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Formation and removal of *Listeria monocytogenes* and *Lactococcus lactis* biofilms

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Abstract. Since the presence of biofilms causes significant problems in food industry and human pathogenic bacteria can take part in biofilm formation, within the frame of this study, we wanted to determine the biofilm-forming ability of *Listeria monocytogenes* in monoculture and mixed culture under industrial circumstances. As lactic acid bacteria used in different technological processes can inhibit the growth of pathogens, the effect of *Lactococcus lactis* on the biofilm formation of *L. monocytogenes* was tested. The multiplication ability of two *L. monocytogenes* was investigated in BH broth and UHT milk. As differences in growth of the tested strains were found at 10 °C, it is supposed that their biofilm-forming capacity differs at low temperature as well. During biofilm formation in monoculture, *L. monocytogenes* cells attached to the surface of metal coupon in higher number than those of *Lactococcus lactis*, but in the case of mixed culture lactic acid bacteria inhibited the biofilm formation of *L. monocytogenes*. The destructive effect of Florasept (a chlorinated disinfectant) was higher in biofilms of *L. monocytogenes*, since resistance to the disinfectant was significant in *Lactococcus*

Key words and phrases: biofilm, cleaning, *Listeria monocytogenes*, *Lactococcus lactis*.

lactis biofilms. Based on our results, it can be concluded that – though *L. monocytogenes* can attach to metal surfaces under industrial circumstances – its cell number is low in biofilms. Nevertheless, lactic acid bacteria can reduce or inhibit the formation of biofilms of pathogenic bacteria.

1 Introduction

Bacteria in aqueous environment are rarely planktonic; instead they tend to colonize solid surfaces, forming biofilms. A biofilm is a complex and heterogeneous structure of cells that is surrounded by an extracellular matrix of exopolysaccharide (EPS) (called glycocalyx) secreted by those cells. Both spoilage organisms and foodborne pathogens, e.g., *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* are able to form biofilms on abiotic surfaces and have enhanced tolerance to different antimicrobial agents, e.g., disinfectants when their cells form biofilms (*Costerton et al.*, 1987, *Stewart et al.*, 2000).

L. monocytogenes is a foodborne pathogen of particular concern in the food industry, ubiquitous in the environment, on plant materials and in the soil. As a consequence, the occurrence of *Listeria monocytogenes* biofilms can cause post-processing contamination and a considerable risk to food safety.

Several studies have demonstrated that *L. monocytogene*s can be present in food-processing environments (*Chmielewski & Frank*, 2003; *Martin & Fisher*, 1999; *Tompkin*, 2002) and it is able to persist in processing factories, sometimes for many years with consequent contamination of food products (*Senczek et al.*, 2000; *Bagge-Ravn et al.*, 2003). This leads to lowered shelf-life of products and transmission of diseases (*Carpentier & Cerf*, 1993; *Mittelman*, 1998). The vast majority of organisms in the natural environment and in the food-processing environment occur in multispecies biofilms (*Costerton et al.*, 1987). *Listeria monocytogenes* will also most likely grow with other microorganisms in mixed species biofilms in food-processing environments (*Carpentier & Chassaing*, 2004; *Habimana et al.*, 2009). The presence and the diversity of microbial groups can facilitate or limit the persistence of *L. monocytogenes* on surfaces (*Carpentier* and *Chassaing*, 2004; *Zhao et al.*, 2004). When growing in co-culture biofilm with strains of the genus *Pseudomonas*, *Staphylococcus* or *Flavobacterium*, the population of *L. monocytogenes* could be increased or decreased depending on the strain (*Carpentier* and *Chassaing*, 2004; *Norwood & Gilmour*, 2000, 2001).

Several methods have been evaluated to kill and remove biofilm organisms from food-processing facilities. These methods include various physical cleaning methods such as high pressure cleaning (*Meyer*, 2003) or ultrasound (*Mott et al.*, 1998). Chemical methods are widely used for the inactivation of biofilm organisms. These include alkali/acid wash (*Parkar et al.*, 2004), chlorine (*Meyer*, 2003), peracid sanitizer (*Fatemi & Frank*, 1999) and acidic electrolysed water (*Ayebah et al.*, 2006). However, these methods are limited to small areas and have not yet found their way to practical application. The antimicrobial activity of nisin on planktonic cells of *Listeria monocytogenes* has been well-documented (*Budde & Jacobsen*, 2000). However, studies addressing the effect of nisin-producing strains on *L. monocytogenes* biofilms are scarce (*Leriche et al.*, 1999).

In our study, we investigated the formation of mixed species biofilms of *L. monocytogenes* in combination with *Lactococcus lactis* and their resistance to disinfection treatment.

2 Materials and methods

Cultures

Two strains of *Listeria monocytogenes* (H10 and H24) isolated from meat industrial plant were used in the experiments together with a nisin-producing culture collection strain of *Lactococcus lactis* (ATCC 11454). All bacterial strains were stored at refrigerated temperatures (4 °C). *L. monocytogenes* strains were maintained on BH agar slants (brain extract, heart extract and peptons 17.5 g; glucose 2.0 g; yeast extract 2.5 g; sodium chloride 5.0 g; di-sodium hydrogen phosphate 2.5 g; agar agar 15.0 g; distilled water 1.0 l) (Merck 1.10493)), while *L. lactis* was stored on MRS agar slants.

Overnight cultures of *Listeria strains* (incubated at 37 °C) were used in the experiments. *L. lactis* was cultivated in BH broth and incubated at 30 °C for 24 hours. A stock suspension of 10^9 cells ml^{-1} was prepared from each strain and used after appropriate dilutions.

Growth measurements

The growth of *Listeria monocytogenes* strains and *Lactococcus lactis* was examined individually and in mixed cultures at 10 °C to simulate the cold manufacturing temperatures of a milk (fat content 2.8%) factory. Growth curves were recorded in BH broth and in milk. Ten ml of the growth medium was

inoculated with the bacteria at the level of 10^6 cells ml^{-1}. When biofilm formation was followed in mixed cultures, the initial proportion of *L. monocytogenes* and *L. lactis* was 1:1000. Samples were taken at 0, 24, 120 and 144 hours. Cell numbers were determined by pour-plating with TGE agar (5.0 g Tripton, 1.0 g Glucose, 2.5 g yeast extract, 1.0 l distilled water) in the case of *Listeria* strains, while in the case of *Lactococcus* strain MRS agar was used in double layer.

Biofilm formation

Biofilm formation was carried out in BH broth. Petri dishes containing 20 ml BH broth were inoculated with *Listeria monocytogenes* strains and *Lactococcus lactis* individually or in mixed cultures with an initial number of 10^7 cells ml^{-1}. Two sizes of stainless steel coupons were used in the experiments for the detection of viable cell counts (30×9 mm and 75×9 mm). Metal coupons were immersed and left into the inoculated growth medium for an hour to attach to the test microorganisms. Then coupons were removed from the Petri dishes and rinsed with sterile distilled water to remove the unattached cells. Coupons were then placed into other Petri dishes containing sterile uninoculated BH broth. Attached cells served as "inocula" for biofilm initiation. The number of biofilm-producing cells was determined by pour-plating and fluorescent microscopy after 0, 24 and 144 hours.

Detection of viable cell counts in biofilms

At sampling times, metal coupons were removed from the Petri dishes containing BH broth, and rinsed with sterile water to remove unattached cells. Small coupons were placed into test tubes containing sterile glass beads (diameter $0.4 - 0.6$ mm) and 10 ml sterile diluents (1 g pepton, 8.5 g NaCl, 1 l distilled water). Tubes were vortexed for 2 minutes to remove attached cells from the surface of the coupons. Appropriate dilutions of these suspensions were pour-plated and incubated at 30 °C for $24 - 48$ hours. All measurements were carried out in duplicates.

Microscopic investigations

Microscopic slide size coupons were investigated with epifluorescent microscope (Olympus BH-2, Olympus Optical Gmbh, Germany) after acridine orange (AO, 0.02 g/100 ml water, Merck 14281) staining for 2 minutes.

Biofilm removal

After sampling, coupons were rinsed with sterile water to remove unattached cells and placed into disinfectant solution (8 ml Florasept in 250 ml distilled water). Coupons, after rinsing with sterile distilled water, were placed into Petri dishes containing 15 ml Florasept suspension. They were left there for 15 minutes' contact time and then rinsed with sterile neutralizer (3 g lecitin, 30 ml Tween 80.5 g Na-tiosulphate, 1 g α-hisztidin, 10 ml phosphate buffer (34 g KH_2PO_4 in 500 ml destilled water), 1000 ml destilled water)) to stop the effect of the disinfectant. Control coupons without disinfection and disinfected coupons were examined under fluorescent microscope (see Section 2.5) and the number of surviving cells was determined as described in Section 2.4 in the case of individual cells. When mixed cultures were examined, samples were also spread-plated on *Listeria*-selective Palcam agar (MERCK, 1.11755.0500).

Fluorescent *In situ* hybridization (FISH)

Fluorescent in situ hybridization technique was used to separate *Listeria* cells from *L. lactis* in biofilms of mixed cultures under fluorescent microscope. The VIT-Listeria kit (Vermicon, Munich) was used for the analysis by the instructions of the manufacturer.

3 Results and discussion

Comparison of the multiplication ability of *Listeria monocytogenes* and *Lactococcus lactis* strains in BH broth and UHT milk

To be able to determine the biofilm-forming ability of *L. monocytogenes* strains in BH broth and UHT milk, their growing capacities were tested in different culture media. Changes of the colony-forming units of *L. monocytogenes* H10 and H24, depending on the sampling time, are summarized in Table 1.

L. monocytogenes H10 could propagate well in both media. Its initial cell number grew two orders of magnitude during the 144 hours (6-day-long) incubation time and, as it can be seen from Table 1, it could multiply better in UHT milk since the cell concentration was ten times higher in milk than in BH broth after 24 hours of incubation. In contrast with these observations, *L. monocytogenes* H24 could not multiply efficiently either in milk or in BH broth as its cell number remained 10^6 ml^{-1} during the incubation time.

Table 1: Changes of colony-forming units of *L. monocytogenes* H10 and H24 depending on the sampling time

Sampling time (hours)	*L. monocytogenes* H10 (log_{10} CFU ml^{-1})		*L. monocytogenes* H24 (log_{10} CFU ml^{-1})		*Lactococcus lactis* ATCC 11454 (log_{10} CFU ml^{-1})	
	BH broth	UHT milk	BH broth	UHT milk	BH broth	UHT milk
0	6.2	6.2	6.1	6.1	6.6	6.4
24	6.3	7.2	6.1	6.3	6.4	6.5
120	7.7	7.6	6.5	6.7	8.2	8.2
144	8.4	8.3	6.7	6.9	8.6	8.7

Although both *L. monocytogenes* strains originate from meat samples and were isolated from similar conditions (data are not shown), differences in their growing capacity could be observed. Since *L. monocytogenes* H10 showed better multiplication ability at 10 °C, it was chosen for further investigations.

Lactococcus lactis ATCC 11454 could grow well in both BH broth and UHT milk. The initial cell concentration (10^6 CFU ml^{-1}) reached 10^8 CFU ml^{-1} during 120 hours of incubation and did not change significantly until the 6^{th} day of the experiment.

Culture media used for the propagation of the tested bacteria did not have significant effect on the multiplication ability of the strains; thus, BH broth was used as culture medium in studying biofilm formation.

Biofilm formation of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 strains in monoculture

Determination of biofilm-forming ability for *Listeria monocytogenes* H10 and Lactococcus lactis ATCC 11454 was performed in separate experiments from monocultures. As it can be seen in Figure 1, until the first sampling time (after 1 hour of incubation), more *L. monocytogenes* cells were adhered to the surfaces of the metal coupons than that of *Lactococcus lactis*. Furthermore, in the course of the analysis, higher numbers of *L. monocytogenes* H10 were characteristic of biofilm formation (104-105 cells ml^{-1}), while it was only 101-103 cells ml^{-1} in the case of *Lactococcus lactis* ATCC11454. This observation can correlate with the elevated adherence capacity of *L. monocytogenes*, the significant resistance to different stress factors and the ability to propagate even at lower temperature.

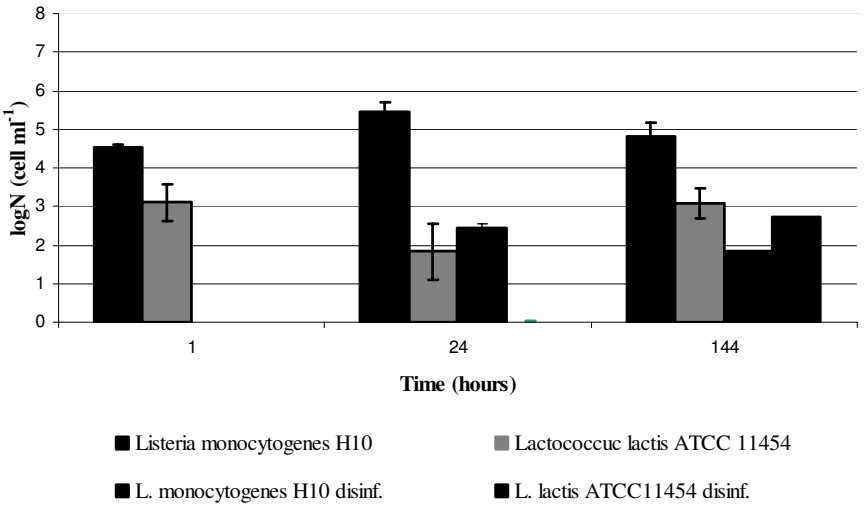

Figure 1: The concentrations of adhered *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 cells took part in the biofilm formation separately. Changes in cell numbers are indicated in the case of control and disinfected samples

Disinfection proved to be adequate in the case of biofilms of the tested bacteria since significant changes could be observed in the cell numbers of treated samples after 24 hours of incubation. Cell concentration decreased three orders of magnitude in the case of *Listeria monocytogenes*, while the presence of Lactococcus lactis living cells was not detected. Since efficiency of chlorinated disinfectants against L. monocytogenes biofilm had been previously observed (Alasri et al., 1992; Green, 1993), the effectiveness of Florasept was confirmed. After six days, a similar decline could be seen in *L. monocytogenes* cell concentration; however, biofilm formed by Lactococcus lactis could not be removed as intensively as before (Figure 1). Presumably, the resistance of the cells that constituted the biofilm increased significantly, which feature is well-known in the process of biofilm formation.

Results of modelling for biofilm formation by fluorescent microscopy

Biofilm formation of the cells was followed by fluorescent microscopy, parallel with viable cell counting. After staining the metal coupons with acridine orange, the results were evaluated by microscopic observation of the cells at-

taching together. As it can be seen in Figure 2 (A/1-A/3), the number of the attached cells in the case of the control sample increased continuously during incubation time. Nevertheless, a relatively low number of *L. monocytogenes* cells took part in biofilm formation. This observation confirms the tendency obtained by the culturing of viable cell counts in 3.2.

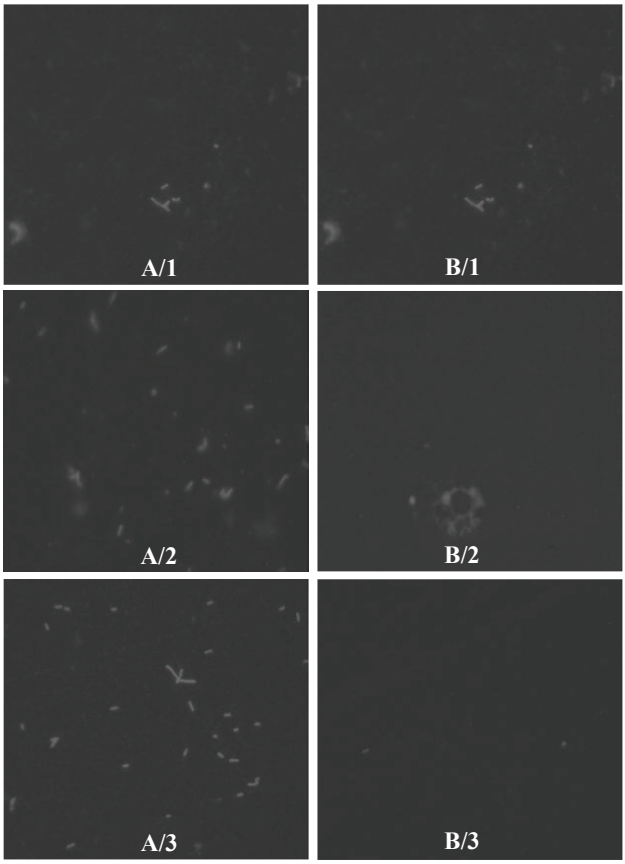

Figure 2: Results of fluorescent microscopic investigation for control and disinfected samples of *Listeria monocytogenes* biofilm at different sampling times.

A/1: control sample after 1 hour, A/2: control sample after 24 hours, A/3: control sample after 144 hours; B/1: disinfected sample after 1 hour, B/2: disinfected sample after 24 hours, B/3: disinfected sample after 144 hours

Based on pictures of metal coupons treated with Florasept (Figure 2. B/1-B/3), it can be observed that the applied disinfectant was effective in the removal of *L. monocytogenes* biofilm. On the surfaces of the sanitized coupons, the number of biofilm-forming cells decreased significantly at both sampling times.

Development of biofilm from the mixed culture of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 strains

After investigating the biofilm-forming capacity of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC 11454 in monocultures, the development of the biofilm from mixed culture was analysed. The inoculation ratio of the two bacteria (*L. monocytogenes* and *Lactococcus lactis*) was 1:1000.

As it is shown in Figure 3, the cell concentration of *Listeria monocytogenes* was relatively low during the experiment ($10^1 - 10^2$ CFU ml^{-1}), but the total cell number was fairly high (10^7 CFU ml^{-1} after 1 hour of incubation), which indicates the dominance of *Lactococcus lactis* cells in biofilms.

