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On the Role of Short-Chain Free Fatty Acids for the Development of a Cheese-like Off-Note in Pasteurized Yoghurt

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ABSTRACT

Free fatty acids were quantified in fresh and stored, pasteurized yoghurt samples by gas chromatography without derivatization after clean-up on anion exchange SPE cartridges. After storage for nine months the yoghurts elicited a cheese-like off-flavor, the intensity of which increased in parallel with the increase in free fatty acids. Their generation was more pronounced at higher storage temperatures and was attributed to the activity of a heat-resistant lipase occurring in the yoghurt. Descriptive sensory experiments performed by adding free fatty acids to fresh yoghurt demonstrated that these compounds were responsible for the off-flavour. Furthermore, odour thresholds of free fatty acids in fresh yoghurt were determined and revealed higher thresholds of the respective compounds than their concentrations. It was, therefore, concluded that the acids produced the cheese-like flavour by synergistic sensory behaviour.

Key words: Free Fatty Acids, Off-Flavour; Yoghurt

INTRODUCTION

Since about 40 years several reports on the volatiles of yoghurt have been published. Up to date, around 60 volatile compounds have been identified, most of which belonging to the chemical classes of aldehydes, ketones, acids, lactones and furans [Viani et al. 1973; Badings et al., 1985]. However, only in one of these studies an attempt was made to evaluate the odour-active compounds by applying gas chromatography-olfactometry (Ott et al., 1997). These authors identified 21 compounds as important flavour contributors, among which also free fatty acids were identified, namely acetic, 3-methyl butanoic and hexanoic acid. Free fatty acids are also often suggested to play a significant role in the typical flavour of cheeses and systematic studies on the flavour of Emmentaler (Preininger et al. 1994, 1996; Preininger and Grosch, 1994) and Gruyere cheeses (Rychlik and Bosset, 2001a, 2001b) have confirmed the importance of acetic, propanoic, 3-methyl butanoic and butanoic acid for cheese flavour.

Usually, the shelf-life of yoghurt is restricted to about 3 weeks in cold storage and to 3 days at 20 °C (Driessen, 1984). After storage periods that exceed these times, the product is mainly spoiled by growth of yeasts or moulds (Lacroix and Lachance, 1990). To gain more freedom with respect to storage conditions, so-called shelf-stable yoghurt is becoming increasingly popular. To ensure microbiological safety, such yoghurts are usually pasteurized and proposed to be stable for up to nine months. However, during storage, sometimes off-flavours are perceived, which are described as rancid and cheese-like.

The aim of the present investigation was, therefore, to use a fast and accurate method for free fatty acid quantification in yoghurts and to evaluate the impact of these compounds on the observed cheese-like off-flavour in stored products.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained commercially from the sources given in parentheses: acetic acid, decanoic acid, dichloro methane, diethyl ether, dodecanoic acid, ethanol, formic acid, heptanoic acid, methyl propanoic acid, potassium dihydrogen phosphate, 2-propanol, sulfuric acid (Merck, Darmstadt, Germany); di-sodium hydrogen phosphate, heptane, 4-methylumbelliferon, sodium sulfate. Triton® X-100 (Fluka, Buchs, Switzerland). Hexanoic acid, 4-methylumbelliferyl butanoate, octanoic acid, wheat germ lipase (Sigma, Steinheim, Germany). Butanoic acid (Aldrich, Steinheim, Germany).

Yoghurt production

Milk, as obtained from local farms, was skimmed, pasteurized and homogenized. Skimmed milk and cream were mixed to obtain a total fat content of 3.5 g/ 100g. Subsequently, milk was mixed with corn starch (1g /100 g; Cerestar, Sas van Gent, Netherlands), pectin (0.2 g / 100 g; CP Kelco, Lille Skensved, Denmark) and sucrose (5g / 100 g; Merck, Darmstadt, Germany), then homogenized at 58 °C and 100 bar, pasteurized at 93 °C for 10 minutes and cooled down to 42 °C. The following incubation with classical starting cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Optiferm, Kempten, Germany) was stopped at a pH of 4.1. After the fermentation process, the yoghurt was pasteurized in a plate heat exchanger to inactivate the microorganisms. To prevent a recontamination, the following mixing and filling process was conducted under sterile conditions in an aseptic extender. All production steps were performed in a pilot plant.

Determination of Free Fatty Acids in Yoghurt

Yoghurt (15 g) was mixed with ethanol (15 mL), H_2SO_4 (1 mL, 2.5 mol/L), and the internal standards methyl propanoic acid and heptanoic acid (1 µg and 0.5 µg, respectively, dissolved in 1 mL of ethanol). Extraction was carried out by stirring for 1 h with diethyl ether/heptane (15 mL, 1:1, v/v) in a screw-capped centrifuge tube. After centrifugation at 2500 rpm for two minutes at room temperature, the upper layer was transfered to a 100 mL conical flask containing anhydrous Na_2SO_4 (2 g). The extraction procedure was repeated twice with diethyl ether/heptane (total volume: 20 mL).

