermodynamic control (ΔH^0_{hydrog} = –4.1 kJ/mol) (Astruc 2007; Housecroft & Sharpe 2014).

Our improvement proposals (Figure 3) were based on batch isomerisation performed in the glass reactor equipped with porous frit (S4, pore diameter 5–15 μm) and inert gas exhaust in the upper part. The isomerisation apparatus, operating under mild conditions (60°C), did not need any mechanical agitation, because biphasic or simple monophasic homogeneous systems were vigorously mixed by the stream of nitrogen bubbles (20–100 dm^3/h according to the flow control). CLA products in the form of free fatty acids or corresponding methyl esters were separated by simple decantation of the ethanol phase at the end of catalytic process.

A reaction drop of the linoleic acid concentration followed a first-order decrease whereas the mono-*trans*

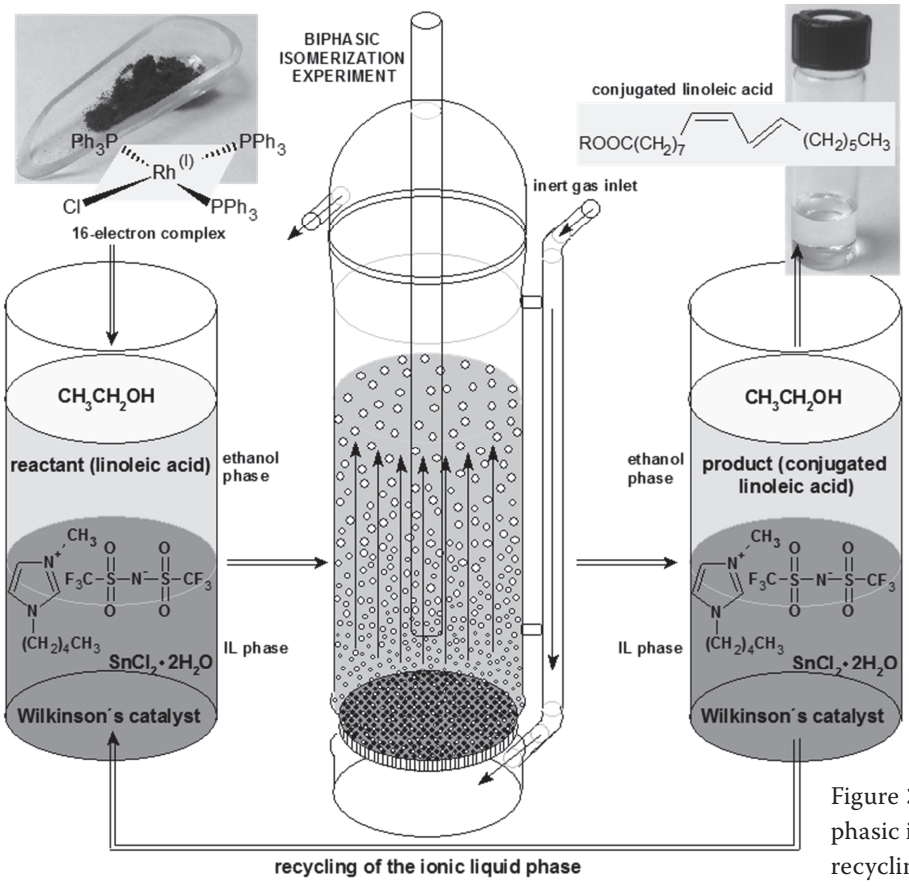

Figure 3. The scheme of laboratory biphasic isomerisation experiments with recycling of Wilkinson's catalyst

doi: 10.17221/362/2016-CJFS

isomers started with a corresponding increase at the same time. Consecutive geometrical isomerisation of rumenic and (10*E*,12*Z*)-octadeca-10,12-dienoic acid to (*E*,*E*)-octadecadienoic acids occurred after 30 min of biphasic isomerisation. Almost quantitative conversion of linoleic acid methyl esters (96–97%, first-order rate constant 2.3×10^{-3}; Figure 4A) was observed in the presence of biphasic systems, 0.51mol % of $RhCl(PPh_3)_3$, and 1.25mol% of $SnCl_2 \cdot 2H_2O$. The presence of Lewis acid $SnCl_2 \cdot 2H_2O$ was essential because it improved the reaction rate and the conversion as a cocatalyst. On the other hand, the reaction proceeded more slowly with significantly lower conversion (35–40%, first-order rate constant 1.0×10^{-5}; Figure 4B) in the absence of ionic liquid (1-butyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide). Undesired transesterification reactions in the excess Lewis acid catalyst (Consorti *et al*. 2009) and increasing proportion of conjugated *E*,*E*-octadecadienoic acid isomers occurred with prolonged conversion period (Figure 4). Therefore, the isomerisation time of methyl linoleate should not exceed 20–30 min in a designed vessel.

