Antimicrobial Properties of 11-Cyclohexylundecanoic Acid

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Abstract

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The fact that milk fat contains many minor fatty acids is mostly neglected. It is known that 11-cyclohexylundecanoic acid is contained in cow's milk fat in the amount of 0.1–0.2%. It is also contained in human milk. 11-Cyclohexylundecanoic acid was synthesised, purified to 99.5% (GC) and its antimicrobial properties were investigated. It was found that 11-cyclohexylundecanoic acid has inhibitory effects on *Bacillus cereus, Escherichia coli*, and *Fusarium culmorum* from the concentration of 0.05 mmol/l, however, no antimicrobial effect on *Saccharomyces cerevisiae* has been determined. 11-Cyclohexylundecanoic acid was found also in goat and cow milk fat on the level of 0.1–0.2%. It is convenient to hydrogenate the sample of fat before the determination of 11-cyclohexylundecanoic acid by GC method.

Keywords: milk fat; fatty acids; antimicrobial properties; 11-cyclohexylundecanoic acid; GC

Milk fat (Vanhatalo *et al*. 2007) contains a high amount of saturated fatty acids (70–75%), a lower one of monounsaturated fatty acids (20–25%), and a small amount of polyunsaturated fatty acids (0–5%). Besides the major fatty acids, milk fat contains also a wide range of minor fatty acids (cca 40 compounds, gross content cca 10%). Particular minor fatty acids are contained in amounts from 0.01% (which is the lowest detectability limit of GC) to cca 1%. These less usual fatty acids have 12–26 carbon atoms, they are saturated or unsaturated (they contain up to 6 double bonds), and linear or branched.

The minor fatty acids given here differentiate milk fat (butter) from emulsified vegetable fats (margarines). According to the presence of these acids, adulteration of butter can be proved. There is minimum information about their functions in milk and their importance in the human nutrition.

Emulsified fats (margarines) producers emphasise that these lipids are a source of omega-3 and omega-6 unsaturated fatty acids (which are contained in higher amounts in native vegetable oils and form the liquid component of margarine blends). The published works are also concerned mainly with the nutritional importance of the major fatty acids (Williams 2000; Lock & Bauman 2004; Wahle *et al.* 2004).

Minor fatty acids of milk fat *trans*-acids form almost one half (4%) of the milk fat minor fatty acids (Vanhatalo *et al*. 2007) (of that cca 0.3% *trans*-C16:1 and 3.6% *trans*-C18:1). From today's point of view, *trans*-acids are discussed very much, therefore the margarine producers declare their amounts being close to zero. Nevertheless, milk fat *trans*-acids have apparently also additional biological effects, for example vaccenic acid (Figure 1), which is contained in milk fat in the amount of

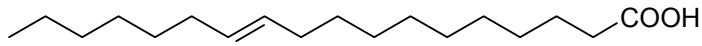
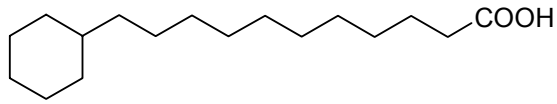
Acid	Content in milk fat (%)	Formula
vaccenic	1.04	
11-cyclohexylundecanoic	0.18	

Figure 1. Some minority acids of milk fat (Vanhatalo *et al.* 2007)

1%, is a rat growth factor (Boer *et al.* 1947; Lock & Bauman 2004).

11-Cyclohexylundecanoic acid is unique among the milk fat minor fatty acids (its content in milk fat is 0.1–0.2%), being the only cyclic fatty acid in milk fat (Vanhatalo *et al.* 2007; Schogt & Begemann 1965). It was first isolated from milk fat by Dutch authors in 1965 (Schogt & Begemann 1965). 11-Cyclohexylundecanoic acid is together with phytanic acid a minor component of human milk (Egge *et al.* 1969). The lipids produced by bacteria *Bacillus acidocaldarius* (DeRosa *et al.* 1971) and *Curtobacterium pusillum* (Suzuki *et al.* 1981) contain 11-cyclohexylundecanoic acid as a major component (up to 96%; Suzuki *et al.* 1981).