Aminopropyl columns Strata NH_2 (Phenomenex, Aschaffenburg, Germany) were conditioned with heptane (10 mL) and then the extract was applied. Following, the columns were washed with dichloro methane/2-propanol (4 mL, 3:1, v/v) and the free fatty acids were eluted with diethyl ether/formic acid (4 mL, 49:1, v/v). An aliquot (2.0 µl) was then analyzed by gas chromatography. Each quantification was performed in triplicate.

Gas Chromatography

Gas chromatography (GC) was performed using a type 5890 series II gas chromatograph (Hewlett Packard, Germany) and a Permabond® FFAP, fused silica capillary column (30 m x 0.32 mm, d_f = 0.25 µm, Macherey-Nagel, Düren, Germany).

The samples were applied by the cold on-column technique at 40 °C. Two min after injecting the sample, the temperature of the oven was raised to 60 °C at a rate of 40 °C/min, and then raised at 10 °C/min to 180 °C followed by increasing the oven temperature at a rate of 6 °C/min to 240 °C and maintaining this temperature for 15 min. The flow rate and inlet pressure of the carrier gas, helium, was 2.4 mL/min and 178 kPa, respectively.

Determination of Lipase activity

Yoghurt (10 g) was suspended in phosphate buffer (50 mL, 0.2 mol/L, pH 7.2) containing 1 g / 100 g Triton® X-100. After shaking, the suspension was homogenized for 30 seconds in an ice bath using an Ultraturrax (IKA, Staufen, Germany) at low velocity. Subsequently, the extract was centrifuged at 11400 rpm for 20 minutes at 4 °C (Heraeus-Sepatech, Osterode, Germany). Then, phosphate buffer (2.6 mL, 0.2 mol/L, pH 7.2) and 0.2 mL of the supernatant were mixed in a cuvette. After 5 minutes at 37 °C, 0.2 mL of a solution of 4-methylumbelliferyl butanoate (51.8 mg/L) in phosphate buffer (0.2 mol/L, pH 7.2) were added and fluorescence (excitation 364 nm, emission 447 nm) was measured against 2.8 mL phosphate buffer containing 1g / 100 g Triton® X-100 by means of a spectrofluorometer SAFAS flx-Xenius (Safas, Monaco).

Descriptive Sensory Profiling

A trained sensory panel consisting of eight persons determined the odour attributes sour, cheese-like and yoghurt-like of a pasteurized, fresh yoghurt and a pasteurized, nine-months-stored yoghurt. The panellists had been trained for several years in the sensory testing of milk products. Acidic acid, ripened hard cheese and a nonpasteurized, fresh yoghurt were used as reference materials for the odour attributes sour, cheese-like and yoghurt-like, respectively. The products were served in closable cups at a temperature of 20 °C.

The assessors scored the odour intensity of each attribute on an intensity scale ranging from 0 to 100 % between the anchors "absent" and "extreme". Analoguously, sensory profiling was performed after adding fatty acids to a fresh yoghurt.

Determination of Odour Threshold Values

Fresh yoghurt was used for the determination of the thresholds of butanoic, hexanoic and octanoic acid in yoghurt. Additionally, the odour threshold of butanoic acid was determined in three months-stored yoghurt. The respective fatty acid was dissolved in ethanol and added to the yoghurt in six different concentrations ranging from 0 to 50 mg/kg. The assessors evaluated the samples in order of increasing concentrations starting with the zero option. Each sample was compared to the previous. Threshold values were calculated according to Bundesgesundheitsamt (1993).

RESULTS AND DISCUSSION

Quantitation of free fatty acids

Preliminary experiments were run to optimize the extraction procedure for free fatty acids (FFA) from yoghurt. Earlier studies on cheese reported on extracting the fat and methylating FFA with tetramethyl ammonium hydroxide (Chavarri et al. 1997) or on steam distilling the acids prior to derivatization (Bachmann et al., 1997). Based on our experience with Gruyere cheese (Rychlik and Bosset, 2001b), an effective and fast clean-up might be achieved by anion exchange solid phase extraction according to the method of De Jong et al. [1990]. To compensate for losses during extraction and clean-up, 2-methyl propanoic acid and heptanoic acid were used as internal standards (IS). Analyses of the stored yoghurt had ensured that both acids were absent.

The gas chromatogram on a FFAP capillary shown in Fig. 1. was devoid of contaminations interfering with the fatty acids. In validation studies definite amounts of fatty acids were added to fresh yoghurt samples and recoveries of 101.9%, 96.4%, 97.8%, 96.3%, 89.0% and 87.6% were calculated for acetic, butanoic, hexanoic, octanoic, decanoic and dodecanoic acid, respectively. These results exceeded the data of 70 % for butanoic acid reported by De Jong et al. [1990], which might be due to the use of the better suited internal standard 2-methyl propanoic acid. The latter authors used pentanioc acid as the internal standard, which obviously caused discriminations of the lower boiling butanoic acid.