Mass spectrometry of Diels-Alder cycloadducts of conjugated linoleic acid methyl esters. The determination of double bond positions of CLA isomers required a specific chemical modification of the octadecadiene system. Conjugated linoleic acid derivatives undergo the Diels-Alder reaction with normal electron demand. Three powerful dienophiles were selected for [4+2] cycloaddition between the highest occupied molecular orbital (HOMO) of the CLA methyl esters and the lowest unoccupied molecular orbital (LUMO) of electron-poor dienophiles (4-phenyl-1,2,4-triazoline-3,5-dione, maleic anhydride, and 1,4-benzoquinone) (Young & Vouros 1987; Dobson 1998; Biermann *et al*. 2007).

4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD), which is one of the strongest known enophiles and dienophiles, was even more reactive than maleic anhydride or benzoquinone. (9*E*,11*E*)- and (10*E*,12*E*)-octadecadienoic acid methyl esters, which could adopt the *s-cis* conformation, reacted via a concerted pathway (Figure 5, scheme A) according to the Woodward-Hoffmann rules. Other geometrical and positional CLA isomers with *s-trans* conformation were much more hindered and the reaction was not symmetry allowed (Young & Vouros 1987; Dobson 1998; Biermann *et al*. 2007). Therefore, PTAD afforded *endo* cycloadducts with (*E*,*Z*)-, (*Z*,*E*) and (*Z*,*Z*)-octadecadienoic acid derivatives through the stepwise pathway with diradical intermediate in the transition structure (Figure 5, scheme B). Unfortunately, triazolinedione derivatisations were found not to be selective when PTAD provided products of the ene-reactions (4-phenylurazoles) or [2+2] cycloaddition (1,2-diazetidines) with linoleic acid methyl ester and its isolated geometrical isomers. The simple electron-impact mass spectrum of rumenic acid *endo* cycloadduct with PTAD showed an intense molecular ion 469 *m*/*z* (Figure 6, scheme C). The most intense ions at 312 and 384 *m*/*z* indicated double bond positions of rumenic acid methyl ester after the α-cleavage fragmentation to the neighbouring nitrogen heteroatoms. Another possible mechanism was allylic α-cleavage