11-Cyclohexylundecanoic acid is structurally similar to chaulmoogric acid and dihydrochaulmoogric acid and hydnocarpic and dihydrohydnocarpic acid (Figure 2). Chaulmoogric acid is a part of chaulmoogric oil (*Hydnocarpus kurzii* (King) Warb.; *Flacourtiaceae*), which used to be utilised against leprosy in medicine (*Mycobacterium leprae*; Levy 1975). In terms of this structural analogy it can be assumed that this 11-cyclohexylundecanoic acid can also have antimicrobial properties.

Schogt and Begemann (1965) isolated 11-cyclohexylundecanoic acid from milk fat and determined its structure not only spectrally, but also by the comparison of the isolated compound with a synthetically prepared product.

MATERIAL AND METHODS

11-Cyclohexylundecanoic acid was prepared using a modified method of German authors (Biermann & Metzger 2004) (Figure 3).

11-Cyclohexylundecanoic acid. A solution of ethylaluminium sesquichloride in hexane (0.4 mol/l, 75 ml, 30 mmol) and triethylsilane (3.5 g, 30 mmol) was added to a stirred and cooled (–15°C) solution of cyclohexyl chloroformate (4.9 g, 30 mmol) and 10-undecenoic acid (5.6 g, 30 mmol) in absolute dichloromethane (90 ml) under argon during 90 minutes. The temperature was maintained at –15°C. The resulting reaction mixture was stirred for 30 min (–15°C), then allowed to warm up to room temperature and further stirred for 1 hour.

The reaction mixture was then poured into ether (600 ml) and a mixture of water (150 ml), ice (150 g), and hydrochloric acid (5 mol/l, 30 ml, 150 mmol) was added. After hydrolysis and dissolution of aluminium salts, the organic phase was washed with distilled water (3 × 200 ml), dried with sodium sulphate, and evaporated *in vacuo*.

The obtained oil (10.1 g) was purified by column chromatography (Kieselgel 60, 0.063–0.200 mm, 600 g, column ø 4.6 × 75 cm), mobile phase hexane-ethyl acetate, 7:3, v/v) to colourless crystals (4.1 g), which were crystallised from methanol. 11-Cyclohexylundecanoic acid was obtained as colourless short needles (2.1 g, 26%; purity 99.5%, determined by GC of methylester), m.p. 55.5–57.0°C.