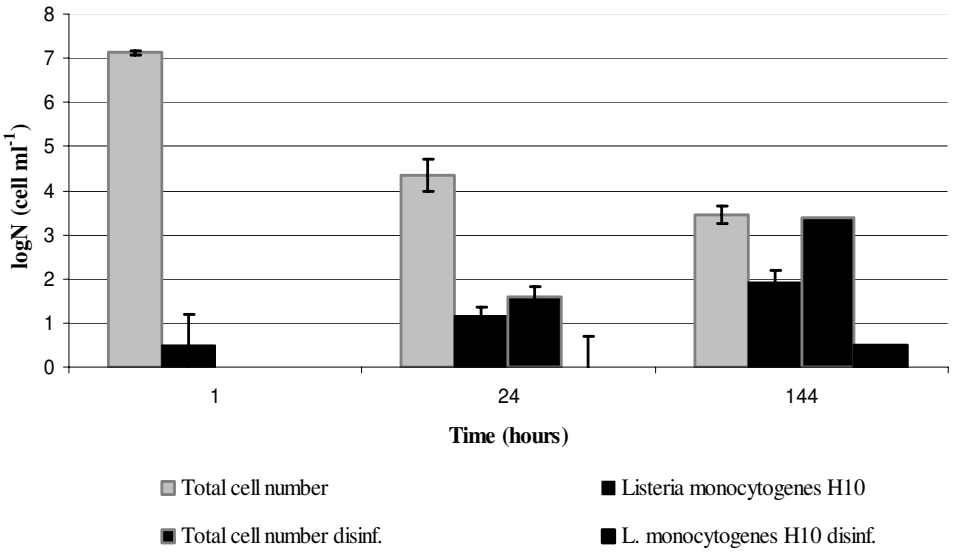

Figure 3: Changes of cell numbers for mixed cultures of *Listeria monocytogenes* H10 and *Lactococcus lactis* ATCC11454 in control and disinfected samples

The concentration of *Listeria monocytogenes* increased through the incubation; however, the total bacterium count decreased (from 10^7 to 10^3 CFU ml^{-1}), which highlights the reduction of *Lactococcus lactis* cells in the biofilm and the higher resistance of *L. monocytogenes* to lower temperature.

Treatment with disinfectant proved to be efficient in the case of *Listeria monocytogenes*, but lactic acid bacteria were resistant to sanitation, since after six days of incubation their number was approximately 10^3 cells ml^{-1}.

Results of fluorescent in situ hybridization

Biofilm formation of mixed culture for *L. monocytogenes* and *Lactococcus lactis* was to be detected by FISH method that can differentiate the cells of *Listeria monocytogenes* from non-*monocytogenes Listeria* and other bacteria during in situ analysis.

As it was observed in 3.4, only a low number of *L. monocytogenes* cells ($10^1 - 10^2$ CFU ml^{-1}) took part in biofilm formation when mixed cultures were investigated. Since the detection level of FISH is 4×10^5 CFU ml^{-1} (*Hogardt et al.*, 2000), *L. monocytogenes* cells were not detected on metal coupons; however, they were present in form of biofilm.

4 Conclusions

In the last 20 years, biofilms have had a particular concern with food industry as bacteria can colonize different surfaces of food-processing plants, thus contributing not only to economic losses but also to the development of food-borne illnesses. Resistance of biofilms to different cleaning and disinfection processes increases; therefore, the removing of biofilms is a big challenge for food industry.

In our study, the biofilm-forming capacity of *Listeria monocytogenes* and *Lactococcus lactis* was investigated in monoculture and mixed culture. *L. monocytogenes* was able to form biofilm at 10 °C on metal surfaces in relatively high number, while *Lactococcus lactis* biofilm formation was partially inhibited. In mixed culture, lactic acid bacteria inhibited the biofilm formation of *L. monocytogenes* when a comparatively high number of *Lactococcus lactis* was used. The effect of a chlorinated disinfectant (Florasept) was higher in biofilms of *L. monocytogenes*, since resistance to the disinfectant was noteworthy in *Lactococcus lactis* biofilms. Lactic acid bacteria applied in technological processes of food industry can help us inhibit the attachment and colonization of harmful microorganisms, amongst them: human pathogenic bacteria.

Removal of biofilms is a complicated task for food industry; thus, cleaning and disinfection of tools and equipments used for food processing are essential. Application of surfaces without scratches is also important in confining hidden bacteria which can take part in biofilm formation.

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References

[1] C. Alasri, C. Roques, G. M. Cabassus, P. Aptel, Effects of different biocides on a mixed biofilm produced on a Tygon tube and on ultrafiltration membranes, *Spectra*, 168 (1992) 21–24.

[2] Y. C. Ayebah, C. Kim Hung, J. F. Frank, Efficacy of electrolyzed water in the inactivation of planktonic and biofilm Listeria monocytogenes in the presence of organic matter, *Journal of Food Protection*, 69 (2006) 2143–2150.

[3] K. Bagge-Ravn, L. Gardshodn, Gram, B. F. Vogel, Comparison of sodium hypochlorite-based foam and peroxyacetic acid-based fog sanitizing procedures in a salmon smokehouse: Survival of the general microXora and Listeria monocytogenes, *Journal of Food Protection*, 66 (2003) 592–598.

[4] B. B. Budde, M. Jacobsen, Real-time measurements of the interaction between single cells of Listeria monocytogenes and nisin on a solid surface, *Applied and Environmental Microbiology*, 66 (2000) 3586–3591.

[5] B. Carpentier, D. Chassaing, Interactions in biofilms between Listeria monocytogenes and resident microorganisms from food industry premises, *International Journal of Food Microbiology*, 97 (2004) 111–122.

[6] R. A. N. Chmielewski, J. F. Frank, Biofilm formation and control in processing facilities, *Comprehensive Reviews in Food Science and Food Safety*, 2 (2003) 22–32.

[7] J. W. Costerton, K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, T. J., Marrie, Bacterial biofilms in nature and disease, *Annual Review of Microbiology*, 41 (1987) 435–464.

[8] P. N. Green, Efficacy of biocides on laboratory generated *Legionella* biofilms, *Letters in Applied Microbiology*, 17 (1993) 158–161.

[9] O. Habimana, M. Meyrand, T. Meylheuc, S. Kulakauskas, R. Briandet, Genetic features of resident biofilms determine attachment of Listeria monocytogenes, *Applied and Environmental Microbiology*, 75, (2009) 7814–7821.

[10] M. Hogardt, K. Trebesius, A. M. Geiger, M. Hornef, J. Rosenecker, J. Heesemann, Specific and rapid detection by fluorescent in situ hybridization of bacteria in clinical samples obtained from cystic fibrosis patients, *J. Clin. Microbiol.*, 38, (2000) 818–825.

[11] B. Carpentier, O. Cerf, A review: Biofilms and their consequences, with particular reference to hygiene in the food industry, *Journal of Applied Bacteriology*, 75, (1993) 499–511.

[12] V. Leriche, D. Chassaing, B. Carpentier, Behaviour of L. monocytogenes in an artificially made biofilm of a nisin-producing strain of Lactococcus lactis, *International Journal of Food Microbiology*, 51, (1999) 169–182.

[13] P. Fatemi, J. F. Frank, Inactivation of Listeria monocytogenes/Pseudomonas biofilms by peracid sanitizers, *Journal of Food Protection*, 62 (1999) 761–765.

[14] M. W. Mittelman, Structure and functional characteristics of bacterial biofilms in fluid processing operations, *Journal of Dairy Science*, 81 (1998) 2760–2764.

[15] S. E. Martin, C. W. Fisher, Listeria monocytogenes, In: *The encyclopedia of food microbiology*, ed. R. Robinson, C. Batt, & P. Patel, 1999 New York: Academic Press. 1228–1238.

[16] B. Meyer, Approaches to prevention, removal and killing of biofilms, *International Biodeterioration & Biodegradation*, 51 (2003) 249–253.

[17] I. E. C. Mott, D. J. Stickler, W. T. Coakley, T. R. Bott, The removal of bacterial biofilm from water-filled tubes using axially propagated ultrasound, *Journal of Applied Microbiology*, 84 (1998) 509–514.

[18] D. E. Norwood, A. Gilmour, The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm, *Journal of Applied Microbiology*, 88 (2000) 512–520.

[19] D. E. Norwood, A. Gilmour, The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature, *Letters in Applied Microbiology*, 33 (2001) 320–324.

[20] S. G. Parkar, S. H. Flint, J. D. Brooks, Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel, *Journal of Applied Microbiology*, 96 (2004) 110–116.

[21] Senczek, R. Stephan, F. Untermann, Pulsed-field gel electrophoresis (PFGE) typing of Listeria strains isolated from a meat processing plant over a 2-year period, *International Journal of Food Microbiology*, 62 (2000) 155–159.

[22] P. S. Stewart, G. A. McFeters, C. T. Huang, Biofilm control by antimicrobial agents, In: *Biofilms II: Process Analysis and Applications*, ed. Bryers, J. D. 2000. New York: Wiley-Liss, 373–405.

[23] R. B. Tompkin, Control of Listeria monocytogenes in the food-processing environment, *Journal of Food Protection* 65 (2002) 709–725.

[24] T. Zhao, M. P. Doyle, P. Zhao, Control of Listeria monocytogenes in a biofilm by competitive-exclusion microorganisms, *Applied and Environmental Microbiology*, 70 (2004) 3996–4003.

Acta Univ. Sapientiae, Alimentaria, **5** (2012) 18–31

Influence of photon flux density and high salinity on the level of some components of the antioxidative defence system in lettuce leaves

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Abstract. The amount of health-promoting substances biosynthesized in lettuce leaves is influenced by environmental stress factors such as photon flux density and salinity. As ubiquitous protective metabolites, carotenoid pigments and ascorbic acid (vitamin C) accumulate in higher concentrations when lettuce plants are exposed to high irradiance and salt stress, in relation with the oxidative damage caused by the environmental constraints. Accumulation of reactive oxygen species in photosynthetic cells induces an enhanced activity of the antioxidative protective system. This is reflected by changes in the catalytic activity of ascorbate peroxidase, responsible for the regulation of hydrogen

Key words and phrases: lettuce leaves, flux density, high salinity, carotenoid pigments, ascorbic acid, antioxidative defence

peroxide level in different cell compartments, through a reaction which involves oxidation of ascorbate. High photon flux density increases the carotenoid content of young leaves and salt stress further enhances this increment, although by itself it does not cause the accumulation of these photoprotective pigments. Water-soluble sugars, which may play a role in osmoregulation and represent a source for the synthesis of ascorbate, are present in higher amount under intense light, but their concentration decreases when enhanced irradiation is associated with salt stress. High photon flux density and high salinity do not exhibit synergism concerning the increment of vitamin C content of lettuce leaves. A good molecular marker of antioxidative defence is the increased enzymatic activity of ascorbate peroxidase under high irradiance, this being further enhanced by the exposure of plants to 80 mM sodium chloride. In conclusion, the cultivation of lettuce under moderately high photon flux density (660 micromole photons m^{-2} s^{-1}) and under mild salt stress (80 mM NaCl) increases vitamin C and the carotenoid pigment content of its leaves, which is a beneficial property for consumers.

1 Introduction

Lettuce is a widespread vegetable that represents a valuable source of vitamins, inorganic nutrients and other health-promoting substances. The amount of different primary and secondary metabolites largely depends on the prevailing growth conditions. When environmental stress factors impair the steady state of different vital functions, acclimatization processes result in concerted metabolic changes that prevent or compensate damages caused by adverse developmental conditions. For example, it was demonstrated UV-B radiation, high light intensity, water stress, mild heat shock and chilling stress induce the biosynthesis of some phenolic compounds (e.g., quercetin-3-O-glucoside, chlorogenic acid, chicoric acid) with health-promoting qualities in the human diet (*Oh et al.*, 2009). An increased antioxidative capacity is conferred by activation of protective enzymes that reduce membrane damage (*Mahmoudi et al.*, 2010), as well as by accumulation of α-tocopherol (vitamin E) and glutathione, which also contribute to the nutritive value of lettuce leaves (*Rios et al.*, 2008). Specific phytochemicals produced through the shikimic acid pathway may accumulate due to a large increase in the expression of the gene encoding phenylalanine ammonia-lyase, the key enzyme for the biosynthesis of secondary phenolic compounds with protective properties (e.g., flavonoids). The transcription of this enzyme is especially enhanced by a series of abiotic stress factors (*Oh et al.*, 2009). The activities of the above-mentioned

key enzyme and of polyphenol oxidase are also responsible for the chemical browning, which reduces the visual quality of cut lettuce (*Altunkaya et al.*, 2008; *Roura et al.*, 2008). In red lettuce cultivars, environmental stress factors also enhance the production of anthocyanins (cyanidin-3-O-(6"-malonyl-β-glucopyranoside) and its methyl ester), which are absent from green lettuce plants and exhibit antioxidative properties, which results in a significantly reduced lipid peroxidation and cycloxigenase activity in red lettuce leaves (*Mulabagal et al.*, 2010). This is also the reason why red lettuce varieties were found to be better dietary sources of natural antioxidants, with higher levels of flavonols, which play a role in preventing cardiovascular diseases (*Llorach et al.*, 2008). Phenolic antioxidants generally exhibit a certain synergism with other reducing compounds, such as tocopherols, ascorbic acid, cysteine and carotenoids, which also contribute to the overall antioxidant capacity of leafy vegetables (*Altunkaya et al.*, 2009). For greenhouse-grown broccoli, it was shown that temperature increase under low light intensity affects the amount of phytochemicals such as antioxidative carotenoids, prooxidative chlorophylls and protective glucosinolates (*Schonhof et al.*, 2007).

The photon flux density of the incident light is a major environmental factor that determines the entire metabolic activity and vitality of plants, not only because it is the crucial energy source for the photosynthetic biomass production, but also because it may be the main source of photo-oxidative damage that triggers a network of concerted photoprotective mechanisms. While low light causes an imbalance in the energetic metabolism of plants that limits growth and development, excessively high photon flux densities may cause the photoinhibition of photosynthetic components and may lead to the overproduction of reactive oxygen species in chloroplasts. As a defence mechanism, overexpression of antioxidative enzymes and enhanced production of non-enzymatic antioxidants occur, and various repair processes take place in the thylakoid membranes, in metabolones and in the genetic material (*Pogány et al.*, 2006).

For greenhouse-grown and field-grown vegetables, such as lettuce, the salinity of irrigation water may represent a major environmental stress factor that limits growth and induces metabolic tolerance. Because lettuce cultivars are moderately salt-sensitive plants, high salinity is one of the most frequent limiting factors of growth and development in the case of lettuce. On a shorter time scale, salt stress inhibits growth of stem and leaves, mainly because of its osmotic effect (it causes difficulties in water supply, leading to the partial dehydration of leaves). This effect can be overcome by osmoregulation (accumulation of compatible solutes, such as proline, sucrose, trehalose, sugar

alcohols etc.) and by the downregulation of stomatal conductance, which has negative secondary effects on carbon assimilation and on the aquisition of mineral nutrients (*Ünlükara et al.*, 2008; *Younis et al.*, 2009). If salt stress lasts for longer periods (weeks and months), the chemical toxicity of excessive sodium ions becomes prevailing over the osmotic effect. Accumulation of sodium ions impairs the homeostasis of potassium and calcium in cell compartments, inhibits different enzymes and induces an oxidative stress by the generation of high amounts of reactive oxygen species. This chemical stress caused by high salinity is counteracted by the sequestration of sodium ions in the vacuole, by the enhancement of the antioxidative activity and by a series of secondary defence mechanisms that may vary among species and varieties, according to their degree of salt sensitivity, tolerance or resistance (*Ahmad et al.*, 2012; *Eraslan et al.*, 2007; *Kohler et al.*, 2009).

Many external adverse factors induce similar negative effects in living organisms, manifested as oxidative stress. This is why plants may develop cross-tolerance towards different environmental stressors by the activation of the antioxidative protective system. Radical scavengers and reducing agents (such as ascorbate, tocopherol, glutathione, simple phenolic compounds, flavonoids, carotenes and xanthophylls) represent the non-enzymatic component of the antioxidative system, while the enzymatic component includes the different isoenzymes of superoxide dismutase, catalase, peroxiredoxins, glutathione reductase, dehydroascorbate reductase, ascorbate peroxidase and other peroxidases (*Pogány et al.*, 2006; *Shigeoka et al.*, 2002; *Xu et al.*, 2008).

The aim of this research is to investigate the influence of moderately high (non-inhibitory) photon flux density and moderately high salinity, as well as of the interaction of these abiotic environmental factors on the concentration of some health-promoting metabolites, such as ascorbic acid and carotenoid pigments, as well as on a representative enzyme of the antioxidative system (i.e. ascorbate peroxidase) and on the water-soluble sugar content of fresh lettuce leaves, in order to improve nutritive value by modulating growth conditions.

2 Material and methods

Lettuce (*Lactuca sativa* L.) plantlets belonging to the May King cultivar (one of the most widespread varieties, cultivated all around Europe) were grown hydroponically in Hoagland's inorganic nutrient solution, in a growth chamber (Versatile Environmental Test Chamber, Sanyo) with a light intensity of 220 μM photons m^{-2} s^{-1} of photosynthetically active radiation, at 20 °C and a

constant atmospheric humidity of 70%. Four-week-old plants were separated into four experimental groups: the control group was grown for one more week under the above-mentioned conditions, a second group was illuminated with high light of 660 μM photons m^{-2} s^{-1}, a third one was put in Hoagland solution supplemented with 80 mM sodium chloride (p.a.), while the fourth group was subjected to the combined action of 660 μM photons m^{-2} s^{-1} and 80 mM NaCl. Measurements were performed on young but fully expanded leaves exposed for seven days to the above-mentioned conditions.

Carotenoid pigments were extracted with dimethylformamide and determined spectrophoto-metrically. 0.1 g fresh leaf material was immersed in 4 ml dimethylformamide and kept for 48 hrs in darkness for the complete extraction of photosynthetic pigments; then the extract was centrifuged for 5 min at 7000 g, and the absorbance of the supernatant was measured at 470 nm (*Bartha et al.*, 2010).

Water-soluble sugars were determined based on the absorbance of the green product resulting from the incubation of leaf extracts with anthrone solution. 0.5 g fresh leaves were homogenized in a final volume of 10 ml of 96% ethanol (v/v); the extract was centrifuged for 10 min at 5500 g, then 100 μL of supernatant was mixed with 3 μL freshly prepared anthrone solution in 72% H_2SO_4; the mixture was incubated for 10 min in boiling water and the absorbance of the green product was measured at 620 nm. Standard curve was obtained with glucose (*Bartha et al.*, 2010).

Ascorbic acid content was determined photometrically by the change in absorbance at 265 nm upon addition of 4 units (10 μL) of ascorbate oxidase. 1 g fresh leaf material was ground in a chilled mortar with a final volume of 4 ml 6% trichloroacetic acid; the mixture was centrifuged for 15 min at 4 °C and 15600 g, then 200 μL of supernatant was mixed with 790 μL of 0.1 mM phosphate buffer (pH 5.6). Upon the addition of 10 μL ascorbate oxidase solution, the absorption decrease at 265 nm was measured using an extinction coefficient of 14 mM^{-1} cm^{-1} (*Xu et al.*, 2008).