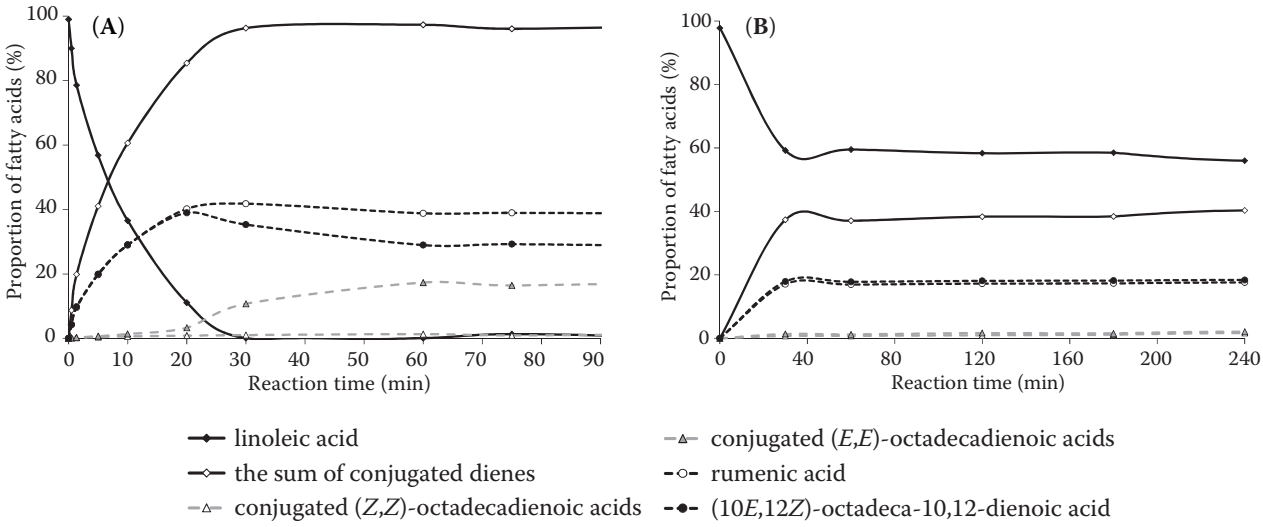

Figure 4. Homogeneous biphasic isomerisation of methyl linoleate by Wilkinson's catalyst and $SnCl_2 \cdot H_2O$ in the presence of (A) ionic liquid and anhydrous ethanol and (B) anhydrous ethanol at 60°C

doi: 10.17221/362/2016-CJFS

fragmentation of the substituted cyclohexene derivative (Young & Vouros 1987; Dobson 1998; Biermann *et al*. 2007; Pavia *et al*. 2009).

Maleic anhydride and benzoquinone were other strong electron acceptors readily forming cycloadducts with CLA isomers. The mechanism of thermal cycloaddition was the combination of concerted and stepwise pathway like in the case of PTAD (Figure 5, scheme A, B). The high temperature interconversion of *endo* Diels-Alder adduct to *exo* product resulted in the observation of two peaks eluted very close to each other (retention time 18.600 and 18.716 min) with similar EI-MS spectra (Young & Vouros 1987; Dobson 1998; Biermann *et al*. 2007). Maleic anhydride and benzoquinone were shown to be powerful dienophiles, which reacted more stereo- and regioselectively with conjugated diene systems and benzoquinone was the only reagent suitable to determine (*E,E*)-octadecadienoic acid derivatives (conversion ≤ 95%) in the presence of other geometrical isomers. Furthermore, the ene-reactions were inhibited to minimum (conversion of isolated fatty acid isomers lower than 20% by maleic anhydride). The EI-MS spectrum of the rumenic acid methyl ester *endo* cycloadduct with benzoquinone showed an intense molecular ion 402 *m/z* and fragmentation ion 371 *m/z* corresponding to the loss of alkoxy group by α-cleavage (Figure 5, scheme D). The most intense ion at 245 *m/z* indicated double bond positions of rumenic acid methyl ester after the allylic α-cleavage fragmentation at ω-6 position (Young & Vouros 1987; Dobson 1998; Biermann *et al*. 2007; Pavia *et al*. 2009).

Preparation of CLA-enriched triacylglycerols (CLA-TAG). Dietary supplements enriched with conjugated linoleic acid (NordPharma, MedPharma products) correspond to the mixture of rumenic and (10*E*,12*Z*)-octadeca-10,12-dienoic acid (74.79–75.12%) accompanied by minor conjugated (*E,E*)-octadecadienoic acid and (*Z,Z*)-octadecadienoic acid isomers. However, the most appropriate nutrition products should be available in the form of CLA-enriched triacylglycerols with high efficiency of their digestion and absorption. The aim of this study was to prepare conjugated linoleic acid enriched acylglycerols.

In the present paper, the reaction sequence was initiated by the preparation of 1-monolinolein from isopropylidene glycerol. The yield of Steglich esterification was almost 95%. However, a high loss on purification was observed after the removal of protecting group with the final yield of the two-stage