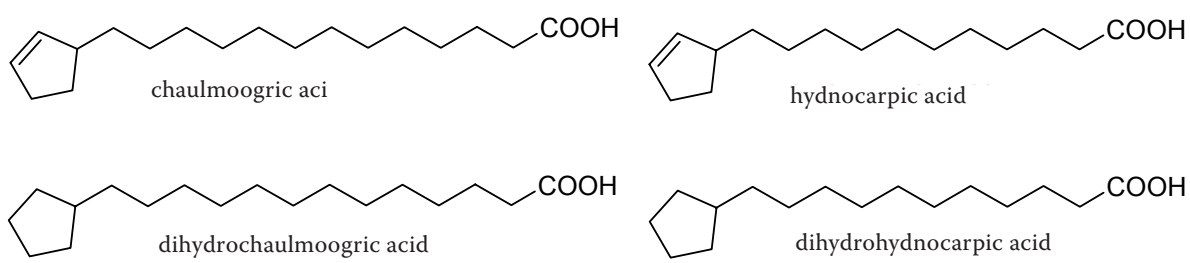


Figure 2. Structure analogues of 11-cyclohexylundecanoic acid

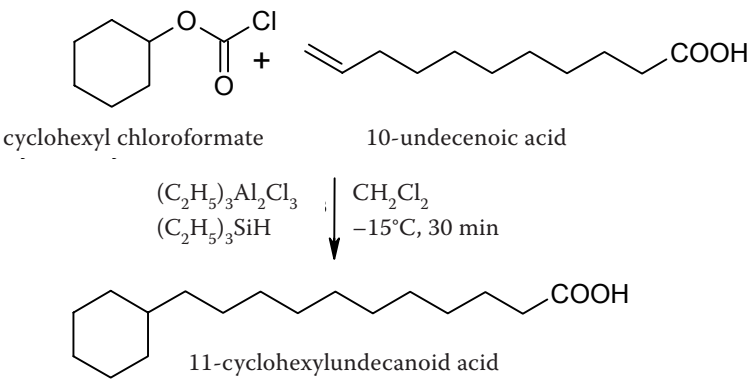


Figure 3. Synthesis of 11-cyclohexylundecanoic acid

The crystallisation of the concentrated mother liquor gave another part (0.7 g, 9%; purity 98.4%), m.p. 55.5–56.0°C. Schogt and Begemann (1965) reported m.p. 55.4–56.6°C (synthetic), 53.4–54.0°C (isolated); Hiers and Adams (1926) reported m.p. 58–59°C; Biermann and Metzger (2004) reported m.p. 35–37°C.

The course of the column chromatography was monitored by TLC (Kieselgel 60 F_{254}, hexane-ethyl acetate, 7:3, v/v, TLC plates were sprayed with a 5% solution of phosphomolybdic acid in ethanol and then warmed). 11-Cyclohexylundecanoic acid had R_F = 0.45 and the starting 10-undecenoic acid had R_F = 0.40.

MS (*m*/*z* (%)): 268 (49, M^+), 186 (36), 143 (18), 129 (31), 97 (25), 83 (91), 55 (100), 41 (73), 29 (20)

Methyl 11-cyclohexylundecanoate. To a stirred solution of 11-cyclohexylundecanoic acid (537 mg, 2 mmol) in hexane, a solution of diazomethane in ether was added (0.5 mmol/ml, 8 ml, 4 mmol). The reaction mixture was stirred at room temperature for 2 hours. After evaporating *in vacuo*, the residue was dissolved in hexane, dried with sodium sulphate, and evaporated. Methyl 11-cyclohexylundecanoate was obtained as a colourless oil (524 mg, 93%), which was crystallised in a refrigerator, m.p. 20–21°C (R_F = 0.65, conditions of TLC are mentioned above; purity of 99.5% was determined by GC).

The conditions of gas chromatography of methyl-esters: Chromatograph 6890N (Agilent Technology, USA), column SP^{TM}-2560 (Supelco, Bellefonte, USA), 0.25 × 100 000 mm, film thickness 0.2 µm, carrier gas helium, flow rate 1 ml/min, temperature of FID 300°C, flow rate of hydrogen 40 ml/min, flow rate of air 300 ml/min, flow rate of make up gas (nitrogen) 25 ml/min. The temperature of the inlet was 220°C, in oven 175°C.

MS (*m*/*z* (%)): 282 (60, M^+), 239 (13), 199 (13), 143 (28), 97 (13), 87 (86), 74 (100), 55 (72), 41 (35), 29 (8)

HRMS: calculated 282.2559, found 282.2553

11-Cyclohexylundecanoic acid in cow, goat, and sheep milk fats. The lipids were isolated from milk by the normalised method (IDF 1996). The fatty acids of milk fat were converted to methyl esters (Christopherson & Glass 1969). Methyl ester of 11-cyclohexylundecanoic acid was determined by CGC under the conditions described above. Fatty acids content, in g/100 g ∑fatty acids, was calculated from the composition of fatty acid methyl esters according to DGF-Einheitsmethoden. Milk fat was hydrogenated in the presence of Pd catalyst (Palladium on activated Charcoal, Fluka) under standard temperature and pressure: 500 mg of fat were dissolved in n-hexane, 20 mg of catalyst were added and the fat was hydrogenised under overpressure of 20 hPa and stirring of 250 rpm for several hours – until hydrogen content changed.

Determination of antimicrobial properties. The antimicrobial effects of 11-cyclohexylundecanoic acid were compared with those of saturated fatty acids which occur in milk fat (decanoic, undecanoic, dodecanoic, tridecanoic, tetradecanoic and hexadecanoic acids – Sigma-Aldrich, St. Louis, USA; purity > 98%) and then compared with that of 10-undecenoic acid (Sigma-Aldrich, purity 98%) which is not a constituent of lipids but is known for its antimicrobial properties. The purity of these compounds was at least 99%. As the test microorganisms were used *Bacillus cereus* DMF 2001 as a member of Gram-positive bacteria, *Escherichia coli* DMF 7503 as a Gram-negative bacteria, *Saccharomyces cerevisiae* DMF 2880 as a member of yeasts, and *Fusarium culmorum* DMF 0103 from filamentous fungi.