The contents of the free fatty acids in yoghurt (table 1) were, however, quite similar to those found by De Jong et al. [1990].

Release of free fatty acids during storage

During storage of yoghurt at 20 °C, all acids increased within the first 50 days, but did not go beyond the figures thereafter (Fig. 2). In particular, butanoic acid increased significantly between day 20 and day 50 of the trial, whereas the increase was much less pronounced than for the other acids. As shown for butanoic acid in fig. 3, an increase in the temperature from 4 °C to 30 °C slightly increased the liberation of this acid. For all temperatures, the increase between day 20 and 50 was most pronounced.

Lipase activity in yoghurt

The liberation of free fatty acids in milk products is known to be caused by the activity of lipases being delivered by the starter cultures used. Therefore, the activity of this enzyme was determined photometrically by monitoring the formation of fluorescent 4-methyl umbelliferone from its butanoic acid ester. The fresh yoghurt revealed an activity of 15.5 U/g supporting the assumption of fatty acids being liberated by this enzyme. To provide further evidence, a lipase from wheat germ was added to the yoghurt to give a total lipase activity of 25 U/g. As expected, the increase of fatty acids was accelerated (Fig. 4).

In order to evaluate whether the lipase activity can be lowered by a thermal treatment, the yoghurt was heated in single experiments in a plate heat exchanger a) for 10 sec at 140 ° and b) for 10 min at 140 °C. Both treatments reduced lipase activity a) to 12.7 and b) to 7.8 U/g, respectively. Therefore, the lipase present in the yoghurt sample appeared to be heat-resistant as previously observed for microbial lipases of different bacteria in milk (Cogan, 1977).

Impact of free fatty acids on the cheese-like off-flavour of yoghurt

The results of an aroma profile analysis of a spoiled and a yoghurt without off-flavour by a sensory panel is displayed in fig. 5. While the fresh yoghurt exhibited an intense sour and yoghurt-like odour, the stored yoghurt smelled less yoghurt-like, but more intensely cheese-like and sour. Taking into consideration the standard deviation of sensory ratings of about 10 %, only the increased cheese-like odour of the stored yoghurt was found to be significantly different from that of fresh yoghurt according to the student's t-test (α=5%). To evaluate the impact of the fatty acids on this off-note, their odour thresholds were determined. As the other odorants of yoghurt are likely to have an impact on the odour perception of FFA, odour thresholds were determined by adding increasing amounts to either a fresh yoghurt or a yoghurt that had been stored for three months (table 2). This approach has already been successfully applied in a study evaluating a metallic off-flavour in buttermilk (Heiler and Schieberle, 1997). Comparing these “difference” thresholds with the actual concentrations in the yoghurt resulted in the interesting finding that none of the single acids exceeded its threshold in the yoghurt showing an off-flavor.

In another sensory experiment, all six fatty acids were added in single (addition A) or double concentration (addition B) to fresh yoghurts, which were then evaluated by the sensory panel. For experiment A the resulting concentrations of butanoic acid in the yoghurt were slightly below and for experiment B higher than its difference threshold, whereas the concentrations of the other acids were below their thresholds (table 3). Interestingly, both additions of FFA lowered significantly (α=5%) the intensity of the yoghurt-like odour and increased significantly both the intensity of the cheese-like (α=5%) and the sour flavour (α=5%) (Fig. 6). As to be expected, these effects were more pronounced in the samples with the higher amounts added.

CONCLUSION

As the addition of the fatty acids to fresh yoghurt led to a more pronounced cheese-like odour impression, it can be assumed that these play an important role in this type of off-flavour. Two possible explanations for this finding are conceivable: on the one hand, other odourants than FFA present in fresh yoghurt might mask the off-note and the off-flavour is not detectable until their contents decrease during storage. On the other hand, a synergistic action of the short-chain acids might be suggested, i.e. compounds with similar odour attributes occurring in sub-threshold concentration may enhance each other and are thus detected. As the threshold of butanoic acid did not change during yoghurt storage (table 2), the synergistic actions become plausible. A similar effect was observed for the aldehyde (E)-2-nonenal and its geometric isomer, (Z)-2-nonenal. Widder and Grosch (1994) showed that in butter oil both compounds did not exceed their odour thresholds, but were responsible for a cardboard-like off-flavor. However, for yoghurt the impact of other odourants than FFA on the flavor defect still has to be evaluated. Studies using GC-olfactometry, as already shown for the metallic off-note in buttermilk (Heiler and Schieberle, 1996) or a potato-like off-flavour in Gruyere cheese (Rychlik and Bosset, 2001a) have been proven to be valuable means to screen for the compounds responsible for a flavour defect. Thus, quantification of all odorants and sensory studies using an odour-less matrix have to be performed to clarify whether the free fatty acids are the only odourants responsible for the off-flavour.