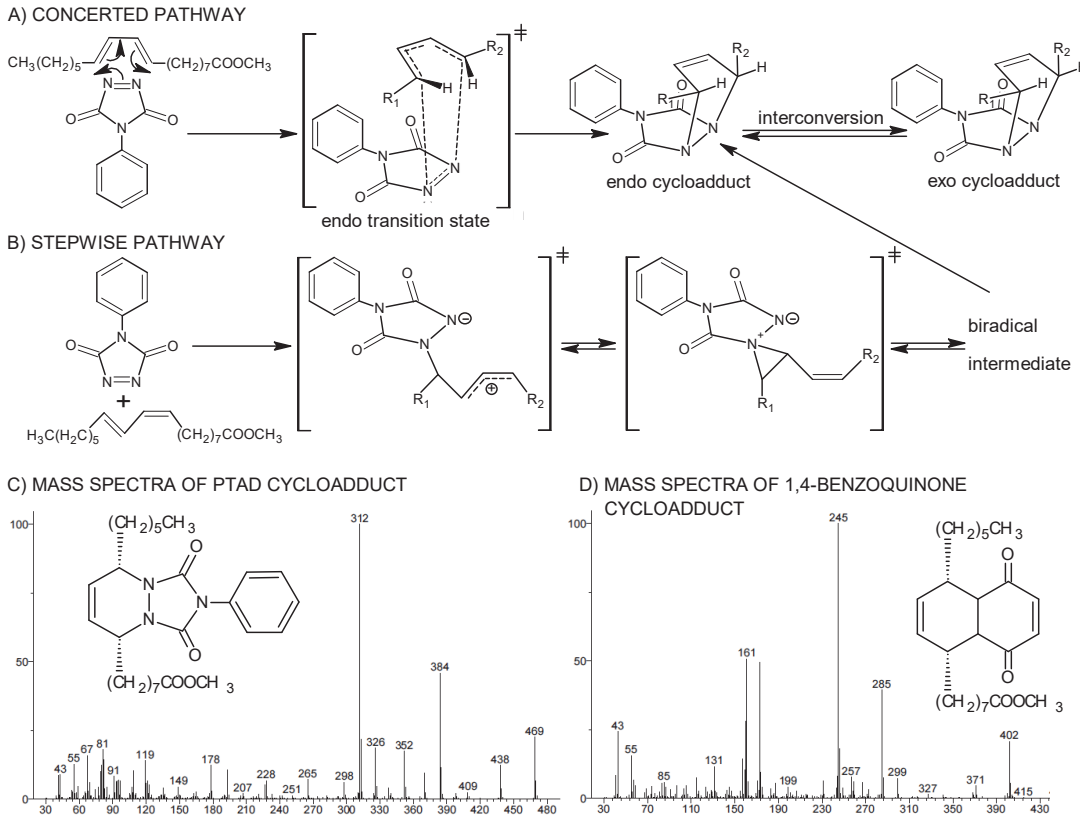

Figure 5. Electron impact mass spectrometry of Diels-Alder cycloadducts of conjugated linoleic acid (CLA) methyl esters

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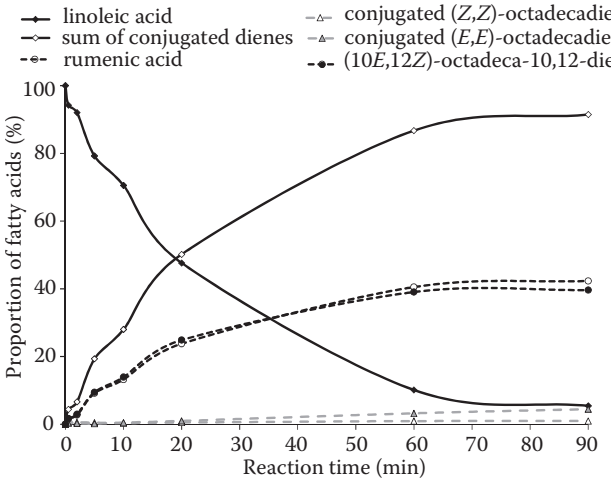

Figure 6. Homogeneous biphasic isomerisation of 1-monolinolein by Wilkinson's catalyst and $SnCl_2 \cdot H_2O$ in the presence of ionic liquid and anhydrous ethanol at 60°C

process: 50.6%. Gas chromatography analysis of silylated 1-monolinolein resulted in the observation of two peaks eluted very close to each other with similar EI-MS spectra, which showed an intense molecular ion 498 *m*/*z*. We have suggested that about 5% of 1-monolinolein was transformed into 2-monolinolein as a result of spontaneous acyl migration.