Ascorbate peroxidase activity was determined spectrophotometrically, based on the oxidation of ascorbic acid initiated by addition of hydrogen peroxide, and measured through decrease in the absorbance of the reaction mixture at 290 nm. 1 g leaf material was ground in a prechilled mortar with 5 ml extraction solution consisting of 50 mM phosphate buffer (pH 7.8), 1 mM Na_2-EDTA, 1 mM ascorbic acid and 2% water-soluble polyvinyl-pyrrolidone. The homogenate was centrifuged for 20 min at 15000 g. 50 μL of the supernatant (as plant enzyme extract) was mixed with 1.75 mL phosphate buffer (pH 7.8) containing 1 mM Na_2-EDTA and 100 μL of 10 mM ascorbic acid. The reaction

was started with the addition of 100 µL of 20 mM hydrogen peroxide, and after a lag period of 40 s the decrease of absorbance at 290 nm was registered for 3 min. A molar extinction coefficient of 2.8 mM^{-1} cm^{-1} was used for the ascorbic acid and the reference mixture contained distilled water instead of hydrogen peroxide. Enzyme activity was expressed as consumed hydrogen peroxide per minute per mg protein, considering that 1 mole ascorbic acid reduces 1 mole of hydrogen peroxide. The protein content of leaf materials was assayed according to Bradford's method, with bovine serum albumine as a standard (*Bartha et al.*, 2009).

Every experimental setup had 4 repetitions. Statistical analyses of experimental data were performed in R environment (version 2.14.1), using the Shapiro-Wilk test for normality, Bartlett's test for homogeneity of variances and the post-hoc Tukey HSD test for the significance of differences between treatments (at $P < 0.05$).

3 Results

Environmental factors, such as light, temperature, water availability and mineral nutrient supply, have a determining influence on plant metabolism and development. Adverse external parameters are perceived as stress factors that induce changes in the main physiological functions like photosynthesis, mineral nutrition, water relations, secondary metabolism, growth and development. Metabolic plasticity enables plants to develop tolerance to stress factors, while physiological processes reach new steady state levels through a complex network of down- and upregulation mechanisms. Because of a considerable similarity between biochemical processes in the different living organisms, defence products that enable the survival of plants under changed environmental conditions may have benefic influence also on consumer organisms. For example, metabolites used by plants to overcome oxidative stress caused by reactive oxygen species have a demonstrated health-promoting action in humans upon consumption of plant products (*Mulabagal et al.*, 2010). In this context, lettuce is an important vegetable as a source of useful phytochemicals, especially when its fresh leaves are consumed in salads. A common group of metabolites synthesized by plants and related to photoprotection of chlorophylls in the photosynthetic apparatus of leaf cells is represented by the carotenoid pigments (carotenes and xanthophylls). Their role in preventing the generation of singlet oxygen in illuminated thylakoid membranes and in neutralizing the already produced singlet oxygen is well-established. Zeaxanthin and anther-

axanthin, as the two photoprotective pigments of the xanthophylls cycle, but also lutein, β-carotene, licopene and other carotenoids have an important antioxidative effect on all types of cells by protecting vital molecules (proteins, nucleic acids, unsaturated fatty acids in lipids, pigments) from oxidative damage (*Younis et al.*, 2009). These carotenoids cannot be produced by the human organism, so our carotenoid supply depends on plants. The present experiments showed that the carotenoid pigment content of lettuce leaves increases considerably if photon flux density is elevated from 220 to 660 μM photons m^{-2} s^{-1}. Even though mild salt stress exerted by 80 mM sodium chloride did not influence significantly the overall carotenoid content of the leaves, if salt stress was associated with increased light intensity, it acted synergistically with higher photon flux density and resulted in the further increment of the carotenoid content (Figure 1).

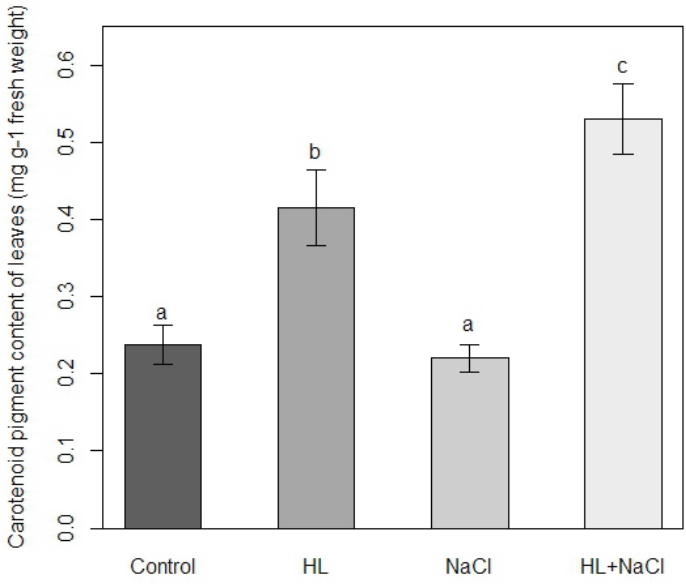

Figure 1: Influence of high light (HL) and salt stress – separately and in combination – on the carotenoid pigment content of lettuce leaves. For experimental conditions, see the *Material and methods* section. Bars represent means ± SE (n = 4). Different letters indicate significant differences at P < 0.05

Considering that carotenoid pigments are helth-promoting phytochemicals, their increased amount improves the nutritive value of lettuce grown under high light intensity and under mild salinity stress. *Oh et al.* (2009) have

demonstrated that chilling stress, heat shock, high light intensity (800 μM photons m^{-2} s^{-1} for one day) and other mild environmental stresses improve the health-promoting quality of lettuce with no significant adverse effects on its growth and fresh biomass yield. *Mahmoudi et al.* (2010) have found that treatment with 100 mM NaCl enhanced carotenoid biosynthesis in salt tolerant lettuce varieties. This enhancement was not observed in our experiments with the May King cultivar.

Water-soluble sugars are important products of photosynthetic carbon assimilation; furthermore, they represent intermediates for the biosynthesis of different defence products (e.g., biosynthesis of ascorbic acid involves D-mannose and L-galactose), and some simple sugars may play an important role in osmoregulation as compatible solutes of plant cells (e.g., sucrose, trehalose). This is the reason why we have investigated the influence of increased photon flux density and of high salinity on the water-soluble sugar content of lettuce leaves. While intense light caused a significant increase in the sugar content, salt stress had no effect on it, and it anihilated the effect of high light when they were applied in combination (Figure 2).

This may reflect that water-soluble sugars are not the main osmoregulators in lettuce leaves, thus their synthesis is not enhanced by the osmotic component of salt stress. While higher light energy input results in the accumulation of simple sugars in leaf cells (most probably as sucrose produced in the cytoplasm of mesophyll cells and loaded into the phloem vessels of leaf veins), salt stress disturbs carbohydrate metabolism and exerts an indirect inhibitory influence on water-soluble sugar accumulation. In other experiments, performed under generally similar conditions, it was found that different degrees of salt stress have no significant influence on the simple sugar content of several lettuce varieties (*Bartha et al.*, 2010). It is worth mentioning that salt stress did not cause any significant dehydration of leaves (their water content was around 94% in the control and around 91% under the influence of 80 mM NaCl – data not shown), so there is an efficient osmoregulation in lettuce, but this is probably not achieved with a significant contribution of water-soluble sugars.

Along carotenoids, which are lipophylic antioxidants occurring in biomembranes (thylakoid membranes) and in plastoglobuli as metabolic inclusions of the plastid stroma, ascorbic acid (vitamin C) is a major water-soluble antioxidant, being present in different compartments of the plant leaf cells in average concentrations of around 5 mM (with an average of 25 mM in the stroma of chloroplasts). It cannot be produced by the human organism, being supplied through diet. Because of its reducing property, ascorbic acid detoxifies

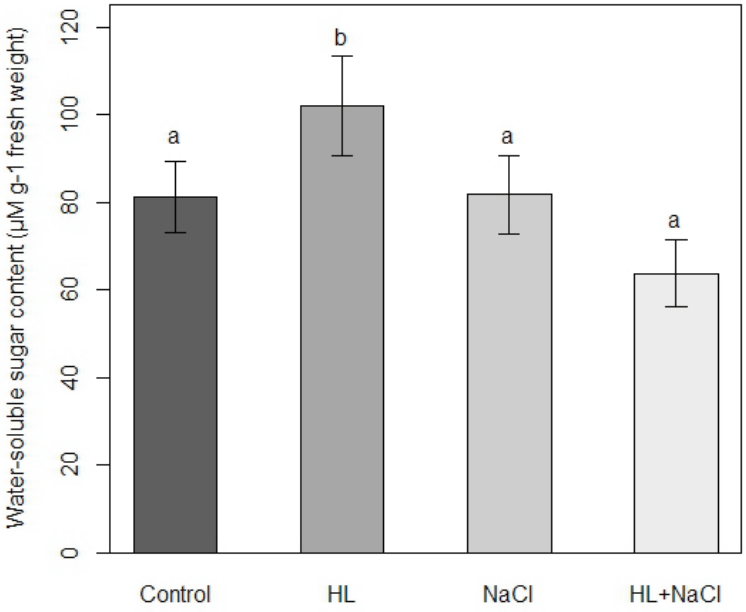

Figure 2: Influence of high light (HL) and salt stress (80 mM NaCl) on the water-soluble sugar content of lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at P < 0.05, according to the post-hoc Tukey HSD test

hydrogen peroxide and it also contributes to the regeneration of vitamin E during the neutralization of hydroxyl radical, which is one of the most potent reactive oxygen species along hydrogen peroxide and the superoxide radical anion (*Pogány et al.*, 2006). In cell compartments where there is no catalase enzyme, the reduced form of vitamin C is used by ascorbate peroxidase to convert hydrogen peroxide into two molecules of harmless water. During this process, ascorbate is oxidized to dehydroascorbate, and the enzyme dehydroascorbate reductase ensures the regeneration of reduced vitamin C. For an efficient defence against oxidative stress generated by accumulation of hydrogen peroxide, a sufficiently high amount of vitamin C and a constantly high molar ratio between its reduced and oxidized form is needed. For this reason, the vitamin C content of plant organs is a molecular indicator of oxidative stress tolerance, and it also increases the health-promoting quality of food plants, considering that vitamin C has the same benefic action in the human organism. Under our experimental conditions, high photon flux density and salt stress both increased the ascorbic acid content of lettuce leaves, but no

synergistic or antagonistic interaction could be observed between the two environmental factors (Figure 3). Probably because the applied light intensity caused a significant photo-oxidative influence in leaf cells, it resulted in a higher vitamin C accumulation than salt stress exerted by 80 mM sodium chloride. The results are in agreement with several other findings, which demonstrates that various abiotic and biotic stress factors that induce oxidative damage increase the antioxidative capacity of plants through a higher ascorbic acid content (*Altunkaya and Gokmen*, 2008; *Kohler et al.*, 2009; *Rios et al.*, 2008; *Schonhof et al.*, 2007; *Xu et al.*, 2008).

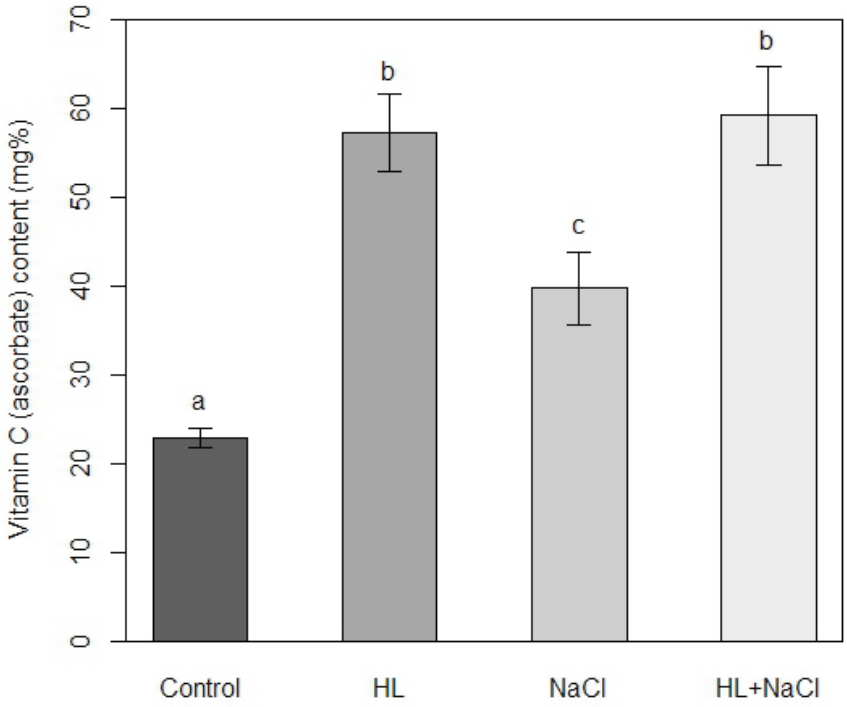

Figure 3: Influence of high light (HL) and salt stress on vitamin C content of fresh lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$, according to the Tukey HSD test

The main antioxidative protective enzyme, which regulates the level of hydrogen peroxide in the micromolar concentration range by converting the excessive amount into water through reduction with ascorbic acid, is represented by the different isoforms of ascorbate peroxidase. It accumulates upon oxida-

tive stress in tolerant plants and its enzymatic activity decreases in sensitive species or intraspecific varieties; thus, it is considered a good biochemical marker of stress tolerance (*Pogány et al.*, 2006; *Shigeoka et al.*, 2002; *Younis et al.*, 2009). Its activity increased in lettuce leaves exposed to elevated light intensity; it was not influenced significantly by mild salt stress exerted by 80 mM sodium chloride, but salt stress enhanced the effect of high light when the combination of the two factors was applied (Figure 4). This suggests that high salinity acts synergistically with high light intensity in triggering this enzymatic component of the antioxidative defence system of plant cells, and the interaction of high irradiance with salt stress results in a higher catalytic activity of ascorbate peroxidase. This is in accordance with the elevated vitamin C level of leaves exposed to high photon flux density, vitamin C being the organic substrate which accomplishes the reduction of hydrogen peroxide.

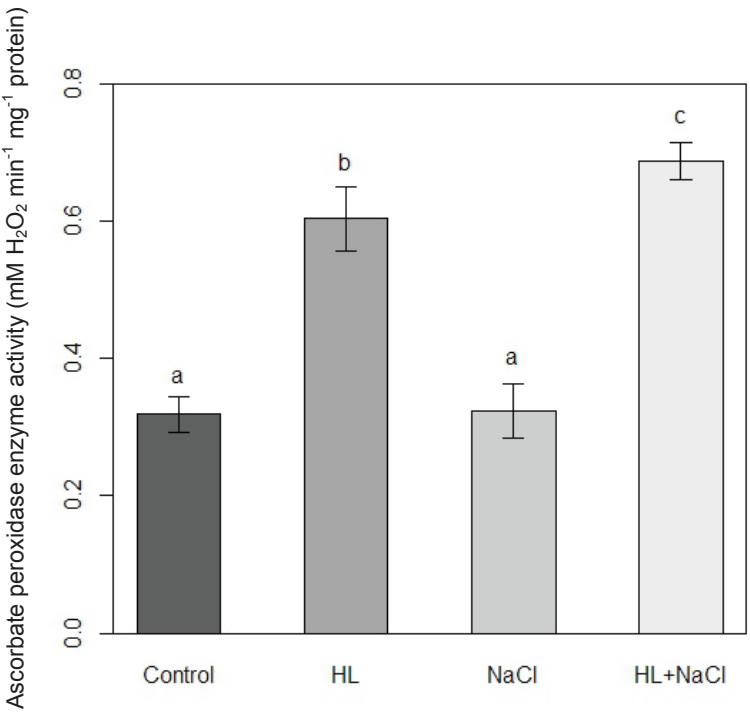

Figure 4: Influence of high light (HL) and salt stress on the enzymatic activity of ascorbate peroxidase in lettuce leaves. Bars represent means ± SE (n = 4). Different letters indicate significant differences at $P < 0.05$

4 Conclusions

Higher photon flux density increases carotenoid pigment content, water-soluble sugar concentration, vitamin C content and ascorbate peroxidase activity in lettuce leaves, thus conferring them a higher nutritive value related to enhanced antioxidant properties. Even though salt stress has no stimulating effect on carotenoid and simple sugar content of leaves, high salinity enhances the positive influence of high light on the accumulation of carotenoids and on the antioxidative activity of ascorbate peroxidase, and it reverses the action of elevated irradiance on water-soluble sugar content. Combination of moderately high photon flux density (660 μM photons $\mathrm{m}^{-2}\mathrm{s}^{-1}$) and mild salt stress (80 mM NaCl) improves the content of healthy phytochemicals in fresh lettuce leaves.

References

[1] P. Ahmad, M. M. Azooz, M. N. V. Prasad, *Ecophysiology and Responses of Plants under Salt Stress*, Springer (2012) 525.

[2] Altunkaya, V. Gokmen, Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce (*Lactuca sativa*), *Food Chemistry*, 107 (2008) 1173–1179.

[3] A. Altunkaya, E. M. Becker, V. Gokmen, L. H. Skibsted, Antioxidant activity of lettuce extract (*Lactuca sativa*) and synergism with added phenolic antioxidants, *Food Chemistry*, 115 (2009) 163–168.

[4] Cs. Bartha, L. Fodorpataki, E. Nagy, Zs. Gy. Keresztes, Gy. Székely, O. Popescu, Photosynthesis and water relations of leaf cells exposed to salt stress, *Annals of Romanian Society of Cell Biology*, 15 1 (2010) 211–218.

[5] Cs. Bartha, M. C. Martinez-Ballesta, L. Fodorpataki, O. Popescu, M. Carvajal, Screening parameters for salt stress tolerance of lettuce cultivars, based on physiological and biochemical responses, *Current Opinion in Biotechnology*, 22 1 (2011) 136–137.

[6] F. Eraslan, A. Inal, O. Savasturk, A. Gunes, Changes in antioxidative system and membrane damage of lettuce in response to salinity and boron toxicity, *Scientia Horticulturae*, 114 1 (2007) 5–10.

[7] J. J. Irigoyen, D. W. Emerich, M. Sanchez-Diaz, Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants, *Physiologia Plantarum*, 84 (1992) 67–72.

[8] J. Kohler, J. A. Hernandez, F. Caravaca, A. Roldan, Induction of antioxidant enzymes involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress, *Environmental and Experimental Botany*, 65 (2009) 245–252.

[9] R. Llorach, A. Martinez-Sanchez, F. A. Tomas-Barberan, M. I. Gil, F. Ferreres, Characterization of polyphenols and antioxidant properties of five lettuce varieties and escarole, *Food Chemistry*, 108 (2008) 1028–1038.

[10] H. Mahmoudi, J. Huang, M. Gruber, R. Kaddour, M. Lachaal, Z. Ouerghi, A. Hannoufa, The impact of genotype and salinity on physiological function, secondary metabolite accumulation, and antioxidative responses in lettuce, *Journal of Agricultural and Food Chemistry*, 58 (2010) 5122–5130.