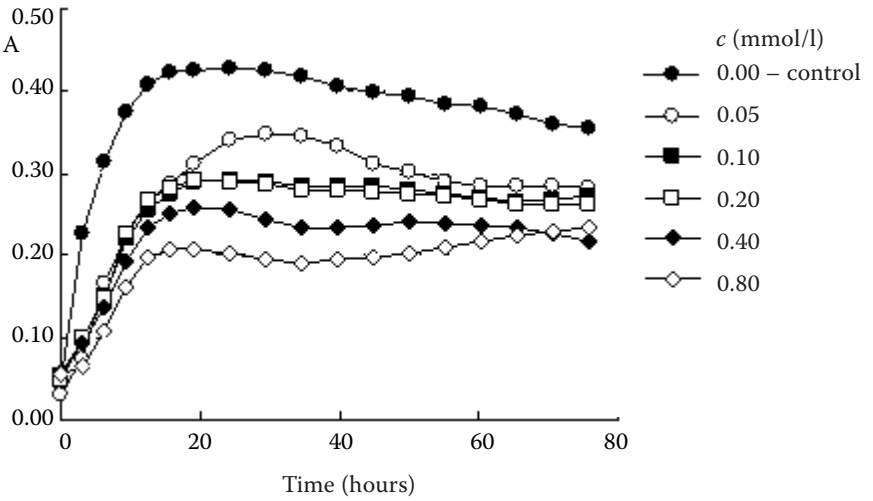


Figure 4. The relation between rate of growth of *Escherichia coli* DMF 7503 and concentration of 11-cyclohexylundecanoic acid

A spectrophotometric method was used for the determination of the antimicrobial activity of the agents tested. The inoculum or spore suspension was inoculated into a set of flasks containing liquid medium (Nutrient broth for bacteria, Malt extract broth for yeast and mould) with a given concentration of the carboxylic acid tested (carboxylic acids were dissolved in ethanol which have a final concentration of 2% in the medium. The antimicrobial effect of ethanol was eliminated with a blank test). From these flasks were filled sterile microtitrate plates in which took place the cultivation and measuring of the absorbance using an automatic reader PowerWave XS (650 nm – bacteria, 630 nm

Bacillus cereus DMF 2001

	c [mmol/l]				
Fatty acid	0.05	0.10	0.20	0.40	0.80
C11:1					
C10:0					
C11:0					
C12:0					
C13:0					
C14:0					
C16:0					
C11-cyclo					

Escherichia coli DMF 7503

	c [mmol/l]				
Fatty acid	0.05	0.10	0.20	0.40	0.80
C11:1					
C10:0					
C11:0					
C12:0					
C13:0					
C14:0					
C16:0					
C11-cyclo					

Saccharomyces cerevisiae DMF 2880

	c [mmol/l]				
Fatty acid	0.05	0.10	0.20	0.40	0.80
C11:1					
C10:0					
C11:0					
C12:0					
C13:0					
C14:0					
C16:0					
C11-cyclo					

Fusarium culmorum DMF 0103

	c [mmol/l]				
Fatty acid	0.05	0.10	0.20	0.40	0.80
C11:1					
C10:0					
C11:0					
C12:0					
C13:0					
C14:0					
C16:0					
C11-cyclo					