Isomerisation in the presence of a noble metal complex was the next step. High conversion of 1-monolinolein (91.45%, first-order rate constant 6.0×10^{-4}; Figure 6) to CLA-enriched monoacylglycerol (CLA-MAG) has been observed in the presence of biphasic systems, 0.51mol% of $RhCl(PPh_3)_3$, and 1.25mol% of $SnCl_2 \cdot 2H_2O$ after 90 minutes. Experimental results of commercially important processes and biphasic experiments were compared and summarised in Table 1. High reaction rates and quantitative conversions were the main advantages of biphasic isomerisation. On the other hand, the prolonged conversion period caused an increasing proportion of conjugated *E*,*E*-octadecadienoate moieties (4.47%; Figure 6). If biphasic isomerisation was terminated after 20 min, the content of all-*trans* isomers was dramatically lowered to 1% as well as the conversion of total CLA isomers.

Special attention has been paid to recycling of Wilkinson's catalyst and its effective removal from CLA products. ICP-MS analyses showed that the ionic liquid phase showed 89.7–90.7% of the initial rhodium content (906–1082 mg/kg), which made the ionic liquid applicable for the re-use of the noble metal catalyst with slightly decreased catalytic activity. Fresh monolinolein could be conjugated effectively in the next three cycles without Wilkinson's catalyst regeneration, the conversion decreased from 95% through 73% to final 48%. CLA-enriched fatty acid methyl ester or monolinolein contained

Table 1. Experimental results and comparison of simple monophasic isomerisation, biphasic experiments, and commercial important processes

Product structure	CLA-FFA		CLA MAG			CLA-FAME	
Synthetical method	alkali-isomerisation	alkali cat. dehydration	biphasic isomerisation			homogeneous catalysis	
Isomerisation catalyst	excess of KOH	excess of KOH	$RhCl(PPh_3)_3$ (Wilkinson's catalyst)			$RhCl(PPh_3)_3$	
Solvent/solvent system	ethylene glycol	ethylene glycol	[BMIM]·[NTf_2] + anhydrous EtOH (5 : 3, v/v)			ionic liquid	anhydr. EtOH
Temperature (°C)	180	100	60			60	
Reaction time (h)	12	12	2	1.5	0.33	0.5	0.5
Rate constant (s^{-1})	–	–	–	6.0×10^{-4}	2.3×10^{-3}	7.0×10^{-4}	1.0×10^{-5}
Yield* (%)	49.9	53.4	77.6	91.5	79.9	80.1	32.8
9*Z*,11*E*-C18:2 (%)	26.8	47.0	25.6	42.3	40.2	39.3	17.0
10*E*,12*Z*-C18:2 (%)	28.2	3.0	24.9	39.6	39.0	33.7	17.9
Conj. *E*,*E*-C18:2 (%)	2.6	1.6	2.4	4.5	3.3	7.1	1.3
Conj. *Z*,*Z*-C18:2 (%)	1.3	46.0	19.0	1.0	0.8	0.8	-

*Yield was given by dividing the amount of isolated products (actual yield) by theoretical yield; actual yield of finally purified product covered the conversion, losses on separation, and chromatographic purification as well as chemical equilibrium during the reaction including the reversibility of the isomerisation processes; the proportion of isomerised fatty acids after alkali isomerisation, alkali elimination, biphasic isomerisation experiments, and the content of CLA in commercial supplements

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approximately 104–111 mg/kg of rhodium, which is not acceptable for applications in the food industry. Short path distillation and the column chromatography procedures were able to clean up the products from metal contamination below detectable levels.

Finally, palmitoyl chloride was used to prepare pure CLA-enriched triacylglycerols in the form of a white crystalline solid. High temperature GC experiments resulted in the observation of four peaks eluted very close to each other (retention time 41.527, 41.903, 42.506, and 42.636 min). It was proposed that the structures corresponded to major triacylglycerols 1-((9*Z*,11*E*)-octadeca-9,11-dienoyl)-2,3-dipalmitoyl-*sn*-glycerol; 1-((10*E*,12*Z*)-octadeca-10,12-dienoyl)-2,3-dipalmitoyl-*sn*-glycerol accompanied by minor isomers: 1,3-dipalmitoyl-2-((9*Z*,11*E*)-octadeca-9,11-dienoyl)-*sn*-glycerol; 1,3-dipalmitoyl-2-((10*E*,12*Z*)-octadeca-10,12-dienoyl)-*sn*-glycerol. Furthermore, we have shown that trilinolein isomerisation in the biphasic system was another effective transformation. These triacylglycerols were prepared as examples of bioavailable dietary supplements and analytical standards for further research.