[11] V. Mulabagal, M. Ngouajio, A. Nair, Y. Zhang, A. L. Gottumukkala, M. G. Nair, In vitro evaluation of red and green lettuce (*Lactuca sativa*) for functional food properties, *Food Chemistry*, 118 (2010) 300–306.

[12] M. M. Oh, E. E. Carey, C. B. Rajashekar, Environmental stresses induce health-promoting phytochemicals in lettuce, *Plant Physiology and Biochemistry*, 47 (2009) 578–583.

[13] M. M. Oh, H. N. Trick, C. B. Rajashekar, Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce, *Journal of Plant Physiology*, 166 (2009) 180–191.

[14] M. Pogány, B. D. Harrach, Y. M. Hafez, B. Barna, Z. Király, E. Páldi, Role of reactive oxygen species in abiotic and biotic stresses in plants, *Acta Phytopathologica et Entomologica Hungarica*, 41 1–2 (2006) 23–35.

[15] J. J. Rios, M. A. Rosales, B. Blasco, L. M. Cervilla, L. Romero, J. M. Ruiz, Biofortification of Se and induction of the antioxidant capacity in lettuce plants, *Scientia Horticulturae*, 116 (2008) 248–255.

[16] S. I. Roura, L. Pereyra, C. E. del Valle, Phenylalanine ammonia lyase activity in fresh cut lettuce subjected to the combined action of heat mild shocks and chemical additives, *LWT Swiss Society of Food Science and Technology*, 41 (2008) 919–924.

[17] Schonhof, H.-P. Klaring, A. Krumbein, W. Clausen, M. Schreiner, Effect of temperature increase under low radiation conditions on phytochemicals and ascorbic acid in greenhouse grown broccoli, *Agriculture, Ecosystems and Environment*, 119 (2007) 103–111.

[18] S. Shigeoka, T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta, K. Yoshimura, Regulation and function of ascorbate peroxidase isoenzymes, *Journal of Experimental Botany*, 53 372 (2002) 1305–1319.

[19] A. Ünlükara, B. Cemek, S. Karaman, S. Ersahin, Response of lettuce (Lactuca sativa var. crispa) to salinity of irrigation water, *New Zealand Journal of Crop and Horticultural Science*, 36 (2008) 265–273.

[20] A. Xu, S. Natarajan, J. H. Sullivan, Impact of solar ultraviolet-B radiation on the antioxidant defense system in soybean lines differing in flavonoid contents, *Environmental and Experimental Botany*, 63 (2008) 39–48.

[21] M. E. Younis, M. N. A. Hasaneen, S. M. N. Tourky, Plant growth, metabolism and adaptation in relation to stress conditions. XXIV. Salinity-biofertility interactive effects on proline, glycine and various antioxidants in *Lactuca sativa*, *Plant Omics Journal*, 2 5 (2009) 197–205.

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Synthetic methods for obtaining conjugated linoleic acids (CLA) by catalysis

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Abstract. Vegetable oils with high CLA content possess high nutritional value. The amount of the bioactive CLA isomers (cis-9,trans-11 and trans-10,cis-12) is very important from that respect. Lots of methods were developed for CLA synthesis. The most environmental friendly methods are based on microbial biosynthesis, but the isomerization yield is very low. Due to this reason, the microbial CLA synthesis is not competitive with the classical (chemical) alkaline isomerization process. With homogeneous catalysis using organometallic catalysts (Ru and Rh complexes), much higher (approximately 80%) yields were obtained with high selectivity related to bioactive isomers.

Key words and phrases: conjugated linoleic acid, CLA, homogeneous catalysis, heterogeneous catalysis, photocatalytic process, hydrotalcite.

The heterogeneous catalysis has the advantage that there is no need for a supplementary separation operation or the recycling of the catalyst at the end of the reaction. In a heterogeneous process, the maximum yield may even be higher than 90% and the selectivity remains quite high as the reaction conditions are optimized. The substrates for obtaining CLAs are in general linoleic acid or alkyl linoleates and the catalysis is acidic. Yield and selectivity depend on the strength and type of acidic sites as well as on the size distribution of the particles. Based on theoretical considerations, we propose a new hydrotalcite-type alkaline heterogeneous catalyst that may combine the high activity and/or stereoselectivity of the homogeneous alkaline catalyst and the well-known advantages of heterogeneous reaction systems. Besides the existing catalytic methods, a photocatalytic process with UV and visible light irradiation with iodine promoter can be applied, but in this case only highly purified oil substrates are suitable to obtain high CLAs yields.

1 Introduction

Traditionally conjugated linoleic acids (CLAs) were used as additives for oil-based dyes due to the increased reactivity of conjugated double bonds compared to non-conjugated ones, shortening this way the hardening time. Also, the CLAs are precursors (as monomers) for obtaining different biopolymers by radicalic or cationic copolymerization. Recently (*Ha et al.*, 1987, 1989), many new health-benefit effects of the CLAs were discovered. They decrease the body fat quantity and increase muscle mass, possess anti-inflammatory and cancer-preventive effects, exert beneficial effects on the skeletal system, act as immunostimulants and decrease the probability of asthma occurrence.

Theoretically, there are 56 isomers; however, few of them (cis-9,trans-11; trans-10,cis-12 and trans-9,trans-11-CLA) have shown significant biological activity. The bioactivity of the trans-9,trans-11-CLA isomer was discovered two decades ago (*Ha et al.*, 1987,1989) and in some cases its effect is stronger than that of the other isomers.

The predominant isomer (approx. 90%) in the natural products used as foods is the cis-9,trans-11-CLA. The main foods containing this isomer are milk, dairy products, meat and fat of the ruminants, as this isomer is produced by the bacteria of the rumen microflora.

Nowadays, CLAs are obtained for industrial purposes from vegetable oils that are processed at high temperature. The basic method in the industrial approach is the isomerization of linoleic acid (LA) with basic catalysis, using ethylene glycol as solvent and potassium hydroxide or potassium alkoxide as

catalyst. The separation of the catalyst at the end of the process is made by neutralization by orto-phosphoric acid. Through basic catalysis, the amount of the two formed isomers (cis-9,trans-11 and trans-10,cis-12-CLA) is almost equal (selectivity $\approx$ 50%) and the yield is quite high (over 80%). This synthetic method cannot be applied to the direct transformation of vegetable oils into CLA-rich triacylglycerols because ester bonds of triacylglycerols hydrolyze resulting free fatty acids (or salts) and glycerine.

The homogeneous catalytic methods using transition metallic complexes were intensively studied and almost quantitative transformation of linoleic acid into CLAs was reported. Despite the great selectivity and conversion, the main problem in this case is the separation of the catalyst from the products.

By using heterogeneous catalytic method to obtain CLAs, the separation problem is avoided, the catalyst can be separated by sedimentation or filtration (at laboratory scale), or even the separation is not necessary (at industrial scale) when fixed bed catalysts are used. The heterogenic catalysis may be a very attractive and efficient way for industrial processing of CLAs. The isomerization of linoleic acid or methyl linoleate by heterogenic catalysis was described, but the conversions were extremely low. The conclusion was that to achieve an acceptable level of conversion, the catalytic active metallic centres need to be partially saturated by absorption of high amount of hydrogen; however, this way, the selectivity was seriously affected by the appearance of a large amount of saturated by-products such as stearic and oleic acids. To avoid the formation of unwanted by-products, a two-step catalytic method was developed. In the first step, a ruthenium-based catalyst was saturated by hydrogen and then, in the second step, the linoleic acid was isomerized in nitrogen atmosphere. In this way, selectivity rose considerably, but still a great amount of hydrogenated product was formed. Using Ru/C catalyst and preactivation of the catalyst by hydrogen absorption (reaction conditions: temperature 120 °C; duration 6 hours), the amount of conjugated products was 53% and the amount of the hydrogenated products was 24%. Without preactivation, catalytic activity decreased significantly. When the carbon support was changed to zeolite or aluminate, the productivity of CLAs formation decreased comparatively to Ru/C, and the formation of the by-product acetylacetonate and $RuCl_3$ was observed.

As heterogeneous catalysts, silica-supported gold and silver catalyst was used in the presence of hydrogen. These metallic active centres possess low hydrogen-binding energy, and therefore the selectivity for hydrogenation (the secondary reaction with unwanted by-product formation) is quite low. In the light of the above, there may be a great opportunity for the development of

selective and active heterogeneous catalysts for the production of the main physiological active CLAs': cis-9,trans-11, trans-10,cis-12 and trans-9,trans-11-CLA isomers (*Philippaerts et al.*, 2011a). Simultaneously, it can be concluded that the homogeneous alkaline catalysis at high temperature has many drawbacks. Firstly, the catalyst is in solution and must be removed by neutralization at the end of the process, which is disadvantageous from environmental and economic point of view. The second drawback is that the product is a mixture of many different CLA isomers, not only of the health-benefit ones.

As long as the CLAs are used only for industrial purposes (in paints and inks to enhance their hardening properties), the multitude of isomers does not create any problem, but the use of the CLA isomer mixture is unacceptable as a component in functional foods. Finally, the application of CLA-enriched triacylglycerols would be more advantageous in food than free CLA fatty acids, but they cannot be obtained directly from vegetable oils using homogeneous base catalysis because of the concomitant saponification and transesterification reactions. Therefore, any new methods and procedures increasing the yield of production and able to enhance the purity of CLA mixtures are very welcome.

The synthetic methods for obtaining CLA, in general, can be categorized into three groups: microbiologic, catalytic (both homogeneous and heterogeneous) and photocatalytic methods (*Philippaerts et al.*, 2011a). In this review article, we wish to present the different synthetic methods developed for the obtaining of the CLAs.

Microbiologic methods for obtaining CLAs

The best methods for the obtaining of CLAs from environmental point of view are the microbial biosyntheses. Some microorganisms contain specific isomerase enzymes that are able to transform linoleic acid into CLAs. This isomerization occurs in the rumen of the ruminant mammals, where the main CLA isomer formed is the cis-9,trans-11 CLA (*Kim et al.*, 2002). Unfortunately, these bacterial species cannot be utilized for the industrial production of the CLAs because at the end of the biotransformation the intermediate product, that is CLA, is transformed into saturated products such as stearic acid. Moreover, bacteria do not synthetize the isomer cis-9,trans-11 until the linoleic acid concentration is high, producing the inhibition of biohydrogenation. Some microorganisms are known for possessing both linoleic acid isomerase and CLA reductase activity, and these are tolerant for linoleic acid. Other microorganisms produce the cis-9,trans-11 isomer only in small quantity; the main product in this case is the trans-10,cis-12 CLA (*Kemp*, 1984;

Kim et al., 2000; *Fukuda et al.*, 2005).

Some of the microorganisms living in the human intestinal tract are also able to produce the cis-9,trans-11 isomer. There are great variations among the tested microflora, and some of the species are able to produce even vaccenic acid. Also important differences exist between the cell lines, producing many different final products (*Coakley et al.*, 2003).

The majority of the rumen microorganisms are obligate or facultative anaerobes, that is, in a linoleic acid-rich medium, they are able to produce CLA isomers. The composition of the CLA mixture is influenced by the properties of the media and the concentration of the linoleic acid. Given the high isomeric specificity, the bacteria have the ability to produce only a minimal quantity of CLAs as the free linoleic acid is a growth-inhibitor for this type of bacterial strains. The majority of the studied species produce mainly the cis-9,trans-11 CLA, but a small amount of trans-9,trans-11 and cis-10,trans-12 isomers is also formed. Some researchers attempted to produce CLAs from LA in different cell cultures. In these cases, the initial LA concentration was set higher than in usual bacterial media to obtain the acceptable CLA concentration. In general, cell cultures could be used only once; however, some lactobacillus cultures were able to produce CLA in five consecutive cycles. Reusability was achieved by the immobilization of lactobacillus cells, but their activity was quite low, the reaction rate was 0.003 g/(L·min). The final concentration of 175 mg/L for CLA was obtained in an hour with 500 mg/L initial concentration of LA (*Ogawa et al.*, 2001). The mechanism of the bacterial biosynthesis of CLA from LA is described below. The first step of the consecutive reactions is water addition to the linoleic acid, and 10-hydroxi-12-octadecenoic acid is formed. In the second step, CLA is formed by the water loss of the intermediate product. This mechanism of CLA formation is absolutely identical with those of the acid-catalysed synthesis.

Beneath the isomerization of LA, the bacteria have the ability to transform the ricinoleic acid into CLA, the product containing preponderantly cis-9,trans-11 and trans-9,trans-11 isomers; selectivity depends on the reaction conditions. In the presence of the reaction mixture of different substances (serine, glucose, sodium chloride, or silver nitrate), the amount of trans-9,trans-11 isomer decreased, and thus the proportion of cis-9,trans-11 isomer may reach even the proportion of 75%. If the initial LA concentration is very low, the proportion of trans-9,trans-11 CLA may reach the value of 97% after a long reaction time due to the thermodynamic control of the isomerization (*Kishino et al.*, 2002). *Salamon et al.* (2006, 2009a) studied the seasonal variation of the fatty acid composition, the CLA content of the cows' milk and the effect

of milk processing on the CLA content of the different dairy products (*Salamon et al.*, 2007a). Particular attention was paid to the effect of microwave processing on isomerization of unsaturated fatty acids (*Salamon et al.*, 2007b, 2009b). The experiment was focused on cis-9,trans-11 CLA since this is the most abundant isomer present in milk and in dairy products in an amount of approx. 80%. During the experiments – with the aim to increase the CLA content of fermented dairy products –, sunflower oil (with high LA content) was added to raw milk (*Salamon et al.*, 2009c, 2009d, 2009e). The optimal concentration of the added LA was determined and the CLA productivity of strains and mixed cultures commercially utilized for industrial production of fermented dairy products was studied (*Salamon et al.*, 2009d, 2012).

As a conclusion, the CLA production using microbial culture is stereoselective and environmentally friendly in comparison with alkaline methods. Unfortunately, to obtain CLAs by microbiological method is possible only from free LA. For the production of CLA-rich triglycerides, a supplementary reaction step is needed, as triglycerides possess higher bioavailability than free fatty acids. The main problem with the microbiological methods is the low conversion of the reactant, and from that reason the microbiological methods are not competitive with the classical alkaline izomerization method. The best yield obtained microbiologically is 6 mg CLA per minute in a litre of reaction media (*Salamon et al.*, 2009c, 2012), about 500 times lower than in the case of the homogeneous alkaline process. Otherwise, it is obvious that microorganisms have had an important role in obtaining CLA-rich food products instead of the production of pure CLA at large scale. For example, the CLA content of the milk and that of the dairy products could be influenced by changing the fodder composition of the ruminants. The CLA composition of the fermented dairy products or cheeses could be raised by using microbial strains with high CLA-producing potential. Despite of a lot of accumulated knowledge, more studies are needed to elucidate whether the CLA amount produced by lactobacillus or propionobacteria strains during the fermentation process for the obtaining of yoghurt and the ripening of cheeses is sufficient to produce physiological benefit effects in the human organism (*Sieber & Collomb*, 2004; *Philippaert et al.*, 2011a; Salamon et al., 2009c, 2012).

Synthesis of CLAs with metal catalysts

In the metal-catalysed synthesis of CLA, both homogeneous and heterogeneous catalytic methods are developed, but these methods have not been implemented in practice yet. Many researches were focused on the isomer-

ization of linoleic acid or linoleic acid-rich oil using organometallic complexes or supported metal particles as catalyst (*Philippaerts et al.*, 2011a).

Synthesis of CLAs with homogeneous organometallic catalysts

For the obtaining of CLAs and CLA-containing oils, chromium, platinium, ruthenium and rhodium complexes were tested. The highest productivity with such catalysts was 97%. In this case, the substrate for obtaining CLA was methyl linolate (reaction conditions: reaction time 24 hours; temperature 60 °C, ethanol solvent) with ruthenium complex (0.1 mol% $[RhCl(C_8H_{14})_2]$) in the presence of 0.8 mol% $SnCl_2.2H_2O$ and 0.4 mol% $(p\text{-}CH_3C_6H_4)_3P$. At this mild reaction temperature, the substrate is not limited to fatty acids or their alkyl esters like in the base-catalysed or microbial isomerization processes, but triglycerides such as soybean or safflower oils have also been transformed in a single step into their conjugated derivatives. An amount of 100 g soybean oil in 300 mL ethanol solvent was almost completely converted (with selectivity for 95%) into conjugated products within one day, corresponding to a productivity of 0.11 g CLA/(L·min) (*Larock et al.*, 2001).

Homogeneous Ru and Rh complexes used as catalysts are quite active, but their productivity is one order of magnitude lower than in the case of the classic alkaline process. The catalytic turnover is about 20 substrate molecules transformed per metal atom in one hour. Depending on the combination of metal complexes and different solvents, the product is a mixture of CLA isomers with different composition. For example, when three types of rhodium-complex catalysts ($[RhCl(C_8H_{14})_2]_2$, $RhCl(PPh_3)_3$ and $RhCl_3\ 3H_2O$) are used under same reaction conditions in ethanolic solution, in the first two cases the cis-trans and trans-cis isomers are dominant, while in the second case the trans-trans isomer is the dominant one. Thus, in methanol solvent, a higher selectivity towards the desirable cis-9,trans-11 and trans-10,cis-12 CLA isomers is obtained. From methyl linoleate substrate, after 24 hrs of reaction time, the obtained CLA yield was 83% and the cumulated selectivity toward cis-9,trans-11 and trans-10,cis-12 isomers was 79%. Using soybean oil as substrate (under the same reaction conditions), the conversion was almost quantitative, with the selectivity toward the desired isomers being 75%. Similar to the isomerization process performed with strong alkaline catalysts, it is generally observed that with the raising of the temperature, the amount of the trans,trans isomers increases due to their higher thermodynamic stability (*Andjelkovic et al.*, 2006; *Larock et al.*, 2001). Despite their obvious advantages, homogeneous metal complexes are not used at industrial scale due to their very high production

costs. The price of rhodium is approximately ten times the price of ruthenium, while platinum is two to five times more expensive than ruthenium.

When CLAs are destined to use as food additives, the choice of the solvent is very important since there may remain solvent traces in the product. Practically, only GRAS (Generally Regarded as Safe) solvents are accepted since the total separation of the solvent from the product is very complicated. Another difficulty is the removal of the toxic and soluble metal complexes from the product mixture and the reuse of these very expensive catalysts. To bypass the presented drawbacks for the isomerization of soybean oil and methyl linoleate, a biphasic catalytic system with ionic liquids has been suggested recently. At the end of the reaction, the ionic liquid phase containing the metal complex can be easily separated from the product, preventing the CLA from metal contamination (*Consorti el al.*, 2009).