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Table 1. Concentrations of free fatty acids in fresh yoghurt

Fatty acid	**(mg/kg)**
Acetic acid	76.02 (n.d.) [a]
Butanoic acid	3.07 (3.4) [a]
Hexanoic acid	1.65 (2.55) [a]
Octanoic acid	2.03 (2.05) [a]
Decanoic acid	3.62 (1.95) [a]
Dodecanoic acid	5.95 (3.2) [a]

n.d not determined

[a] Data in parentheses reported by De Jong et al. (1990)

Table 2. Orthonasal odour thresholds of free fatty acids in a fresh yoghurt and a three months-old yoghurt.

	fresh yoghurt			**3 months old yoghurt**		
fatty acid	Amount added [mg/kg]	natural content [mg/kg]	**threshold [mg/kg]**	amount added [mg/kg]	natural content [mg/kg]	**threshold [mg/kg]**
Butanoic acid	3.54	3.04	**6.58**	1.73	4.89	**6.62**
Hexanoic acid	11.98	1.65	**13.63**	-		**n.d.**
Octanoic acid	10.98	2.03	**13.23**	-		**n.d.**

n.d. not determined

Table 3. Amounts of free fatty acids added to fresh yoghurt for descriptive sensory profiling

Fatty Acid	**Addition A [mg/kg]**	**Total concentration after addition A (mg/kg)**	**Addition B [mg/kg]**	**Total concentration after addition B (mg/kg)**
Acetic acid	34.87	110.89	69.75	145.77
Butanoic acid	2.79	5.86	5.59	8.66
Hexanoic acid	1.06	3.95	2.11	5.00
Octanoic acid	0.57	5.12	1.13	5.75
Decanoic acid	2.09	6.90	4.19	9.00
Dodecanoic acid	2.67	8.62	5.35	11.30

Legends to the figures:

Fig. 1. Gas chromatogram of free fatty acids in a pasteurized yoghurt stored for four months at 20 °C.

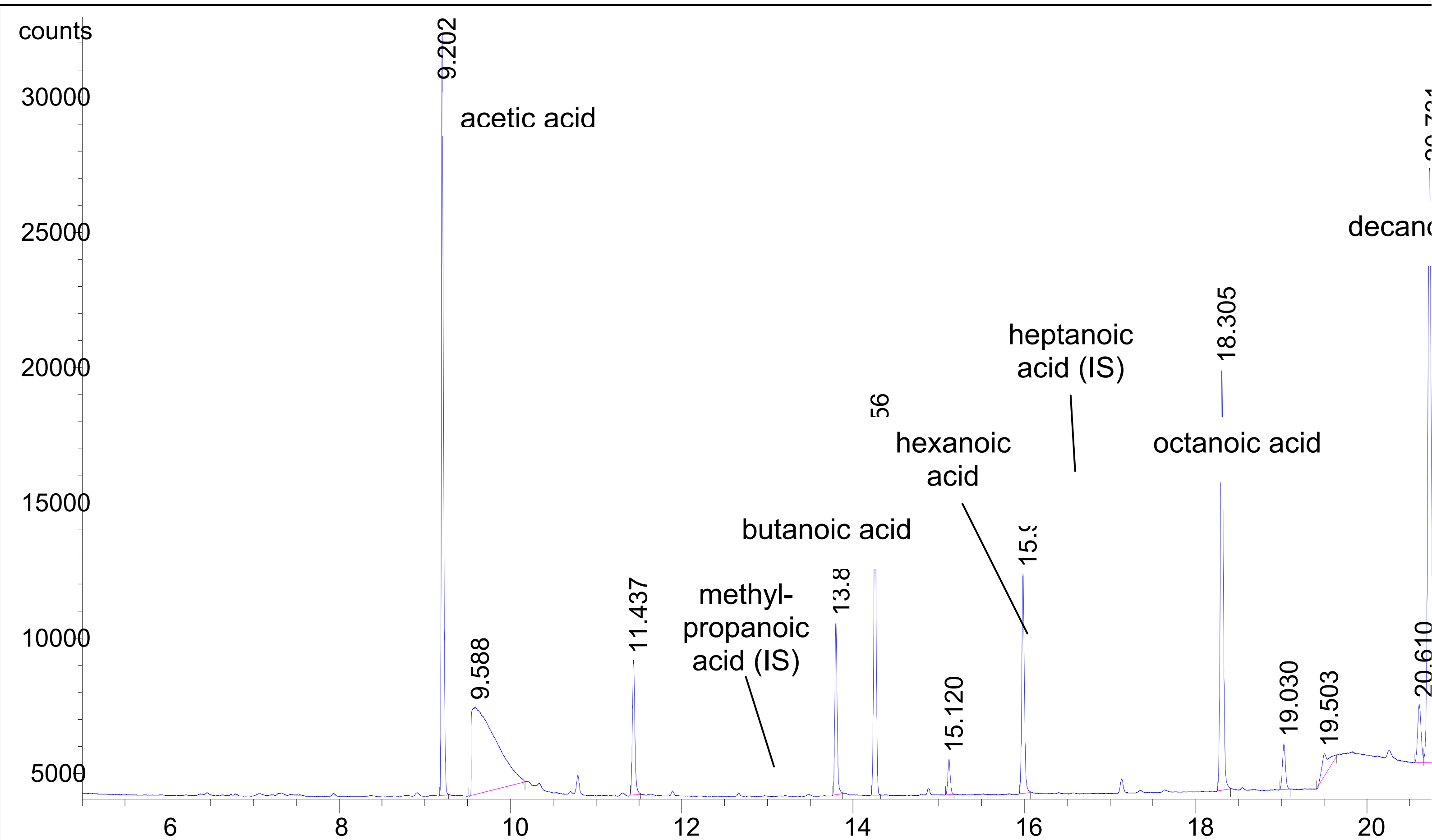
Fig. 2. Changes in the concentrations of free fatty acids in yoghurt during storage at 20 °C Butanoic acid (■), Hexanoic acid (▲), Octanoic acid (+), Decanoic acid (o), Dodecanoic acid(●).