CONCLUSION

In the present paper, we investigated homogeneous organometallic transformations of linoleic acid, methyl linoleate, and 1-monolinolein in order to describe the mechanism of the catalyst action and to find out how to control the isomerisation in a designed reactor. Among the three applied methods, biphasic isomerisation experiments were demonstrated to have the highest yields (77.6–91.5%) and the highest first-order rate constants (from 2.3×10^{-3} s^{-1} to 6.0×10^{-4} s^{-1}) in comparison with conventional alkali-catalysed processes (yield 49.9 and 53.4%, first-order rate constant 4.2×10^{-7} s^{-1}).

Special attention has been paid to undesired transesterification reactions in the excess Lewis acid catalyst and the increasing proportion of conjugated *E*,*E*-octadecadienoate isomers. *p*-Benzoquinone was the only reagent applicable for selective determination of conjugated (*E*,*E*)-octadecadienoic acid methyl esters in the presence of other geometrical isomers.

Our experiments further revealed the time dependence of biphasic isomerisation when the reaction was under thermodynamic control. The double bond shift could be considered as a consecutive reaction caused by changing the hydridisation of alkene carbons with the metallocyclopropane transition structure. Our research suggested that the isomerisation time of methyl linoleate and 1-monolinolein should not exceed 20–30 min in a designed vessel to inhibit the formation of conjugated *E*,*E*-octadecadienoate isomers below 1%. Finally, mixed triacylglycerols [major triacylglycerols 1-((9*Z*,11*E*)-octadeca-9,11-dienoyl)-2,3-dipalmitoyl-*sn*-glycerol; 1-((10*E*,12*Z*)-octadeca-10,12-dienoyl)-2,3-dipalmitoyl-*sn*-glycerol accompanied by minor isomers: 1,3-dipalmitoyl-2-((9*Z*,11*E*)-octadeca-9,11-dienoyl)- -*sn*-glycerol; 1,3-dipalmitoyl-2-((10*E*,12*Z*)-octadeca-10,12-dienoyl)-*sn*-glycerol] and conjugated trilinolein were prepared. In the coming months, we will focus on the preparation of CLA-enriched structured fats or fat blends.

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References

Akoh C.C., Lai O.-M. (2005): Healthful Lipids, Champaign, AOCS Press: 1–24.

Henry C.J.K. (2007): Novel Food Ingredients for Weight Control. 1st Ed. Woodhead Publishing Ltd.: 263–276.

AOCS (1997): Determination of *cis*- and *trans*- fatty acids in hydrogenated and refined oils and fats by capillary GLC, AOCS Official Method Ce 1f-96. Champaign, AOCS Press

Astruc D. (2007): Organometallic Chemistry and Catalysis. Berlin Heidelberg, Springer-Verlag: 367–392.

Bentley P.H., McCrae W. (1970): Efficient synthesis of symmetrical 1,3-diglycerides. Journal of Organic Chemistry, 35: 2082–2083.

Berdeaux O., Voinot L., Angioni E., Juanéda P., Sébédio J.L. (1998): A simple method of preparation of methyl *trans*-10,*cis*-12- and *cis*-9,*trans*-11-octadecadienoates from methyl linoleate. Journal of the American Oil Chemists' Society, 75: 1749–1755.

Biermann U., Butte W., Tarik E., Haase D., Metzger J.O. (2007): Regio- and stereoselective Diels-Alder additions of maleic anhydride to conjugated triene fatty acid methyl esters. European Journal of Organic Chemistry, 23: 3859–3862.

Christie W.W. (1989): Gas Chromatography and Lipids: A Practical Guide. The Oily Press Ltd.: 130–140.

Consorti C.S., Aydos G.L.P., Ebeling G., Dupont J. (2009): Multiphase catalytic isomerisation of linoleic acid by transition metal complexes in ionic liquids. Applied Catalysis A: General, 371: 114–120.

doi: 10.17221/362/2016-CJFS

Devillard E., Andant N., Wallace R.J. (2006): Increased expression of a molecular chaperone GroEL in response to unsaturated fatty acids by the biohydrogenating ruminal bacterium, *Butyrivibrio fibrisolvens*. FEMS Microbiology Letters, 262: 244–248.