The mechanism of isomerization in presence of organometallic complexes is somewhat different from the alkaline-catalysed reaction. The key step is the complexation of the fatty acid olefinic double bond by transition-metal catalytic centre. The next step is hydrogen atom abstraction nearby a double bond, followed by a hydrogen addition reaction. The mechanism, in brief, is a consecutive hydrogen addition-elimination, formally similar with the mechanism of heterogeneous isomerization, opening the possibility towards a competitive hydrogenation with the formation of different isomers (*Frankel*, 1970).

Synthesis of CLAs with heterogeneous catalysis

Most heterogeneous catalysts contain transition metals for the reaction with conjugating double bonds in polyunsaturated fatty acids. The bonding on the substrate on metal surfaces includes the addition-elimination steps (*Kreich & Klaus*, 2005a, 2005b). This mechanism consists of three steps. The first step is the chemisorption of hydrogen on the catalyst surface and its dissociation into two hydrogen atoms; the second step is the chemisorption of the double bond of the substrate (linoleic acid or methyl linoleate) on the catalyst surface, concomitant with the opening of the double bond and the formation of two new bonds with the catalyst, which leads to the formation of a stronger adsorbed complex. The third step is the migration of a hydrogen atom on the catalyst surface toward one of the carbon atoms of the adsorbed substrate complex, forming a semi-hydrogenated intermediate. If the hydrogen load on the catalytic surface is rather low, a hydrogen adjacent to the surface-bonded carbon atom is eliminated and a double bonded adsorbed species is formed. This complex at the surface of the catalyst, however, may adopt another configu-

ration, which leads, after desorption, to the formation of different geometrical and positional isomers of linoleic acid.

When the hydrogen load of the catalytic surface is high, a second hydrogen atom is added to the half-hydrogenated intermediate, leading to double-bond saturation, and this way mono-unsaturated fatty acids are formed. This mechanism can be repeated with the primary intermediates (CLA isomers, non-conjugated linoleic acid isomers and mono-unsaturated fatty acids), and hence other CLA isomers, non-conjugated linoleic acid isomers and stearic acid may be formed. *Kreich* and *Claus* (2005a, 2005b) performed the isomerization of the LA with silver catalyst in presence of hydrogen. The Ag/SiO_2 catalyst was synthesized by incipient wetness impregnation of silver lactate on SiO_2, followed by drying at 80 °C and reduced at 325 °C under hydrogen flow – it resulted in an average Ag particle size of 14 nm (Ag content of catalyst 7.7 wt%). At first, they achieved good results with ruthenium catalysts (Ru/C, Ru/Al_2O_3) – in agreement with literature reports. The use of a typical hydrogenation metal catalyst as nickel promised no improvement (*Bernas et al.*, 2003). Afterwards, they developed the silver-mediated catalyst in presence of hydrogen for the obtaining of CLA. In agreement with literature data, silver is the metal that possesses the lowest hydrogen binding energy, silver crystals bond the hydrogen very weak. In the silver catalysed hydrogenation of the polyunsaturated carbon-carbon double bonds, the efficiency is very low in comparison with other metallic catalysts. Because of this behaviour of the silver, the formation of the oleic and stearic acids is hindered. The isomerization with silver ion catalysis was performed under similar conditions as in the case of ruthenium-doped catalysts, namely, in nitrogen atmosphere after catalyst preactivation with hydrogen. Under these conditions, as conversion was not observed as expected, surprisingly good results were achieved in direct synthesis of CLAs over heterogeneous silver catalysts and in the constant presence of hydrogen: the 90% conversion was achieved after 90 minutes over Ag/SiO_2, the selectivity was approx. 60-67% towards CLAs. It was found that the particle size did not exhibit a noticeable influence on the catalytic process. High amount of the trans-9,trans-11 isomer was obtained at high conversions. The linoleic acid conversion increased with the reaction temperature, while the physiologically important cis-9,trans-11- and trans-10,cis-12 isomers remained always the main components of the products. Furthermore, it was observed that the selectivity towards the undesired saturated hydrogenation products (oleic acid and stearic acid) decreased with decreasing substrate/catalyst mass ratio. The transformation of linoleic acid was also carried out without the preactivation of the Ag catalyst with hydrogen and the same degrees of conversion

and selectivity were obtained as those with preactivation. Similar results were found using supported gold catalysts (Au on Al_2O_3, Fe_2O_3, CeO_2, MnO_2, TiO_2, ZrO_2, activated carbon, silicalite TS-1) in the presence of hydrogen at 165 °C in a batch reactor. The best results were obtained using a catalyst with 2 wt% Au on TS-1, which exhibits high selectivity (78%) towards CLA (*Bauer et al.*, 2009).

The isomerization of linoleic acid or methyl linoleate toward CLAs by heterogeneous catalysis is made through hydrogenated intermediates. The proposed reaction mechanism has six different steps (*Bernas et al.*, 2004): migration of one double bond of linoleic acid, which results in the formation of CLA; positional and geometric isomerization of CLA; hydrogenation of one double bond of linoleic to mono-unsaturated fatty acid; positional and geometric isomerization of mono-unsaturated fatty acids; hydrogenation of the double bond of a mono-unsaturated fatty acid to stearic acid (*Philippaerts et al.*, 2011b). During the heterogeneous catalytic process, the isomerization and hydrogenation reactions are competitive. Since the isomerization is reversible, the process eventually leads to the formation of the thermodynamically most stable trans,trans CLA isomers. The hydrogenation can be considered as an irreversible consecutive reaction that lowers the CLA yield. In the majority of the heterogeneous catalytic isomerization systems, hydrogen is needed for the forming of the half-hydrogenated intermediates, which will finally transform into final products: the CLAs. On the other hand, the elevated level of hydrogen will lead to the formation of unwanted hydrogenated by-products. Hence, the direct production of CLAs by heterogeneous catalysts is a difficult and complicated process. The activity and selectivity for the isomerization are dependent on the surface structure and the hydrogen adsorption capacity of the metal-dopped catalyst. Metals with a high hydrogen binding capacity (such as palladium) show a high activity and selectivity for the double-bond hydrogenation of LA, while other metals (such as silver and gold) with low hydrogen binding activity show a low activity and selectivity, hence the migration process of the carbon-carbon double bonds. The high activity of ruthenium for double bond isomerization is explained by the fact that the ruthenium contains free d-orbitals, with ability to interact with the π-bonds of linoleic acid and being capable of activating the adjacent C-H bond. These steps are necessary for catalytically migrating double bonds (*Bernas et al.*, 2004; *Bernas & Murzin*, 2003, 2005).

The acid and base active sites on the surface of the catalysts also interact with the carbon-carbon double bonds, and facilitate the isomerization. The Brønsted acids may form carbenium ions. Then, the subsequent stabilization

of the ionic intermediate by a proton expulsion occurs, resulting the migration of the double bond, yielding to an initial cis/trans product ratio of around 1:1. Conversion of linoleic acid was strongly affected by the amount and strength of the acidity. Higher amounts of weak-moderate acidic sites (both, Brønsted and Lewis type) favour the conversion of LA over conjugated CLAs and increase the selectivity toward the main isomer of interest, cis9-trans11 CLA. There may exist a synergic effect between Brønsted and Lewis centres. When the acid centres are stronger, the catalytic activity and selectivity decrease faster in time (*Cardó et al.*, 2012). On active basic sites, carbanions are formed, yielding mainly to cis,cis CLA isomers by an allylic mechanism (*Pines & Stalick*, 1977).

Obtaining CLAs from linoleic acid, alkyl linoleates and triglycerides

By utilizing the main advantage of heterogeneous catalysts, i.e. the easy catalyst/product separation, this type of catalysts can be used in a continuous process in a fixed-bed reactor or a continuous stirred tank reactor (CSTR), enabling the simple and cheap synthesis of functional food ingredients. The continuous operation has a major technological advantage with higher productivity. As the active metal particle or site is well-dispersed on a solid material, heterogeneous catalysts are much easier to be taken apart and recycled, as they overcome the separation difficulties of the soluble metal (*Bauer et al.*, 2009; *Philippaerts et al.*, 2011a). For the isomerization of alkyl linoleates to obtain CLAs, several metals (Ni, Ru, Rh) on various supports were used. At high concentration, nickel was the most active, but ruthenium was found the best from an economic point of view. Ruthenium and rhodium were used in a concentration of 5%, immobilized on active carbon surface, and were activated with hydrogen adsorption. The reaction was carried out at the temperature of 180–240°C, in nitrogen atmosphere. If the reaction was carried out in methanol or isopropanol as a solvent, a high activity and selectivity toward saturated by-products were obtained, whereas using hexane or cyclohexane, the selectivity increased toward the desired CLA isomers. Parallel with isomerization, the polymerization reaction occurs, since this process is catalysed also by metallic centres. The addition of a minimal quantity of nickel to the Ru/C catalyst increased the selectivity toward cis-trans CLA isomers (*Deshpande et al.*, 1985; *Narasimhan et al.*, 1985).

Different metal catalysts (Ru, Ni, Pd, Pt, Rh, Ir, Os, and bimetallic Pt-Rh) on various supports (carbon, γ-Al_2O_3, SiO_2/Al_2O_3, and on zeolites) have been screened more systematically (*Bernas et al.*, 2002, 2003, 2004, 2005) for the isomerization of linoleic acid to CLAs. The reactions were carried out in n-decane in the temperature range of 80–120°C. They showed that pretreatment of the metal catalysts with hydrogen is not required for conjugation, but the activity of the catalyst is drastically increased when the catalyst is brought into contact with H_2 at elevated temperature prior to reaction. The main disadvantage of such treatment is the emergence of the competitive hydrogenation due to the presence of activated (chemisorbed) hydrogen on the metal surface. However, quite high CLA concentrations were found in the product mixture and the degree of saturation was low compared with the pressurized hydrogenation. Pd possesses great selectivity for hydrogenation, while Ni, Ru and Pt are more selective for isomerization. In the case of Ru, the activity and selectivity is highly dependent on the distribution of Ru particles and on the nature of ruthenium compounds. Larger Ru-clusters increase the selectivity towards CLA formation. The presence of chlorine together with Ru affects the distribution of the CLA isomers. For example, impregnation of γ-aluminate with ruthenium acetyl acetonate results in a catalyst highly selective towards cis-9,trans-11 and trans-10,cis-12 CLA isomer formation (i.e., 68–75% selectivity), whereas impregnation with $RuCl_3.3H_2O$ shows preference for t9, t11 CLA (40% selectivity) (*Bernas et al.*, 2004). Probably, this selectivity difference is caused by the acidity of chlorine, which – similar to acidic zeolites – increases the selectivity toward to all-trans CLA isomers. The temperature has a major influence on reaction rate and selectivity. Contrary to expectations, reaction rate is decreased with temperature increase; in contrast, selectivity increases drastically toward CLAs. The explanation is that higher temperature promotes the hydrogen desorption from the catalyst surface, favouring isomerization.

Philippaerts and co-workers (2011a) developed a novel catalytic method for obtaining CLAs, using highly dispersed ruthenium-dioxide on zeolite support in hydrogen-free condition. As the hydrogenation step was totally missing, the highest yield and selectivity toward the regarded CLA isomers were obtained. The optimal catalyst was identified using several types of zeolites with different Si/Al ratio in their composition (causing structural modification) and changing the amount of countercations (H^+, Na^+, Cs^+) of the aluminosilicate lattice. The most active and selective isomerization catalyst was the Ru/Cs-doped USY zeolite with a Si/Al ratio of 1:40, at the reaction temperature of 165°C. In this condition, from methyl linoleate substrates, mainly cis-9,trans-

11 and trans-10,cis-12 CLA isomers were formed with an overall yield of 67% and a selectivity of 82% at low catalyst concentrations. The main advantage of this catalyst is that no hydrogen pretreatment or addition of hydrogen donors are required, and the obtained productivity was the industrially relevant 0.7 g/(l·min) of CLAs.

Proposal for a new type of heterogeneous catalyst for CLA formation

Despite that different types of hydrotalcites are known as isomerization catalysts, hydrotalcite catalysts were not utilized until now for obtaining CLAs from LA substrate. Their advantage is – as in general with heterogeneous catalysts – that the separation from the reaction mixture is easy at the end of the reaction. In the case of using a fixed bed reactor, even this step may be omitted, as the reaction mixtures flow through the void space of the catalyst bed. Another advantage of the hydrotalcites is that they are cheap, nontoxic and their structure possesses stereoselectivity. Hydrotalcites (HTs) are mixed hydroxides with natural origins, possessing excellent anion exchange capacity and high specific surface, stable at high temperatures, with metal-oxide active centres. Their industrial applications increased in the second half of the last century in the production of different organic substances as catalysts or catalyst supports. The chemical formula of the natural HTs is $Mg_6Al_2CO_3(OH)_{16}\cdot 4H_2O$, layered double hydroxides, magnesium aluminium hydroxyl-carbonate tetrahydrate. The structure of the polymorphic variants depends on the nature of cations included into the layers. The exchange of Mg^{2+} ions with Al^{3+} ions results a positive charge excess in the layers, which will be compensated by hydroxyl or other anions. The structure of HTs strongly depends on many factors such as the synthesis method, the pH of reaction media, the temperature as well as on the applied washing and drying method. Generally, it can be concluded that the nanolayers are elastic, the crystalline water contained by the interlayers is mobile and reversibly removable. The HTs are soluble in acids, and by thermal treatment macro-, meso- and micropores can be formed in them.

The methods for obtaining layered double hydroxides are based on coprecipitation, sol-gel calcinations/rehydration techniques. In most of the cases, Mg/Al or Mg/Zn containing HTs are obtained, but in this way it is possible to synthetise HTs with various metal ion content in their crystalline structure. The morphology, crystallinity degree and the structure of crystalline phases depend on the technique of coprecipitation, reaction temperature and matura-

tion time. By the addition of different anions and organic molecules during the rehydration process, a large variety of structures were obtained (*Mitsudome et al.*, 2008).

Kishore & Kannan (2002, 2006) used MgAl-HTs for the basic isomerization of eugenol and safrole, and for obtaining anetole from estragole. While no data were found in literature for the utilization of HT-catalysts for obtaining CLAs, we consider this proposal as a novelty.

Obtaining CLAs by photochemical methods

An alternative way besides microbial methods and metal catalysis is photoisomerization. Japanese scientists (*Seki et al.*, 1998) obtained CLA methyl esters with high yield (80%) from a diluted methyl linoleate solution (5-10%) in petrol ether, using iodine as photocatalyst and with intense visible light irradiation, without reporting the isomer composition of the products. Another research group extended the method to the production of CLA-rich vegetable oil by direct isomerization of soybean oil by UV-light irradiation of solventless substrate, with iodine sensitizer. Under irradiation, the I_2 possesses radical forming potential. In the first experiment (*Gangidi & Proctor*, 2004), 6 mg CLA (cis-9,trans-11 CLA isomer) was obtained from 1g soybean oil (0.25 w/w% I_2, irradiation time 85 hrs, irradiation source: mercury lamp, power 100 W). By the optimization of the process and performing the reaction in stirred batch photo-reactor (*Jain & Proctor*, 2006), a production of 240 mg CLA/g substrate was obtained (0.15 w/w% I_2, irradiation time 144 hrs), but the isomeric composition was unfavourable, as the rate of trans,trans and that of cis,cis isomers was about 5:1. The main influencing factors are the irradiation flux, the stirring intensity and the concentration of the catalyst.

Recently, the isomerization of soybean oil was performed in pilot scale (1 L volume) laminar flow photo-reactor, irradiated with UV-Vis lamp (reaction conditions: lamp power: 450 W; I_2 concentration: 0.35% w/w; temperature: 48 °C; reaction time: 12 hrs), with a yield of 220 mg CLA/g soy oil. The main isomer (approx. 80%) was the trans,trans CLA (*Jain et al.*, 2008a). Soybean oil should be refined prior to photoisomerization (*Jain et al.*, 2008b); otherwise, the productivity of CLAs decreases drastically (with approx. 98%). Minor components, particularly peroxides and phospholipids, should be removed. Tocopherols enhanced CLA yield at low level, but decreased it at high level. Lutein and free fatty acids seem to have little effect on CLA production (*Tokle et al.*, 2009). The iodine-catalysed photoisomerization of LA has a radical mechanism: the photolytically formed iodine radicals formed in the

initiation step can react with the unsaturated substrate in two ways. When the iodine radical directly adds the double bond, an unstable iodinated radical intermediate is formed, which allows bond rotation (stereoisomerization) and the formation of the more stable (trans) isomer. Alternatively, when the iodine abstracts a hydrogen atom, forming hydrogen iodide and allylic radical, then positional isomerization occurs, the trans,trans conjugated product (being the most stable isomer) is formed. It is important to emphasize that – due to the radical nature of the photocatalytic reaction – the isomerization needs to be carried out in inert atmosphere to ensure high chemoselectivity; otherwise, the iodine radicals may be inactivated by other compounds (*Philippaerts et al.*, 2011a).

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References

[1] D. D. Andjelkovic, M. Byungrok, D. Ahn, R. C. Larock, Elucidation of structural isomers from the homogeneous rhodium-catalyzed isomerization of vegetable oils, *J Agric Food Chem.*, 54 (2006) 9535–9543.

[2] P. Bauer, P. Horlacher, P. Claus, Direct isomerization of linoleic acid to conjugated linoleic acid using gold catalyst, *Chem Eng Technol.*, 32 (2009) 2005–2010.

[3] A. Bernas, D. Y. Murzin, Influence of hydrogen preactivation on the linoleic acid isomerization properties of supported ruthenium catalyst, *React Kinet Catal Lett.*, 78 (2003) 3–10.

[4] A. Bernas, D. Y. Murzin, Linoleic acid isomerization on Ru/Al_2O_3 catalyst: 1. Conjugation and hydrogenation, *Chem Eng J.*, 115. (2005) 13–22.

[5] A. Bernas, D. Y. Murzin, Linoleic acid isomerization on Ru/Al_2O_3 catalyst: 2. Elementary step mechanism and data fitting, *Chem Eng J.*, 115 (2005) 23–43.

[6] A. Bernas, N. Kumar, P. Laukkanen, J. Vayrynen, D. Y. Murzin, Influence of ruthenium precursor on catalytic activity of Ru/Al2O3 catalyst in selective isomerization of linoleic acid to cis-9,trans-11 and trans-10, cis-12 conjugated linoleic acid, *Appl Catal.*, 267 (2004) 121–133.

[7] A. Bernas, N. Kumar, P. Maki-Arvela, B. Holmbom, T. Salmi, D. Y. Murzin, Heterogeneous catalytic production of conjugated linoleic acid, *Org Proc Res Dev.*, 8 (2004) 341–362.

[8] A. Bernas, N. Kumar, P. Maki-Arvela, B. Holmbom, T. Salmi, D. Y. Murzin, Heterogeneous catalytic production of conjugated linoleic acid, *Org Proc Res Dev.*, 8 (2004) 341–352.