Fig. 3. Changes in the concentrations of butanoic acid in yoghurt during storage at different temperatures 30°C (■), 20 °C (♦), 4 °C (▲).

Fig. 4. Changes in the concentrations of free fatty acids in yoghurt after addition of wheat germ lipase during storage at 30 °C Butanoic acid (■), Hexanoic acid (▲), Octanoic acid (+), Decanoic acid (o), Dodecanoic acid(●).

Fig. 5. Odour discriptions of fresh (——) and nine months-stored (- - -) yoghurts.

Fig. 6. Odour discriptions of fresh yoghurt (——)as well as yoghurt with additions A (- - -) and B (· · · · ·)of fatty acids listed in table 3.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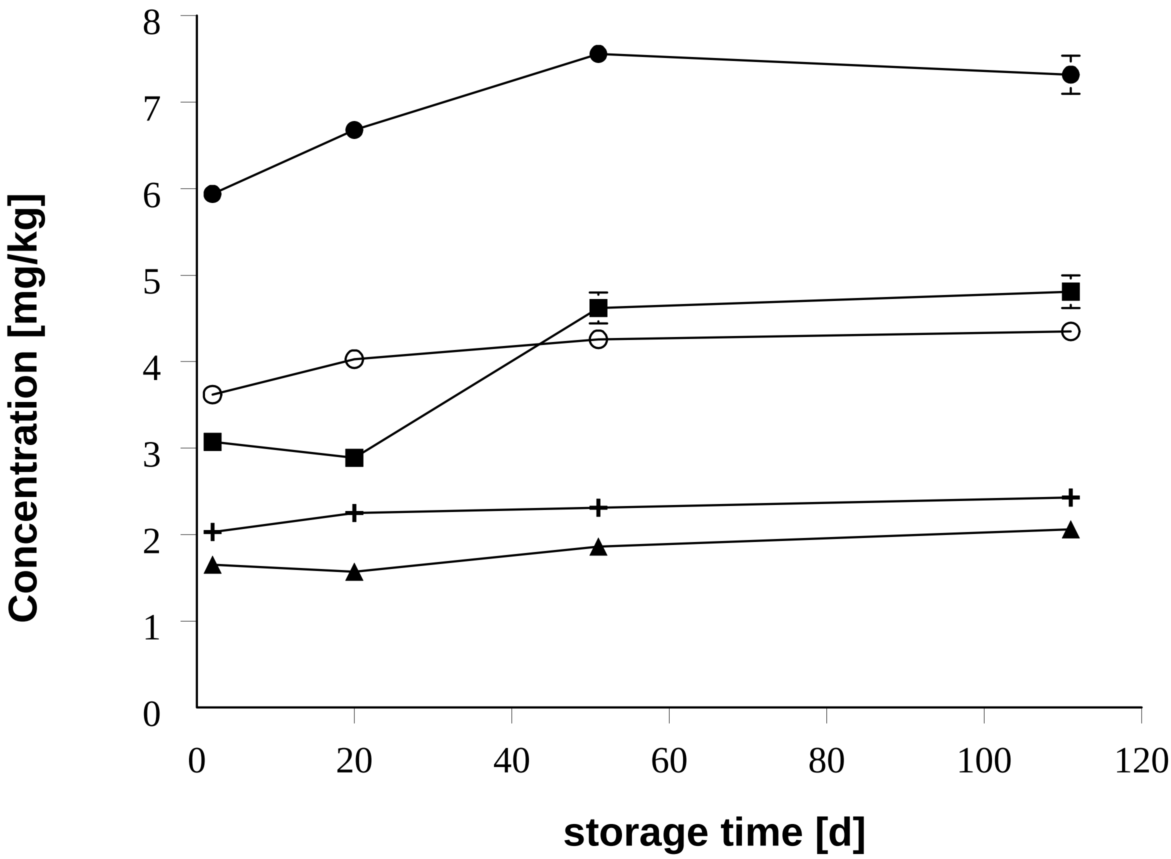
25000
20000
15000
10000
5000
9.202
acetic acid
9.588
11.437
methyl-
propanoic
acid (IS)
butanoic acid
13.8
56
hexanoic
acid
15.120
15.9
heptanoic
acid (IS)
18.305
octanoic acid
19.030
19.503
20.610
decano
6
8
10
12
14
16
18
20