Dobson G. (1998): Identification of conjugated fatty acids by gas chromatography-mass spectrometry of 4-methyl-1,2,4-triazoline-3,5-dione adducts. Journal of the American Oil Chemists' Society, 75: 137–142.

Frankel E.N. (1970): Homogeneous catalytic conjugation of polyunsaturated fats by chromium carbonyls. Journal of the American Oil Chemists' Society, 47: 33–36.

Ha Y.L., Grimm N.K., Pariza M.W. (1987): Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis, 8: 1881–1887.

Housecroft C.E., Sharpe A.G. (2014): Anorganická chemie (Czech translation of 4th Edition). Prague, VSCHT Praha: 917–945.

Hunter W.J., Baker F.C., Rosenfeld I.S., Keyser J.B., Tove S.B. (1976): Biohydrogenation of unsaturated fatty acids. Hydrogenation by cell-free preparations of *Butyrivibrio fibrisolvens*. Journal of Biological Chemistry, 251: 2241–2247.

IUPAC (1987): Determination of di- and tri-unsaturated fatty acids by ultraviolet spectrophotometry. Standard Methods for the Analysis of Oils, Fats and Derivatives, 5th Ed. Method 2.206. USA, IUPAC.

Jenkins T.C., Wallace R.J., Moate P.J., Mosley E.E. (2008): Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. Journal of Animal Science, 86: 397–412.

Kepler C.R., Tucker W.P., Tove S.B. (1971): Biohydrogenation of unsaturated fatty acids. V. Stereospecificity of proton addition and mechanism of action of linoleic acid Δ^{12}-*cis*, Δ^{11}-*trans*-isomerase from *Butyrivibrio fibrisolvens*. Journal of Biological Chemistry, 246: 2765–2771.

Köhler P., Grosch W. (1999): Study of the effect of DATEM. 1. Influence of fatty acid chain length on rheology and baking. Journal of Agricultural and Food Chemistry, 47: 1863–1869.

Larock R.C., Dong X., Chung S., Reddy C.K., Ehlers L.E. (2001): Preparation of conjugated soybean oil and other natural oils and fatty acids by homogeneous transition metal catalysis. Journal of the American Oil Chemists' Society, 78: 447–453.

Liavonchanka A., Feussner I. (2008): Biochemistry of PUFA double bond isomerases producing conjugated linoleic acid. ChemBioChem: European Journal of Chemical Biology, 9: 1867–1872.

Liavonchanka A., Hornung E., Feussner I., Rudolph G. (2006): Structure and mechanism of the *Propionibacterium acnes* polyunsaturated fatty acid isomerase. Proceedings of the National Academy of Sciences of the United States of America, 103: 2576–2581.

Leray C. (2015): Lipids: Nutrition and Health. CRC Press Taylor & Francis Group: 228–235.

Neises B., Steglich W. (1978): Simple method for esterification of carboxylic acids. Angewandte Chemie International Edition in English, 17: 522–524.

Okuyama T., Maskill H. (2014): Organic Chemistry: A Mechanistic Approach. Oxford University Press: 127.

Pavia D.L., Lampman G.M., Kriz G.S., Vyvyan J.R. (2009): Introduction to Spectroscopy. 4th Ed. Brooks/Cole Cengage Learning: 445–492.

Singer H., Seibel R., Mees U. (1977): Die Isomerisierung des Linolsäuremethylesters mit Rhodiumkomplexen. Fette, Seifen, Anstrichmittel, 79: 147–150.

Velíšek J. (2002): Chemie potravin. Tábor, OSSIS: 81–87.

Villeneuve P., Lago R., Barouh N., Barea B., Piombo G., Dupré J.-Y., Le Guillou A., Pina M. (2005): Production of conjugated linoleic acid isomers by dehydration and isomerization of castor bean oil. Journal of the American Oil Chemists' Society, 82: 261–268.

Yang L., Huang Y., Wang H.Q., Chen Z.-Y. (2002): Production of conjugated linoleic acids through KOH-catalyzed dehydration of ricinoleic acid. Chemistry and Physics of Lipids, 119: 23–31.

Young D.C., Vouros P. (1987): Location of conjugated diene position in an aliphatic chain by mass spectrometry of the 4-phenyl-1,2,4-triazoline-3,5-dione adduct. Analytical Chemistry, 59: 1954–1957.

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