Inhibition [%]				
0 - 10	10 - 25	25 - 50	50 - 90	> 90

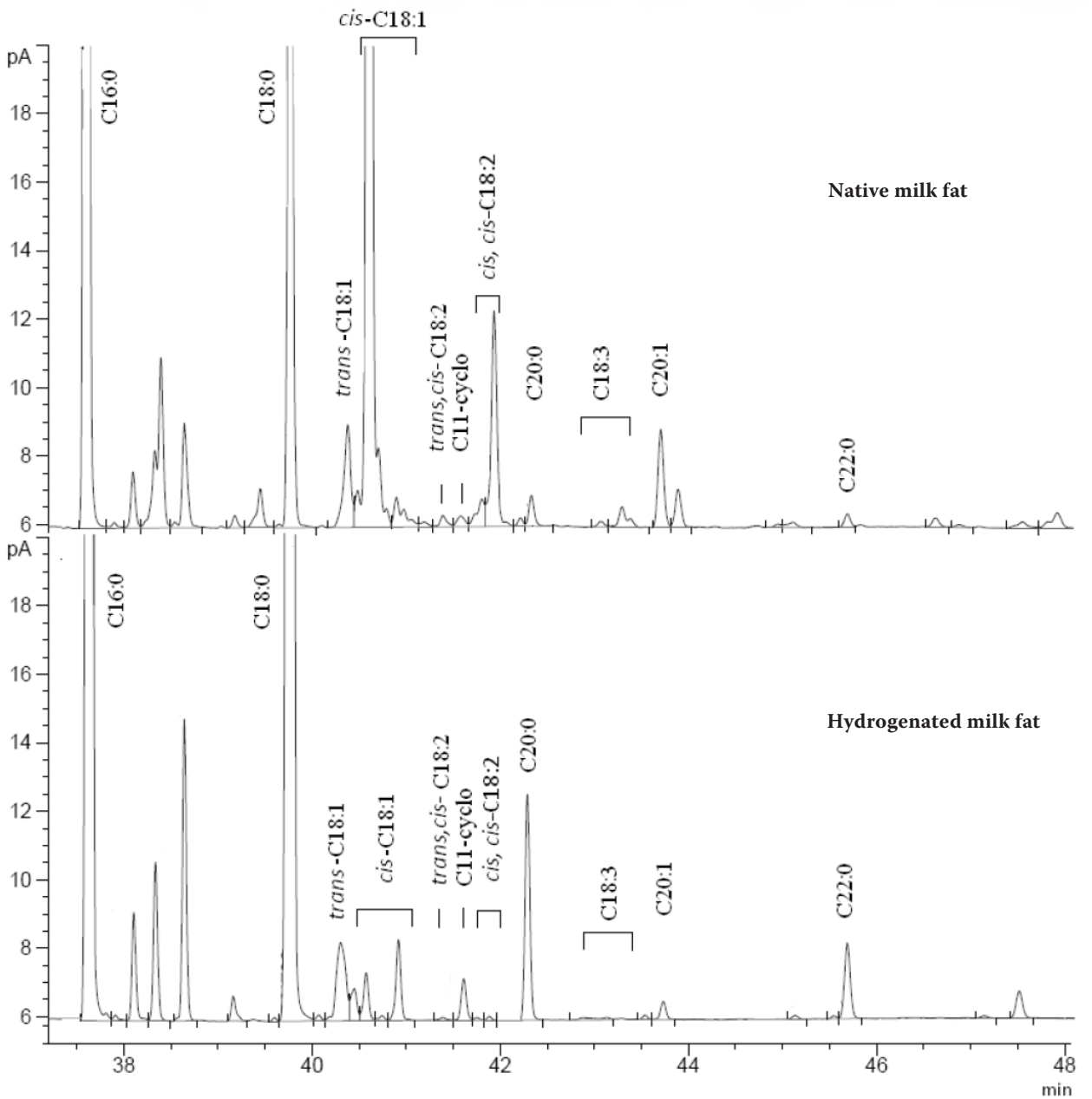
C11:1 = 10-undecenoic acid
C10:0 = decenoic acid
C11-cyclo = 11-cyclohexylundecenoic acid

Figure 5. Antimicrobial properties of fatty acids

– yeast and mould). After certain time intervals the absorbance was measured. 25 times in each well and then averaged in order to increase the measurement accuracy. The results were then statistically analysed. The Dixon test with the significance level of 0.05 was used to eliminate the outlaying values (Jaroš *et al.* 1998). The curves of the dependence of the absorbance on time (72 h – bacteria, 240 h – yeast and mould) represent the data output. Detailed methodology is described in a previous paper (Karlová *et al.* 2010).