Fig. 1


Concentration [mg/kg]
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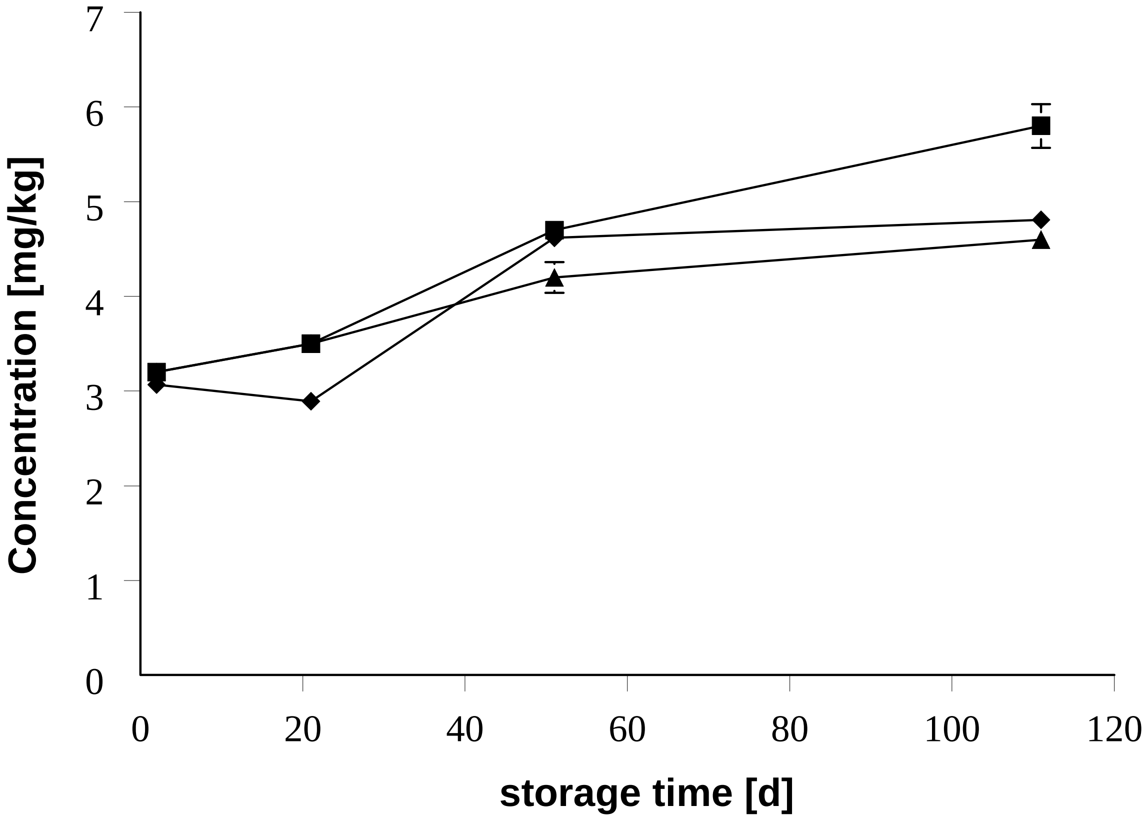
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
storage time [d]