[9] A. Bernas, N. Kumar, P. Maki-Arvela, N. V. Kulkova, B. Holmbom, T. Salmi, D. Y. Murzin, Isomerization of linoleic acid over supported metal catalysts, Appl Catal A., 245. (2003) 257–275.

[10] A. Bernas, P. Laukkanen, N. Kumar, P. Maki-Arvela, J. Vayrynen, E. Laine, B. Holmbom, T. Salmi, D. Y. Murzin, A new heterogeneously catalytic pathway for isomerization of linoleic acid over Ru/C and Ni/H-MCM-41 catalysts, J. Catal., 210 (2002) 354–366.

[11] A. Bernas, P. Maki-Arvela, N. Kumar, B. Holnbom, T. Salmi, D. Y. Murzin, Heterogeneously catalytic isomerization of linoleic acid over supported ruthenium catalysis for production of anticarcinogenic food constituents, *Ind Eng Chem Res.*, 42 (2003) 718–727.

[12] X. Cardo, O. Bergada, Y. Cesteros, P. Salagre, Effect of catalyst acidity and porosity on the catalytic isomerization of linoleic acid to conjugated linoleic acid (CLAs), *Chem Eng J.*, 183 (2012) 459–465.

[13] M. Coakley, R. P. Ross, M. Nordgren, G. Fitzgerald, R. Devery, C. Stanton, Conjugated linoleic acid biosynthesis by human-derived Bifidobacterium species, *J. Appl. Micribiol.*, 94 (2003) 138–145.

[14] C. S. Consorti, G. L. P. Aydos, G. Ebeling, J. Dupont, Multiphase catalytic isomerization of linoleic acid by transition metal complexes in ionic liquids, *Appl. Catal.*, 371 (2009) 114–120.

[15] V. M. Deshpande, R. G. Gadkari, D. Mukesh, C. S. Narasimhan, Studies on kinetics of catalytic isomerization of methyl linoleate, *J. Am. Oil Chem. Soc.*, 62 (1985) 734–738.

[16] E. N. Frankel, Homogeneous catalytic conjugation of polyunsaturated fats by chromium carbonyls, *J Am Oil Chem.*, 47 (1970) 33–36.

[17] S. Fukuda, H. Y. Furuya, N. Sizuki, T. Asanuma, Hino, A new strain of Butyrivibrio fibrisolvens that has high ability to isomerize linoleic acid to conjugated linoleic acid, *J. Gen Appl Microbiol.*, 51 (2005) 105–113.

[18] R. R. Gangidi, A. Proctor, Photochemical production of conjugated linoleic acid from soybean oil, *Lipids*, 39 (2004) 577–582.

[19] Y. L. Ha, N. K. Grimm, M. W. Pariza, Newly recognized anticarcinogenic fatty acids: identification and quantification in natural and processed cheeses, *Journal of Agricultural and Food Chemistry*, 37 (1989) 75–81.

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

[21] V. P. Jain, A. Proctor, Photocatalytic production and processing of conjugated linoleic acid-rich soy oil, *J Agric Food Chem.*, 54 (2006) 5590–5596.

[22] V. P. Jain, A. Proctor, R. Lall, Pilot-scale production of conjugated linoleic acid-rich soy oil by photoirradiation, *J. Food Sci.*, 73 (2008a) E183–E192.

[23] V. P. Jain, T. Tokle, S. Kelkar, A. Proctor, Effect of the degree of processing soy oil conjugated linoleic acid yields, *J Agric Food Chem.*, 56 (2008b) 8174–8178.

[24] P. Kemp, D. J. Landrer, The hydrogenation of the series of methylene-interrupted cis,cis-octadecadienoic acids by pure cultures of six rumen bacteria, *Br J Nutr.*, 52 (1984) 171–177.

[25] Y. J. Kim, R. H. Liu, D. R. Bond, J. B. Russel, Effect of linoleic acid concentration on conjugated linoleic acid production by Butyrivibrio fibrisolvens A38, *Appl Environ Microbiol.*, 66 (2000) 5226–5230.

[26] Y. J. Kim, R. H. Liu, J. L. Rychlik, J. B. Russel, The enrichment of a ruminal bacterium (Megasphaera elsdenii YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid, *J. Appl Microbiol.*, 92 (2002) 976–982.

[27] S. Kishino, J. Ogawa, Y. Omura, K. Matsumura, S. Shimizu, Conjugated linoleic acid production from linoleic acid by lactic acid bacteria, *J. Am. Oil. Chem. Soc.*, 79 (2002) 159–163.

[28] D. Kishore, S. Kannan, Isomerization of eugenol and safrole over MgAl hydrotalcite, a solid base catalyst, *Green Chem.*, 4 (2002) 607–610.

[29] D. Kishore, S. Kannan, Catalytic isomerization of estragole to anethole over hydrotalcites and HT-like compounds, *J. Molec Catal.*, 244 (2006) 83–92.

[30] M. Kreich, P. Claus, Direct conversion of linoleic acid over silver catalyst in the presence of H2: An unsual way toward conjugated linoleic acid, *Angew Chem Int Ed.*, 44 (2005b) 7800–7804.

[31] M. Kreich, P. Claus, Direkte Umsetzung von Linolsäure an Silber-Katalysatoren in Gegenwart von H2: ein ungewöhnlicher Weg zu konjugierten Linolsäuren, *Angew Chem.*, 117 (2005b) 7979–7983.

[32] R. C. Larock, X. Dong, S. Chung, C. K. Reddy, L. E. Ehlers, Preparation of conjugated soybean oil and other natural oils and fatty acids by homogeneous transition metal catalysis, *J Am Oil Chem Soc.*, 78 (2001) 447–453.

[33] T. Mitsudome, Y. Mikami, H. Funai, T. Mizugaki, K. Jitsukawa, K. Kaneda, Oxidant-free alcohol dehydrogenation using a reusable hydrotalcite-supported silver nanoparticle catalyst, *Angew Chem Int Ed.*, 47 (2008) 138–141.

[34] S. Narasimhan, D. Mukesh, R. R. Gadkari, V. M. Deshpande, Kinetics and mathematical modeling of isomerization of methyl linoleate on ruthenium catalyst. 2. Conjugation and polymerization, *Ind Eng Chem Prod Res Dev.*, 24 (1985) 324–326.

[35] J. Ogawa, K. Matsumura, S. Kishino, Y. Omura, S. Shimizu, Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by Lactobacillus acidophilus, *Appl. Environ Microbiol.*, 67 (2001) 1246–1252.

[36] A. Philippaerts, S. Goossens, P. A. Jacobs, B. F. Sels, Catalytic production of conjugated fatty acids and oils, *ChemSusChem*, 4 (2011a) 684–702.

[37] A. Philippaerts, S. Goossens, W. Vermandel, M. Tromp, S. Turner, J. Geboers, G. V. Teldeloo, P. A. Jakobs, B. S. Sels, Design of Ru-zeolites for hydrogen-free production of conjugated linoleic acids, *ChemSusChem*, 4 (2011b) 757–767.

[38] H. Pines, W. M. Stallick, Base catalyzed reactions of hydrocarbons and related compounds, *Academic Press*. (1977)

[39] R. V., Salamon, K. Lóki, Sz. Salamon, P. Sára, B. Albert, Zs. Mándoki, Zs. Csapó-Kiss, A. Győri, Z. Győri, J. Csapó, Changes in the fatty acid composition of different milk products caused by different technology, *Agriculture*, 13 (2007) 189–191.

[40] R. V. Salamon, K. Lóki, Zs. Csapó-Kiss, J. Csapó, Changes in the fatty acid composition and conjugated linoleic acid content of sour dairy products caused by pure cultures, *Acta Universitatis Sapientiae, Alimentaria*, 2 (2009) 276–286.

[41] R. V. Salamon, K, Lóki, Zs., Csapó-Kiss, Sz. Salamon, J. Csapó, Fatty acid profile of sour dairy products produced by different strater cultures, *Acta Agriculturae Slovenica*, 3 (2012) 323–326.

[42] R. V. Salamon, K. Lóki, Sz. Salamon, B. Albert, P. Sára, A. Győri, Z. Győri, Zs. Csapó-Kiss, J. Csapó, Changes in fatty acid composition of foodstuffs during conventional and microwave heat treatment, *Krmiva*, 49 (2007) 23–28.

[43] R. V. Salamon, K. Lóki, É. Varga-Visi, Zs. Mándoki, J. Csapó, Increase of conjugated linoleic acid content of dairy products by adding sunflower oil, *Krmiva*, 51 (2009) 99–103.

[44] R. V. Salamon, K. Lóki, É. Varga-Visi, Zs. Mándoki, J. Csapó, Increase of conjugated linoleic acid content of dairy products by adding sunflower oils, *Acta Universitatis Sapientiae, Alimentaria*, 2 (2009) 287–293.

[45] R. V. Salamon, Zs. Mándoki, Zs. Csapó-Kiss, A. Győri, Z. Győri, J. Csapó, Changes in fatty acid composition of different milk products caused by different technology, *Acta Universitatis Sapientiae, Alimentaria*, 2 (2009) 101–109.

[46] R. V. Salamon, É. Varga-Visi, Zs. Csapó-Kiss, A. Győri, Z. Győri, J. Csapó, The influence of the season on the fatty acid composition and conjugated linoleic acid content of the milk, *Acta Universitatis Sapientiae, Alimentaria*, 2 (2009) 89–100.

[47] R. V. Salamon, É. Varga-Visi, P. Sára, Zs. Csapó-Kiss, J. Csapó, The influence of the season on the fatty acid composition and conjugated linolic acid content of the milk, *Krmiva*, 48 (2006) 193–200.

[48] K. Seki, R. Kaneko, K. Kobayashi, Photoconjugation of methyl linoleate in the presence of iodine as a sensitizer, *Yukagaku*, 38 (1998) 949–954.

[49] R. Sieber, M. Collomb, A. Aeshlimann, P. Jelen, H. Eyer, Impact of microbial cultures on conjugated linoleic acid in dairy products-a review, *Int. Dairy J.*, 14 (2004) 1–15.

[50] T. Tokle, V. P. Jain, A. Proctor, Effect of the minor oil constituents on soy oil conjugated linoleic acid production, *J. Agric Food Chem.*, 57 (2009) 8989–8997.

Acta Univ. Sapientiae, Alimentaria, **5** (2012) 52–62

Gas chromatographic analysis of conjugated linoleic acids

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Abstract. The aim was to determine the conjugated linoleic acids in beef with GC-FID. Perfect separation of all of the isomers cannot be achieved, though conjugated linoleic acid isomers proved to be separable from the other fatty acids that are present in beef in a significant amount related to conjugated linoleic acids. Lipid-extraction was carried out with n-hexane/i-propanol, giving 6.8 percent higher yield than Soxhlet extraction. The variance – due to different parts of the sample preparation process – was quantified. The accuracy of the whole process and the efficiency of triglyceride conversion/fatty acid methyl ester extraction were determined.

1 Introduction

The physiological effects of conjugated linoleic acid isomers (CLA) are in the scope of interest and as a consequence there is an increasing need for the quantification of CLA from food. At the beginning of this decade, it has been

Key words and phrases: conjugated linoleic acid, GC-FID, beef, fatty acid methyl ester.

shown that silver ion liquid chromatography (Ag-HPLC) possesses the best resolving power for the separation of CLA-isomers [1,2]. Now, when it is coming to an end, the question arises why Ag-HPLC does not become the dominant analytical technique for CLA analysis. Authors in the previous years still often used GC alone or Ag-HPLC together with GC [3-5]. The possible cause may be that in Ag-HPLC the potential source of errors could be not only the variations in factors that are common in LC, but other parameters (e.g., sample size, solvent composition and even storage times) could also hamper obtaining reproducible results. Moreover, the batch-to-batch variation in the silver loadings of the columns is also a problem [6]. On the contrary, the gas chromatographic determination of fatty acid composition is easier to implement and proper identifying tools are available. The serious disadvantage of this technique is the improper resolution of CLA-isomers. The best gas chromatographic separation of fatty acid methyl esters (FAME) has been achieved with the use of 100 m long, 100% cyanopropyl polysiloxane stationary phase columns [7-9], and recently fast gas chromatographic methods have been developed in order to achieve the same resolution in much less time [3,4].

Our analytical task was to achieve a reliable method for the determination of the CLA content of beef. Owing to the local conditions, only gas chromatographic analysis was considered, and we tried to carry out the best realizable performance. In one respect, our aim was to determine the possibility and the limits of CLA determination with GC-FID; partly, the reliability of the sample preparation steps was investigated. The main steps of the method were lipid-extraction, transesterification, extraction of fatty acid derivatives and gas chromatographic analysis. Lipid extraction was achieved using a mixture of hexane/isopropanol, because it has been shown to have more advantages over the extraction with chloroform/methanol, which are rapid phase separation, less proteolipid contamination and less toxic solvents [10]. CLA and the other fatty acids present in the glycerides were transesterified by an alkali-catalysed reaction with sodium methoxide in order to avoid changes in the ratio of the CLA-isomers [11].

2 Experimental

Meat samples

Beef samples were obtained from Priváthús Ltd., Kaposvár. Samples originated from four parts of the carcass (thick flank, fore rib, thick rib and neck) were collected five different times. The weight of the individual samples was approx. 100 g. Beef samples were mixed and stored in a freezer (-24 °C).

General scheme of sample preparation and analysis

Lipids were extracted with a mixture of n-hexane/i-propanol 3:2 (v/v)[10]. 80 mL n-hexane/i-propanol mixture (HIP) was added to approx. 10 g meat sample (max. 0.3 g fat); then a suspension was prepared with an Ultra-Turrax T25 basic type dispersion tool (IKA WERKE GMBH, Germany). Solid particles were removed with vacuum filtration. After filtration had been completed, the filter funnel was rinsed three times with 10 mL HIP. Liquid phase was clarified from the soluble non-lipid fraction: it was extracted with 60 mL 0.47 M sodium sulphate in water. The organic phase was separated, dried under water-free Na_2SO_4 and the solvent was then removed under vacuum in a rotary evaporator. The crude lipid extract was dissolved in 10 mL of n-hexane.

Transesterification of glycerides was carried out with sodium methylate in methanol: 0.5 mL lipid extract in n-hexane and 0.5 ml sodium methoxide solution (0.5 M) was mixed and warmed at 50 °C for 30 min. When the reaction had been completed, 1 mL of distilled water was added and FAME was extracted four times with 1 mL hexane and diluted up to the final volume of 5 mL. All solvents and reagents were of analytical grade, the '37-component FAME mix' was obtained from Supelco, while 'conjugated linoleic acid mixture' was purchased from Sigma.

The separation of FAME was accomplished with a Chrompack CP 9000 gas chromatograph. The injection was manual, the split ratio was 16:1 and the injected amount was 6 μL at 270 °C. The column was a CP-Sil 88 (FAME) with a dimension of 100 m × 0.25 mm and the film thickness of the stationary phase was 0.2 μm. The final temperature programme: the temperature of the column was immediately increased from 130 °C to 225 °C at a rate of 2 °C min^{-1}. At 225 °C, isotherm conditions were applied for 20 min; the carrier gas was He (230 kPa, 16.1 cm/s). The temperature of FID was 270 °C. The initial temperature programme that was developed for other FAMEs was changed in order to optimize the resolution of CLA-isomers. The shifts in retention times were observed with the use of the '37-component FAME mix' and also with 'conjugated linoleic acid mixture'. The determination of the limit of detection (LoD) and limit of quantification (LoQ) was based on the slope of calibration curve and the noise of blank (n-hexane).

Checking the reliability of the sample preparation steps

Meat samples were extracted with HIP and the fat contents were determined. These values were compared with the crude fat contents obtained with the Soxhlet method, which is applied for the determination of the fat content of

meat in Hungary (MSZ ISO 1443:2002). In the case of fat determination with HIP-method, sample preparation was carried out as described above, until the evaporation of the solvent from the clarified HIP solution. The residuum was kept at 98 °C for 2 hours in a drying chamber, let to cool down in a desiccator, then the extract was weighed. The drying was repeated until the weight was constant.

In the case of the following two steps of sample preparation, the monitoring of transesterification and FAME extraction were not separated. The precision of transesterification and FAME extraction with hexane was determined with the parallel methylation of the same HIP-extract following extraction with hexane. The accuracy was determined with the transesterification of the known amount of glycerides. The ideal model solution would be an 'artificial beef fat' with similar fatty acid pattern as in beef, or at least CLA glycerol esters with a similar ratio as in beef fat. Due to purchasing difficulties, the efficiency of the above processes was determined with the use of myristic acid glycerol ester (MGE). The methyl ester of myristic acid (MME) appears at the beginning of the chromatogram and its peak area ratio within the sum of the FAMEs in beef is approx. 2%. During the determination of efficiency of transesterification and extraction, 0.150 g MGE was dissolved in 5 mL n-hexane and 0.5 mL from this solution was used for transesterification with 0.5 mL 0.5 M sodium methylate in methanol following the same method as in the case of the meat samples. The amount of the resulting MME derivatives was determined with external standard calibration.

Besides the examination of particular parts of the sample preparation, the reliability of the whole procedure was also evaluated. The MGE stock solution contained 199.2 mg MGE in 100 mL HIP and 2.5 mL of this solution was added to the initial HIP solution, then extracted, clarified, transesterified, and the resulting MME was extracted and measured. The recovery of MGE was determined in the form of MME.

Two-samples T-test was used for the comparison of means, the homogeneity of the variances were checked with F-test and the type of the T-test was chosen according to the results of the F-test. Due to the manual injection and the long cycle time (68 min), the number of measurement/day was restricted according to the up-to-date GC systems; consequently, the number of repetitions was limited.

3 Results

During the examination of the specificity of the analytical method, it was observed – as in the case of the other studies [9] – that with the use of the above means the separation of the CLA-isomers cannot be achieved perfectly, though the CLA-isomers proved to be separable from the other fatty acids that are present in the beef in significant quantities related to CLA. The most abundant isomer, c9,t11-CLA-ME, coelutes with t8,c10-CLA-ME, while the separation of c11,t13-CLA-ME and t10,c12-CLA-ME is adequate; then, the minor c,c- and t,t-isomers come after (Figure 1).