An example of the growth curves of *Escherichia coli* DMF 7503 is shown in Figure 4. It is obvious that an increase of 11-cyclohexylundecanoic acid concentration in the growth medium led to a decrease of the growth rate and a gradual abortion of the microbial growth (decrease of absorbance).

The integrals of the growth curves were counted and, after the comparison with the control (without the addition of antimicrobial compounds), the percentual inhibitions were evaluated.



C11-cyclo = 11-cyclohexyludecanoic acid

Figure 6. Chromatogram CGC – separation of methyl esters of fatty acids, cow milk fat

Table 1. Content of 11-cyclohexylundecanoic acid in native cow, goat and sheep milk fat and after hydrogenation

	Native milk fat (%)			Hydrogenated milk fat (%)		
	cow	goat	sheep	cow	goat	sheep
Saturated FA	62.43	66.13	65.85	95.49	94.59	95.52
Cis-monounsaturated FA	29.39	22.53	20.27	1.79	1.58	0.94
All-*cis*-polyunsaturated FA	3.12	3.15	3.39	0.02	0.04	0.02
Trans-unsaturated FA	2.44	4.58	6.47	0.07	0.31	0.15
Branched-FA	2.39	3.36	3.40	2.41	3.40	3.25
11-Cyclohexylundecanoic acid	0.23	0.25	0.62	0.22	0.08	0.12

The comparison of antimicrobial properties of 11-cyclohexylundecanoic acid with those of fatty acids C11:1 (10-undecenoic acid) and C10:0–C16:0 is shown in Figure 5, where the inhibitory effects of several acids are marked by the intensity of the field colour.

RESULTS AND DISCUSSION

Saturated fatty acids with the chain lengths from 10–14 and 10-undecenoic acid have strong inhibitory effects on the microbial growth (Figure 5). On the other hand, hexadecanoic acid (16 carbon atoms) has almost no inhibitory properties. 11-Cyclohexylundecanoic acid (17 carbon atoms) does not reach the inhibitory efficiency of the C10:0–C14:0 acids but it inhibits *Bacillus cereus, Escherichia coli*, and *Fusarium culmorum* over the whole range of concentrations used. With *Saccharomyces cerevisiae*, the tested compounds were almost ineffective.

The observed antimicrobial effects of 11-cyclohexylundecanoic acid confirm that this fatty acid has obviously some specific function in milk fat. It is generally known that milk not only nourishes the sucklings, but it also protects them from infections. Milk of biological species is optimal and often essential and the minor fatty acids have just the protective function.

The content of 11-cyclohexylundecanoic acid was tentatively determined in cow (origin Central Bohemia) and goat and sheep milk fats (origin Western Slovakia). There is an analytical problem because of the retention time of 11-cyclohexylundecanoic acid being very similar to the retention times of *cis-trans* isomers of octadecadienoic acids, even on 100 m column SP-2560 which is especially suitable for the determination of *cis-trans* isomers and some positional isomers of fatty acids (Figure 6). 11-Cyclohexylundecanoic acid can not be safely identified without the use of standard or eventually GC-MS, there is a coelution; 11-cyclohexylundecanoic acid content is higher in all cases (Table 1). An obvious identification of 11-cyclohexylundecanoic acid is possible in hydrogenated fats, in which it elutes separately. The contents in the milk fats studied are 2–5 times lower – Table 1. The method of the determination as methyl esters after hydrogenation is suitable for the determination of 11-cyclohexylundecanoic acid in complex mixtures of fatty acids of milk fats of ruminants.

CONCLUSIONS

Our results indicate that 11-cyclohexylundecanoic acid has inhibitory effects on *Bacillus cereus, Eschericia coli,* and *Fusarium culmorum* from the concentration 0.05 mmol/l. Especially remarkable is its effect on *Escherichia coli* and *Fusarium culmorum* over the whole range of the concentrations used but no antimicrobial effect on *Saccharomyces cerevisiae* was estimated.

The content of 11-cyclohexylundecanoic acid in cow, goat and sheep milk fats is on the level of 0.1–0.2%. It is convenient to hydrogenate the sample of fat before the determination of 11-cyclohexylundecanoic acid by the GC method .

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