Fig.2


Concentration [mg/kg]
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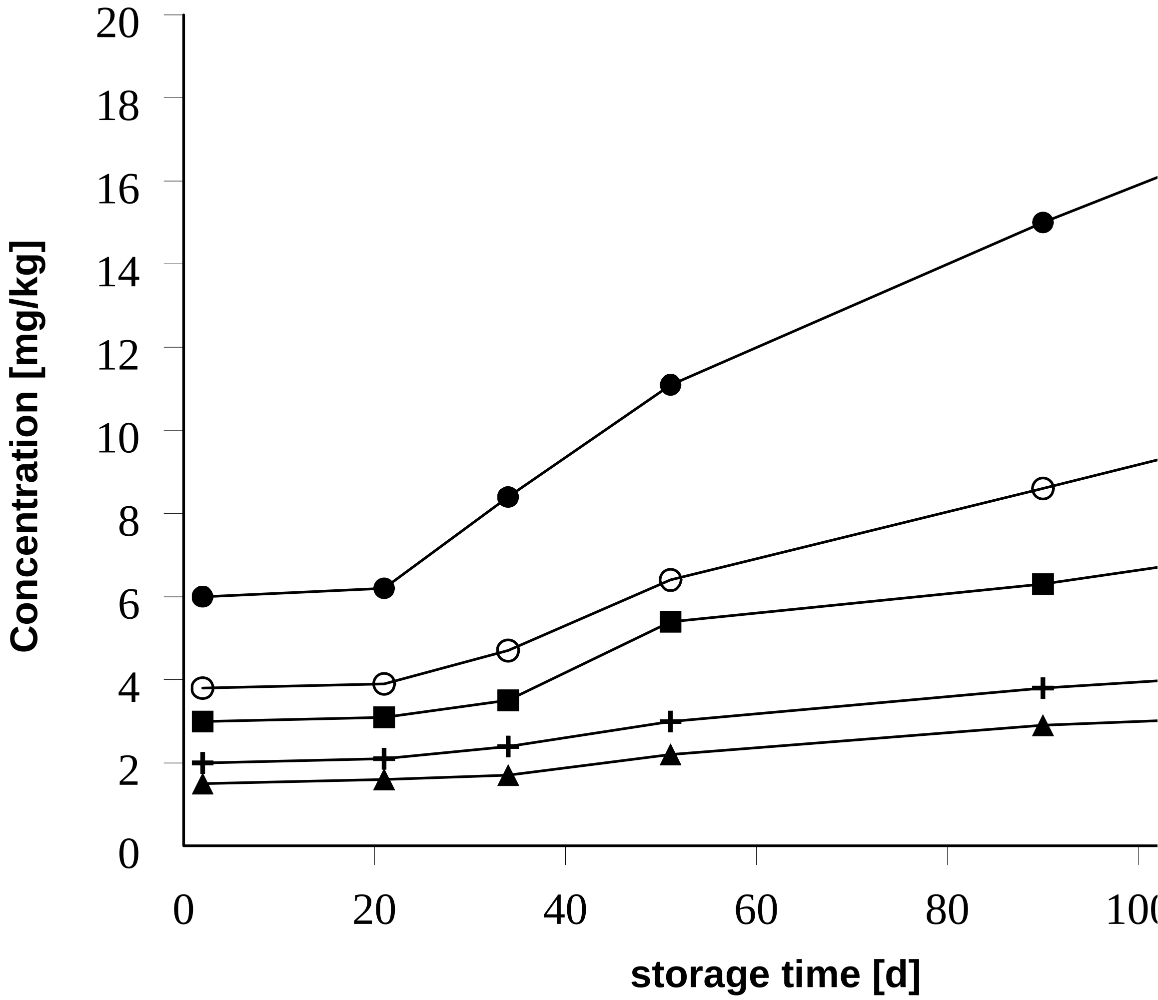
3
4
5
6
7
0
20
40
60
80
100
120
storage time [d]

Fig.3


20
18
16
14
12
10
8
6
4
2
0
Concentration [mg/kg]
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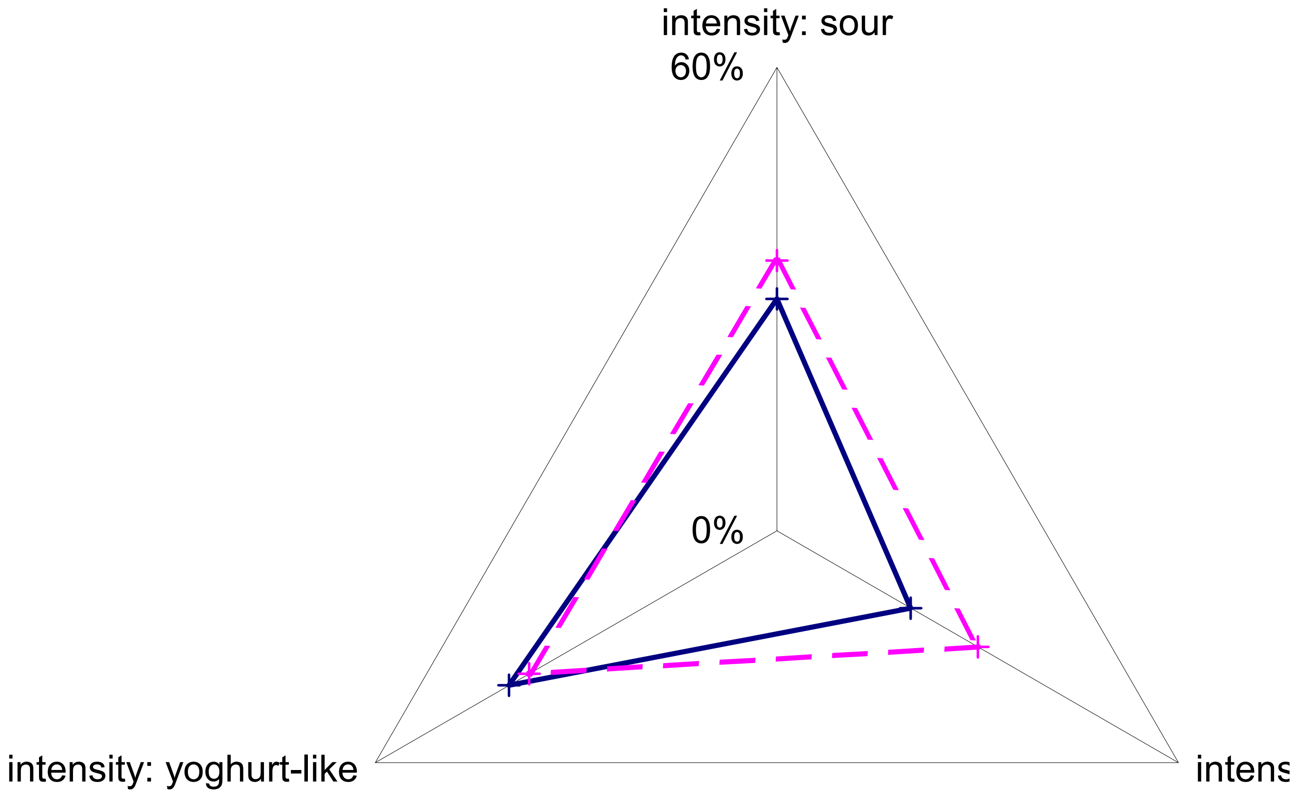
20
40
60
80
100
storage time [d]