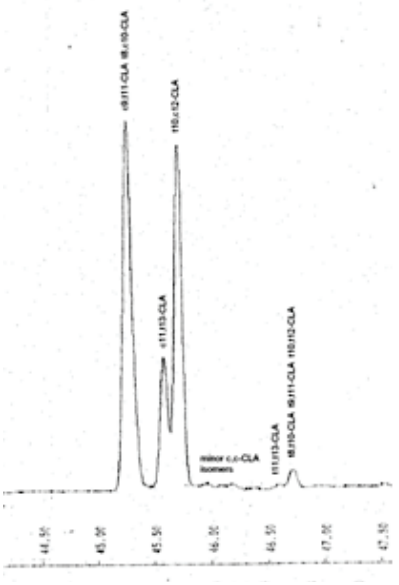

Figure 1: The partial separation of conjugated linoleic acid methyl esters with GC. Standard: 'Conjugated linoleic acids' from Sigma. Chrompack CP 9000 GC; 6 μL manual split (16:1) injection (270 °C); column: 100 m × 0.25 mm CP-Sil 88 (FAME) from 130 °C to 225 °C at a rate of 2 °C min^{-1}, at 225 °C isotherm for 20 min; carrier gas: He (230 kPa, 16.1 cm/s); FID (270 °C)

The resolution could be improved but the cycle time would be too long, more hours. In the chromatogram of the mixture of the two test solutions, it can be seen that heneicosanoic acid methyl ester (C21:0-ME) eluates between the first and the second CLA-ME peaks (Figure 2).

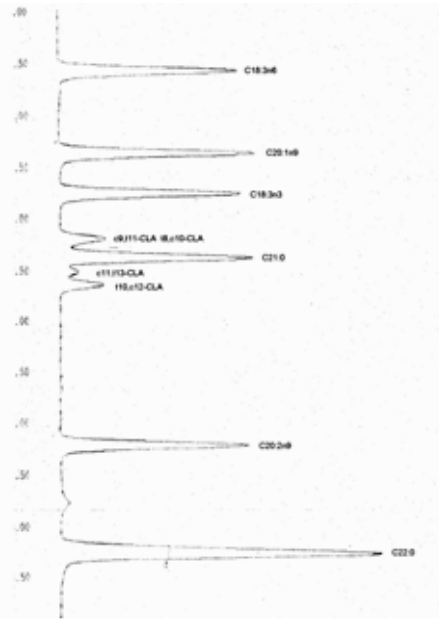

Figure 2: The separation of conjugated linoleic acid methyl esters from the other fatty acid methyl esters with GC. Standards: 'Conjugated linoleic acids', from Sigma and '37 component FAME mix' from Supelco'. GC conditions as Fig. 1

In the beef samples, only the signal of the first CLA peak (c9,t11-CLA-ME/t8,c10-CLA-ME) was big enough for quantification, although more minor isomers were present, but their signal was about or less than the limit of detection (Figure 3).

Table 1 presents the linearity of the examined CLA-isomers.

Table 1: Comparison of the amount of lipids extracted with hexane/isopropanol to the amount of lipids extracted with the Soxhlet method. Sample: fore rib.

Component	t_R	Concentration range (μg mL^{-1})						Slope [S]**	Intercept [I] **	r	LoD (μg mL^{-1})	LoQ (μg mL^{-1})
		No.1	No.2	No.3	No.4	No.5	No.6					
c9,t11-CLA-ME t8,c10-CLA-ME*	45.26	0.511	2.40	4.78	6.90	9.39	18.85	1.461	1.092	0.9992	0.193	0.643
c11,t13-CLA-ME	45.58	0.104	0.771	1.40	2.09	2.87	5.70	1.461	0.333	0.9992	0.193	0.643
t10,c12-CLA-ME	45.71	0.385	1.83	3.82	6.01	7.74	15.45	1.458	0.877	0.9991	0.193	0.645

*c9,t11-CLA and t8,c10-CLA were not separated.

** $A = S \cdot c - I$ A = peak area (mV·s); S = slope; I = intercept; c = concentration (μg mL^{-1})

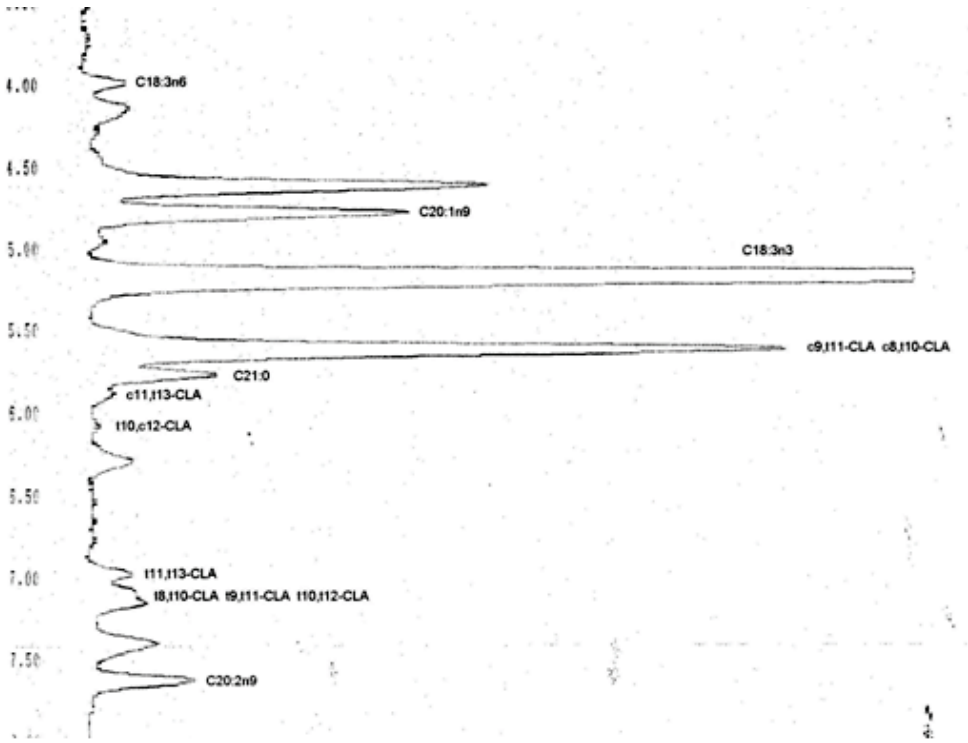

Figure 3: The part of the chromatogram of fatty acid methyl esters of beef, in which conjugated linoleic acid methyl esters are present. Sample: fore rib. GC conditions as Fig. 1

The slope of the equation describing the relationship between the concentrations of isomers and the peak area are similar to each other (1,458-1,461 $mV{\cdot}s{\cdot}mL{\cdot}\mu g^{-1}$); consequently, the LoD and LoQ values of the CLA-isomers are very close. Since slope values of the isomers were very alike, the amount of the two coeluting compounds in the first CLA-peak was evaluated together. The concentration of the most diluted (No.1.) standard solution was below the LoQ, thus standard solutions from No.2. to No.6. were used for calibration and result evaluation. The chromatogram of No.1. standard solution is shown in Figure 4.

The amount of residuum was significantly ($p \leq 0.05$) higher in the case of HIP-extraction than in that of the Soxhlet method (Table 2). With HIP, on average, we extracted 6.8 percent more material than with the other method.

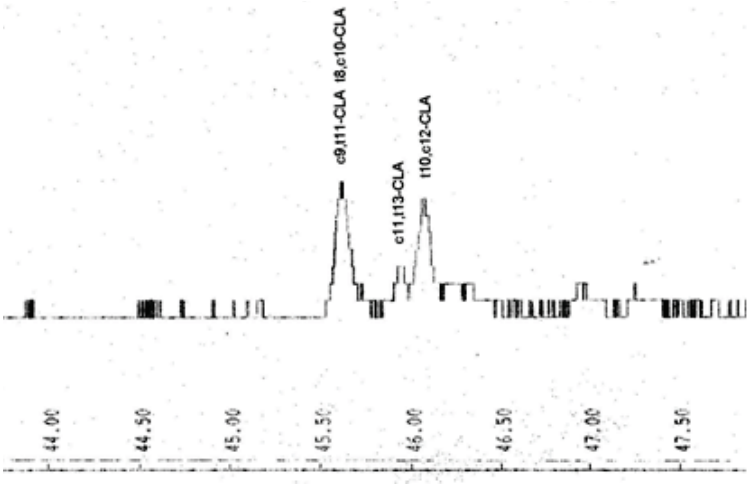

Figure 4: The chromatogram of standard solution No. 1 with concentrations close to the limit of detection. Concentration of conjugated linoleic acid methyl esters are shown in Table 1. GC conditions as Fig. 1

While HIP resolves some parts of carbohydrates and proteolipides, washing with sodium sulphate solution was reported to eliminate most of these compounds, as it was indicated with negative iodine test, and ninhydrine positive substances were also not detected [10]. In our application, the aim of the extraction was not the exact determination of crude fat content, but the extraction of all of the lipid classes containing fatty acids in ester bond; therefore, the possible presence of some other substances does not mean a problem.

Table 2: Comparison of the amount of lipids extracted with hexane/isopropanol to the amount of lipids extracted with the Soxhlet method. Sample: fore rib.

Method	Fat content (g fat/100 g sample)								
	No.1.	No.2	No.3	No.4	No.5	No.6	Mean	SD	RSD (%)
HIP-extraction	3.85	4.13	4.05	4.04	4.28	4.22	4.09	0.151	3.7
Soxhlet method	3.78	3.95	4.00	4.00	3.41	3.72	3.81	0.228	6.0

The precision of transesterification/FAME extraction can be seen in Table 3. The variation coefficient or relative standard deviation (RSD) is smaller for MME than in the case of c9,t11-/t8,c10-CLA-ME. The first component is

present in higher quantities in the fat of the beef. The same tendency can be noticed in the precision data obtained from the whole sample preparation process (Table 3).

Table 3: The precision of transesterification/FAME extraction and that of the total sample preparation and analysis for myristic acid methyl ester (MME) and conjugated linoleic acid methyl esters (CLA-ME). Sample: thick flank.

Component (mg/g sample)	Transesterification/FAME extraction (n=6)			Total sample preparation and analysis (n=3)		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)
MME	0.300	0.0066	2.2	0.284	0.027	9
c9,t11-CLA-ME t8,c10-CLA-ME*	0.074	0.0028	3.8	0.070	0.0073	10

*c9,t11-CLA and t8,c10-CLA were not separated.

The sum of the error variances (s^2) of the individual steps gives the variance of the complete process:

$$s(\text{total})^2 = s(\text{sampling})^2 + s(\text{lipid extraction})^2 + s(\text{transesterification/FAME extraction})^2 + s\ (\text{analysis})^2$$

The above relationship can be described with the square of estimated relative standard deviation. For the extraction of lipids, $RSD^2 = 3.7^2$, but this value estimates the variation in total lipid content, which is not by necessity the same for the examined fatty acids.

In the case of the examination of transesterification/FAME extraction, the effect of GC-analysis on variance was not separated from the effect of sample preparation; thus, for the determination of MME, the next value can be substituted:

$$\text{RSD (transesterification/FAME extraction)}^2 + \text{RSD (analysis)}^2 = 2.2^2$$

For the complete process of MME analysis:

$$9^2 = \text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2 + 2.2^2$$

Thus:

$$[\text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2]^{1/2} = 8.7\%$$

For the determination of c9,t11-/t8,c10-CLA-ME:

$$\text{RSD (transesterification/FAME extraction)}^2 + \text{RSD (analysis)}^2 = 3.8^2$$

The equation describing the variance of the complete process in this case is:

$$10^2 = \text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2 + 3.8^2$$

That is:

$[\text{RSD (sampling)}^2 + \text{RSD (lipid extraction)}^2]^{1/2} = 9.2\%$

Assuming that the variance in the concentration of the analytes – due to lipid extraction – is the same as the variance in lipid content, RSD(sampling) is 7.8% for MME and 8.5% for the examined CLA isomers. It would seem that sampling is the bottle-neck of the process because it is responsible for three-quarters of the total variation. The homogeneity and/or the size of the sample should be increased.

Table 4 presents the recovery of transesterification/FAME extraction expressed as the conversion efficiency of MGE and the extraction efficacy of the resulting MME. On average, 91% of the glycerides were converted into FAME and extracted. The accuracy of the complete process is also shown in Table 4.

Table 4: The accuracy of transesterification/FAME extraction and that of the total sample preparation and analysis. (1) Myristic acid glycerol ester was transesterified and the formed myristic acid methyl ester (MME) was extracted and measured. (2) Myristic acid glycerol ester was extracted with HIP, transesterified and myristic acid methyl ester was extracted and measured.

Transesterification/FAME extraction (n=6) (1)				Total sample preparation and analysis (n=3) (2)			
MME (mg)	Mean	SD	RSD (%)	MME (mg)	Mean	SD	RSD (%)
Nominal *	15.54	–	–	Nominal **	4.93	-	-
Measured	14.17	0.85	6.0	Measured	3.53	0.09	2.6
Recovery %	91	5.4	6.0	Recovery %	72	1.9	2.6

*Nominal: the amount of MME that would form if the efficiency of transesterification and extraction were 100%

**Nominal: the amount of MME that would form if the efficiency of lipid-extraction, transesterification and extraction were 100%

The efficiency of lipid extraction can be estimated based on the following equations:

$$\text{Nominal MME (mg)} \cdot E \cdot A = \text{measured MME (mg)}$$

$$\text{Nominal MME (mg)} \cdot V = \text{measured MME (mg)},$$

where:

E = efficiency of lipid extraction

A = efficiency of transesterification/FAME extraction = 0.91

V = efficiency of the complete process = 0.72

Thus

$$E = V \cdot A^{-1} = 0.72 \cdot 0.91^{-1} = 0.79$$

It seems that the most important part of the losses could be assigned to the incomplete extraction of MGE.

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References

[1] B. Nikolova-Damyanova, S. Momchilova, W. W. J. Christie, *High Resol Chromatogr*, 23 (2000) 348–352.

[2] M. P. Yurawecz, K. M. Morehouse, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[3] C. Moltó-Puigmartí, A. I. Castellote, M. C. López-Sabater, *Anal Chim Acta*, 602 (2007) 122–130.

[4] I. Bondia-Pons, C. Moltó-Puigmartí, A. I. Castellote, M. C. López-Sabater, *J Chromatogr A*, 1157 (2007) 422–429.

[5] P. Luna, M. Juárez, M. A. de la Fuente, *J Chromatogr A*, 1204 (2008) 110–113.

[6] M. Collomb, A. Schmid, R. Sieber, D. Wechsler, E. L. Ryhänen, *Int Dairy J*, 16 (2006) 1347–1361.

[7] N. Sehat, J. K. G. Kramer, M. M. Mossoba, M. P. Yurawecz, J. A. G Roach, K. Eulich, K. M. Morehouse, Y. Ku, *Lipids*, 33 (1998) 963–971.

[8] J. K. G. Kramer, J. Zhou, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[9] J. K. G. Kramer, C. Cruz-Hernandez, Y. Zhou, *Eur J Lipid Sci Technol*, 103 (2001) 594–632.

[10] A. Hara, N. S. Radin, *Anal Biochem*, 90 (1978) 420–426.

[11] Y. Park, K. J. Albright, Z. Y. Cai, M. W. Pariza, *J Agric Food Chem*, 49 (2001) 1158–1164.

Acta Univ. Sapientiae, Alimentaria, **5** (2012) 63–72

Effect of temperature on AFB1, AFB2, AFG2 and T-2 mycotoxins' decomposition in sunflower oil under the irradiation of ultraviolet light

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Abstract. After developing the decontamination method with UV light and establishing the specific analytical procedure with multiple extraction and HPLC, a method was developed to determine the effect of temperature on AFB1, AFB2, AFG2 and T-2 mycotoxins' decomposition in sunflower oil. This paper presents an experimental study concerning the decontamination of mycotoxins in sunflower oil with a new type of photoreactor; the aim of this study was to determine the influence of temperature during the photochemical process. Experiments were conducted at different temperatures, observing the mycotoxin concentration decrease. Our study on the decontamination process shows the relevance of temperature effect; data confirm the differences between AFB1, AFB2, AFG2 and T-2 mycotoxin behaviour. In the photochemical conditions used, obtained mathematical models and specific data can be used to determine the conditions needed by the evolved refining process. Comparing the proposed decontamination process with the classic procedure, photochemical decontamination can be used to increase the nutrient value of the sunflower oil.

Key words and phrases: mycotoxins, photodegradation, sunflower oil, HPLC.

1 Introduction

In the case of the industrial-scale production of sunflower oil, a large portion of mycotoxins, contained previously in oilseeds, is transferred due to the specific solubility in triglycerides. The main manufacturing process uses cold or warm pressing, solvent extraction and later refining, but these stages do not lower the mycotoxin concentrations. As a consequence, the product may become undesirable, usually transferring to bio-fuels. The photochemical decontamination process may be advantageous if added to the end of the refining process, because the obtained product has a higher nutrient value. In contrast with this, other decontamination methods are not convenient due to the minimal intervention principle. In the recent years, several methods have been developed to determine the mycotoxin content of foods, but decontamination process was not considered available (*Hussein-Brasel*, 2001). Reducing or removing mycotoxin concentration from sunflower oil is a food engineering interest because mycotoxins have a multitude of negative health effects on mammals (*Richard*, 2007). Previous studies were conducted to determine the influence of the initial peroxide index of sunflower oil (*Agachi-Gombos*, 2010), the initial concentration of mycotoxins and the influence of bentonite on suspension (*Gombos-Agachi*, 2010).

Scientific publications contain very little useful information and kinetic data on the thermal stability of mycotoxins. In the contaminated sunflower oil's photochemical treatment process is expected that temperature may have small influence on the decontamination process (*Lippolis et al.*, 2008). Taking into account the multitude of contained chemical species, we consider useful to determine the influence of temperature and to find any favourable temperature of the photodegradation of mycotoxins in this composition matrix.

Finding temperature influence on sunflower oil decontamination has two major purposes: to reduce the concentration of mycotoxins, to determine the influence of each mycotoxin decontamination temperature investigated to achieve more efficient decontamination (*Sheppard*, 2008). Later, the sunflower oil refining processes have to use those values of temperatures in the industrial photochemical treatment.

Experimental part

In order to determine temperature effect on mycotoxin decontamination, we performed 4 series of experiments for each mycotoxin, conducted at different temperatures, that is, at 20, 30, 40 and 50 °C. The initial concentration of

mycotoxins was established based on scientific literature (*Sheppard*, 2008), temperature range was chosen based on the need to use small temperature changes, as the sunflower oil temperature at the end of the classical refining process is 25–28 °C. Due to economic reasons, significant temperature changes are not desirable since they would involve massive heat transfer equipments and energy costs. Figure 1 shows the schematic diagram of the experimental photoreactor system.