Fig.4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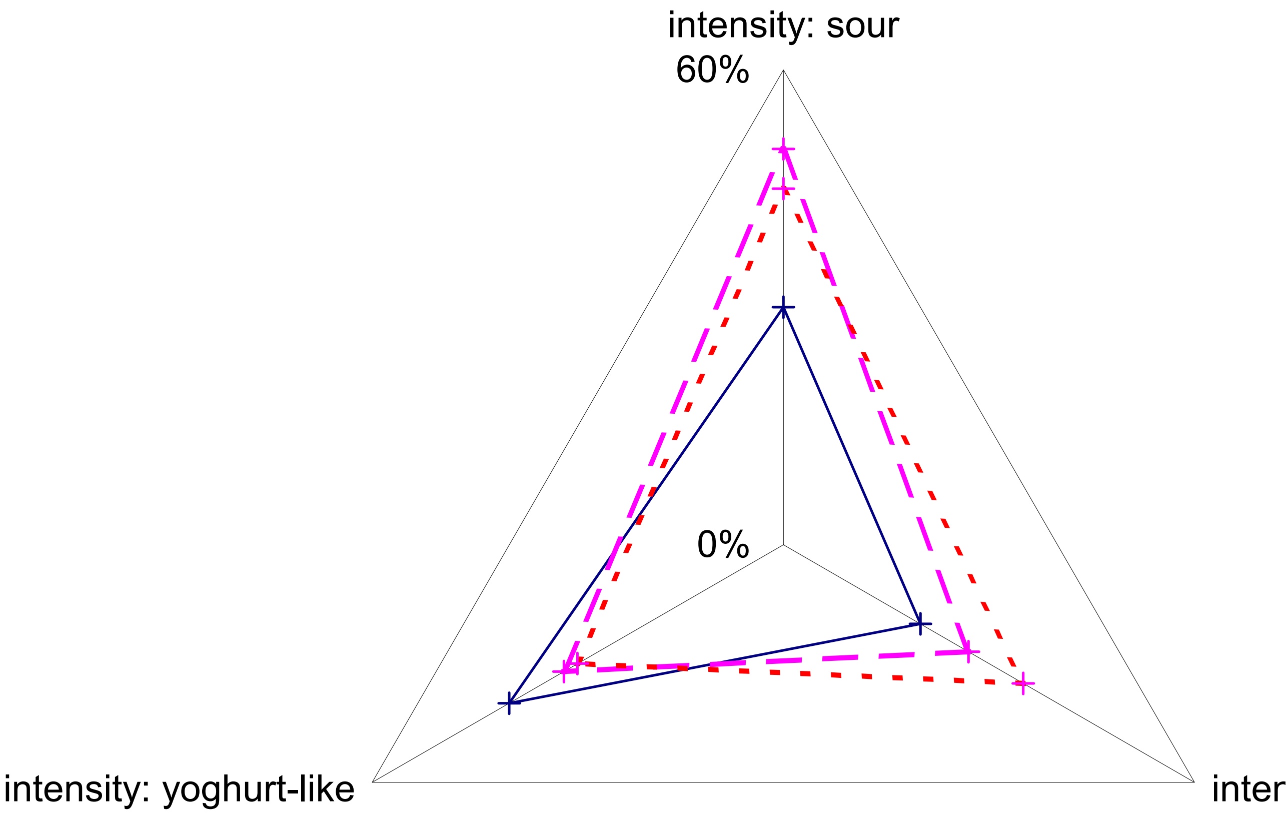
60%
0%
intensity: yoghurt-like
intens


intensity: sour
60%
0%
intensity: yoghurt-like
inter