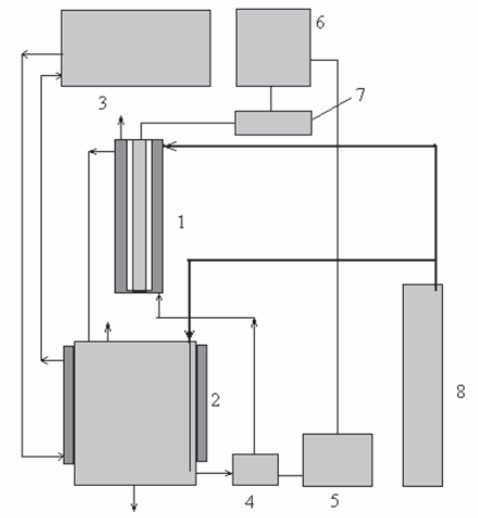

Figure 1: Schematic diagram of the experimental photoreactor system 1 – Plug-flow photoreactor (PFR); 2 – Buffer vessel; 3 – Ultrathermostat; 4 – Adjustable flow pump; 5 – Controller; 6 – Power switch unit; 7 – UV source power control unit; 8 – CO_2 cylinder

Reaction mass samples were collected from the photoreactor's effluent flow, which samples were extracted with methanol in five steps; the extracts were purified and concentrated, later analysed with Varian Star HPLC, using Varian Starn Chromatography Workstation Version 6.00 software, Supelcosil LC 18 column, 0,9 ml/min flow, eluent mixture of water, methanol and acetonitrile (130: 70: 40), excitation at 365 nm, emission at 435 nm, without derivatization (*Turner et al.*, 2009). Data statistical analysis allows observations in more details; for this purpose, experimental data values were processed in Statistica 6.0 software environment, using the Distance-Weighted Least Squares Fitting (DWLSF) method.

2 Results and discussion

Observed AFB1 ($c/c_{0\ AFB1}$), AFB2 ($c/c_{0\ AFB2}$), AFG2 ($c/c_{0\ AFG2}$) and T − 2 ($c/c_{0\ T-2}$) relative concentration values depending on irradiation time (t, minutes) at 20, 30, 40 and 50 °C operating temperatures (corresponding to initial peroxide index value $IP_0 = 1$) are presented in figures 2, 3, 4 and 5.

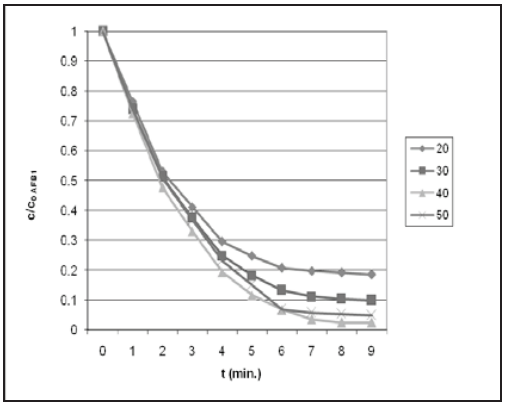

Figure 2: AFB1($c/c_{0\,AFB1}$) **relative concentration depending on irradiation time (t, minutes) at 20, 30, 40 and 50 °C** ($IP_0 = 1, c_{0\,AFB1} = 2$ **μg/kg)**

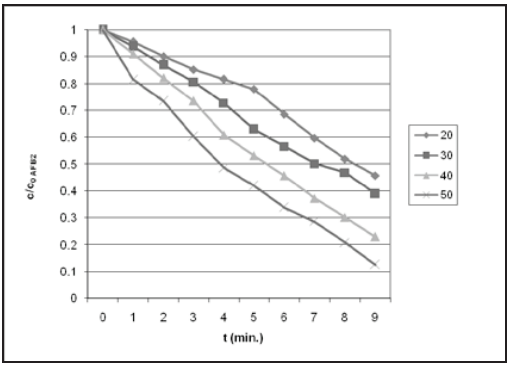

Figure 3: AFB2($c/c_{0\ AFB2}$) **relative concentration depending on irradiation time (t, minutes) at 20, 30, 40 and 50 °C** ($IP_0 = 1, c_{0\,AFB2} = 2$ **μg/kg)**

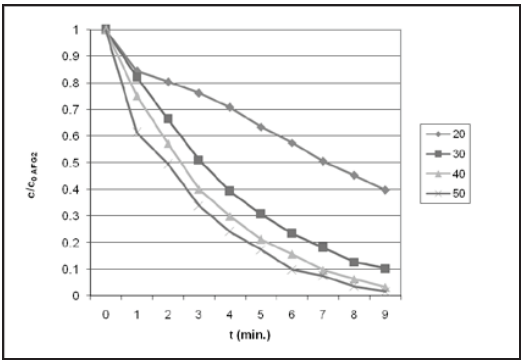

Figure 4: $\mathrm{AFG2}(c/c_{0\ \mathrm{AFG2}})$ **relative concentration depending on irradiation time (t, minutes) at 20, 30, 40 and 50 °C** ($\mathrm{IP}_0 = 1, c_{0\,\mathrm{AFG2}} = 2$ **µg/kg)**

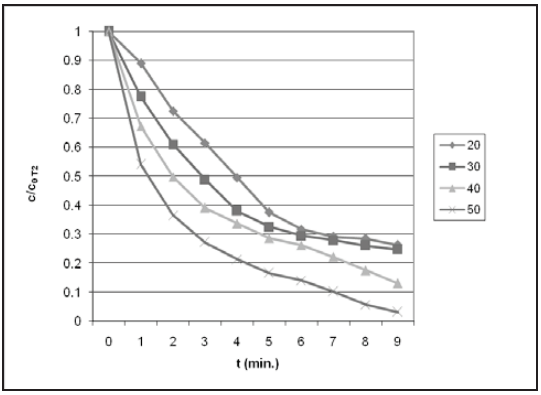

Figure 5: Relative concentration of $\mathrm{T}-2(c/c_{0\ \mathrm{T}-2})$ **depending on irradiation time (t, minutes) at 20, 30, 40 and 50 °C** ($\mathrm{IP}_0 = 1, c_{0\,\mathrm{T2}} = 2$ **µg/kg)**

Variations of $c/c_{0\,\mathrm{AFB1}}$ function of irradiation time at 20, 30, 40 and 50 °C show a relatively poor sensitivity of degradation rate. However, the overall temperature increase has a favourable effect on AFB1 photodegradation. At relatively low UV irradiation periods (up to 3 minutes), the maximum slopes of the curves are relatively similar, and then they decrease. Significant data were collected at 40 and 50 °C; at 50 °C, the value of $c/c_{0\,\mathrm{AFB1}}$ is more favourable than at 40 °C. The polynomial equations of $c/c_{0\,\mathrm{AFB1}}$ function of irradiating time t are:

– at 20 °C : $y = -0.001x^3 + 0.04x^2 - 0.374x + 1.342$ ($R^2 = 0.998$); (1)

– at 30 °C : $y = -0.001x^3 + 0.037x^2 - 0.374x + 1.34$ ($R^2 = 0.999$); (2)

– at 40 °C : $y = -0.001x^3 + 0.039x^2 - 0.395x + 1.36$ ($R^2 = 0.999$); (3)

– at 50 °C : $y = 0.03x^2 - 0.35x + 1.32$ ($R^2 = 0.999$). (4)

Based on the graph (Fig. 2), it is obvious that the favourable temperature range is 20-5 °C; a faster AFB1 photodegradation in sunflower oil is at 41 °C. Taking into account that reaction mass temperature rises inside the photoreactor, typically 0.5–1.5 °C, the favourable initial reaction mass temperature is 40 °C, depending on residence time and irradiative intensity of the UV source. Variations of $c/c_{0\,AFB2}$ indicate a relatively low sensitivity by temperature, but $c/c_{0\,AFB2}$ increase at higher temperatures. Clearly, increasing temperature has favourable effect on decreasing $c/c_{0\,AFB2}$ value, but data indicates nonlinear variations, particularly on 2–5-minute irradiation times, at 40 and 50 °C. The polynomial equations, which describe the variation of $c/c_{0\ AFB2}$ function of irradiation time t, are:

– at 20 °C : $y = -0.002x^2 - 0.032x + 1.028$ ($R^2 = 0.994$); (5)

– at 30 °C : $y = -0.006x^2 - 0.043x + 1.05$ ($R^2 = 0.997$); (6)

– at 40 °C : $y = 0.02x^3 + 0.018x^2 - 0.041x + 1.056$ ($R2 = 0.999$); (7)

– at 50 °C : $y = 0.016x^2 - 0.199x + 1.178$ ($R^2 = 0.997$). (8)

Figure 4 shows low sensitivity of decay rate; the relative concentration decreases at 30, 40 and 50 °C, but differently from 20°C, which indicate even lower sensitivity. The polynomial equations of $c/c_{0\,AFG2}$ dependence on irradiation time t are:

– at 20 °C : $y = -0.008x^3 + 0.069x^2 - 0.277x + 1.207$ ($R^2 = 0.996$); (9)

– at 30 °C : $y = -0.014x^2 - 0.229x + 1.219$ ($R^2 = 0.999$); (10)

– at 40 °C : $y = -0.001x^3 + 0.031x^2 - 0.328x + 1.297$ ($R^2 = 0.999$); (11)

– at 50 °C : $y = -0.012x^3 + 0.12x^2 - 0.607x + 1.482$ ($R^2 = 0.999$). (12)

Figure 5 shows a slightly different sensitivity of $c/c_{0\,T-2}$ by temperature than other (previously studied) mycotoxins, probably due to the specific molecular structure of $T-2$ toxin. Polynomial equations of $c/c_{0\,T-2}$ by irradiation time t are:

– at 20 °C : $y = 0.006x^3 - 0.045x^2 - 0.021x + 1.061$ ($R^2 = 0.998$); (13)

– at 30 °C : $y = -0.001x^3 + 0.033x^2 - 0.313x + 1.281$ ($R^2 = 0.999$); (14)

$$- \text{ at } 40\,^{\circ}\text{C}: \; y = -0.011x^3 + 0.123x^2 - 0.613x + 1.497 \; (R^2 = 0.999); \quad (15)$$

$$- \text{ at } 50\,^{\circ}\text{C}: \; y = -0.021x^3 + 0.209x^2 - 0.908x + 1.708 \; (R^2 = 0.997). \quad (16)$$

Figure 6 illustrates the obtained AFB1 photodegradation's concentration-time-temperature relations with this fitting method.

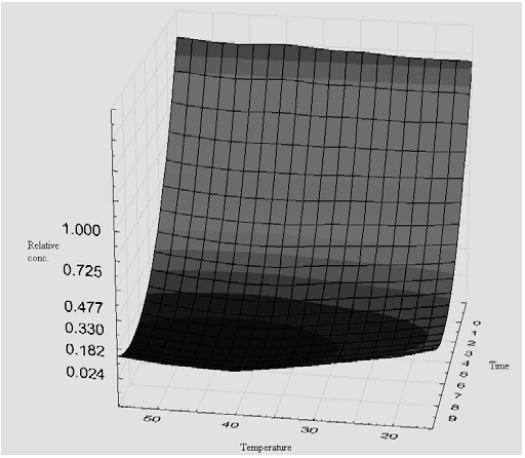

Figure 6: Reduced AFB1 concentration dependence by temperature and irradiation time, using DWLSF method ($IP_0 = 1, c_{0\,AFB1} = 2$ **μg/kg**)

Figure 7 shows the obtained time-temperature-concentration relations for AFB2 photodegradation, using DWLSF method.

AFB2 photodegradation rate increases at higher temperatures; the highest photodegradation rate of AFB2 is at 50 °C. Experimental data were not collected at higher temperatures because it is not favourable to use excessive sunflower oil temperatures. Taking into account the reaction mass temperature rise inside the photoreactor, typically 0.5–1.5 °C, the reaction mass initial temperature has to be 49 °C or even more, depending on residence time and the irradiation intensity. Figure 8 shows AFG2 time-temperature-concentration relations, using DWLSF method.

Based on Figure 8, AFG2 photodegradation rate increases at higher temperatures; the maximum observed rate is at 50 °C. Taking into account reaction mass temperature rise inside the photoreactor, typically 0.5–1.5 °C, the initial temperature may be 49 °C or even more, depending on residence time and the irradiation intensity of the UV light source. Figure 9 illustrates AFG2 time-temperature-concentration relation, using DWLSF fitting method.

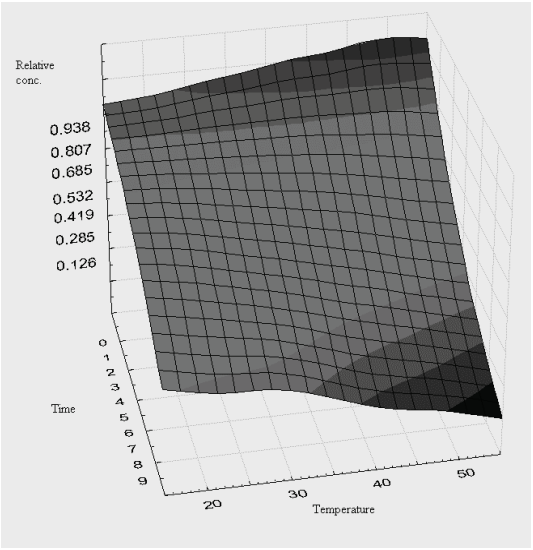

Figure 7: Reduced AFB2 concentration dependence by temperature and irradiation time, using DWLSF method ($IP_0 = 1, c_{0\,AFB2} = 2$ μg/kg)

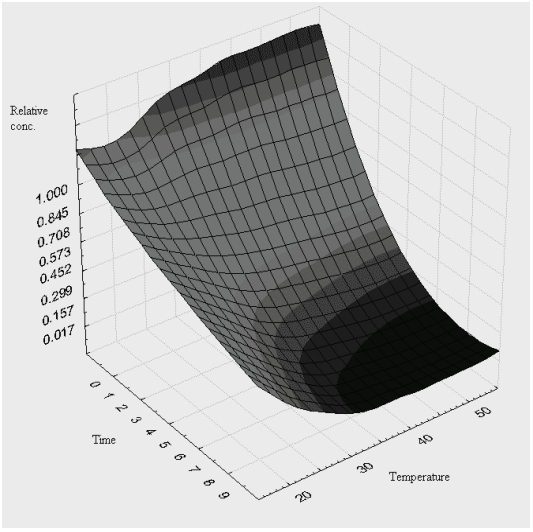

Figure 8: Reduced AFG2 concentration dependence by temperature and irradiation time, using DWLSF method ($IP_0 = 1, c_{0\,AFB2} = 2$ μg/kg)

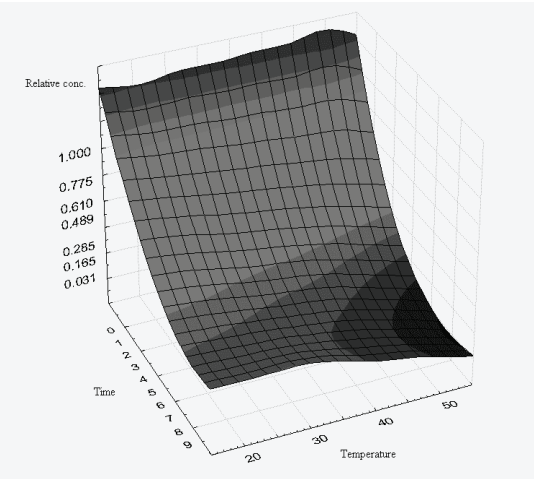

Figure 9: Reduced $T-2$ concentration dependence by temperature and irradiation time, using DWLSF method ($IP0 = 1$, $c_{0\,T2} = 2$ µg/kg)

Based on the graph, it can be concluded that photodegradation rate of $T-2$ toxin increases at higher temperatures; $T-2$ toxin's higher photodegradation rate is at 50 °C. Taking into account the reaction mass temperature rise inside the photoreactor, typically 0.5–1.5 °C, in this case, the reaction mass initial temperature may be 49 °C or even more, depending on residence time and irradiation intensity of UV source. The generalized equations – which describe the studied mycotoxin (MT) concentrations' depleting during the photochemical process by UV radiation and in function of temperature –, using Statistica 6.0 software package, are:

$$AFB1 : c_{AFB1} = 1.187 - 0.2252t - 0.135T + 0.0169t^2 - 0.0006tT + 0.0002T^2; \quad (17)$$

$$AFB2 : c_{AFB2} = 1.0445 - 0.0485t - 0.014T + 0.0012t^2 - 0.0011tT - 6.975E - 5T^2; \quad (18)$$

$$AFB2 : c_{AFB2} = 1.7109 - 0.1408t - 0.0392T + 0.0097t^2 - 0.001tT + 0.0005T^2; \quad (19)$$

$$T-2; c_{T-2} = 1.079 - 0.1974t + 0.0008T + 0.0129t^2 + 4.5455E - 6tT - 0.0001T^2. \quad (20)$$

3 Conclusions

Based on the experimental data on investigated mycotoxins, by comparing experimental data, we may notice some differences and similarities regarding the photochemical behaviour. The highest sensitivity to the effect of increased processing temperature occurs for $T-2$ toxin, followed by AFG2, AFB2 and

finally AFB1. showing the lowest sensitivity. It was observed that the effect of temperature in the photodegradation process may be connected with the behaviour of other chemical species which are present in the sunflower oil. From these experimental data on the effect of temperature, it can be concluded that the most effective decontamination process, the inlet temperature of the contaminated sunflower oil in the photoreactor should be adjusted, depending on the mycotoxin contamination. Increased temperature has a positive effect, except for AFB1. for which has been identified 41 °C, this being more favourable at 20–50 °C. In the photochemical conditions used, obtained mathematical models and specific data can be used to determine the conditions needed by the evolved refining process. Comparing the proposed decontamination process with the classic procedure, photochemical decontamination can be used to increase the nutrient value of the sunflower oil.

References

[1] H. S. Hussein, J. M. Brasel, Review: toxicity, metabolism, and impact of mycotoxins on humans and animals, *Toxicology*, 167 2 (2001) 101–134.

[2] J. L. Richard, Some major mycotoxins and their mycotoxicoses-an overview, *Int. J. Food Microbiol.*, 119 1–2 (2007) 3–10.

[3] P. S. Agachi, S. Gombos, Investigation on initial peroxide index value influence on AFB1. AFB2, AFG2 and T-2 mycotoxins decomposition in sunflower oil, *Studia Chemia*, 229 1 (2010) 229–243.

[4] S. Gombos, P. S. Agachi, The effect of bentonite on AFB1. AFB2, AFG2 and T-2 mycotoxins decomposition in sunflower oil under the irradiation of ultraviolet light, *Studia Chemia*, 229 1 (2010) 249–261.

[5] V. Lippolis, M. Pascale, C. M. Maragos, A. Visconti, Improvement of detection sensitivity of T-2 and HT-2 toxins using different fluorescent labeling reagents by high-performance liquid chromatography, *Talanta*, 74 5 (2008) 1476–1483.

[6] G. S. Sheppard, Determination of mycotoxins in human foods, *Chem. Soc. Rev.*, 37 11 (2008) 2468–2477.

[7] N. W. Turner, S. Subrahmanyam, S. A. Piletsky, Analytical methods for determination of mycotoxins: a review, *Anal. Chim. Acta*, 632 2 (2009) 168–180.

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