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Changes in organic acid contents during mead wort fermentation

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Abstract

Organic acids contained in honey influence the fermentation rate and frequently cause mead fermentation to halt. The purpose of this study was to determine the changes that occur in a group of selected organic acids during the fermentation of mead worts and their impact on the process. Mead worts (1:2 and 1:3 (honey:water)) were fermented using *Saccharomyces cerevisiae* yeast of the Johannisberg-Riesling breed (ŁOCK 105). The worts were supplemented with diammonium hydrogen phosphate (0.4 g/l) and citric acid (0.25 g/l). During fermentation, the contents of selected carboxylic acids were determined using gas chromatography. Mead worts contain relatively high amounts of medium-chain fatty acids, which are believed to inhibit fermentation. The dominant compounds of this group are decanoic (42 mg/l), dodecanoic (31 mg/l) and octanoic (26 mg/l) acids. The experiments demonstrated that during the early days of fermentation, the main acids to form are the acetic and succinic acids, which reduce the wort pH, while the content of fatty acids drops by 70–80%. During fermentation, the amounts of the formic, hexadecanoic and octadecanoic acids also fall.

Keywords: Derivatisation; Fatty acid; Fermentation; GC; Honey; Mead; Organic acid; SPE; SPME; TMS

1. Introduction

Mead is an alcoholic beverage obtained by fermenting mead wort, with the possible addition of various spices and hops, which contains between 9% and 18% of alcohol by volume. Mead wort is produced by diluting bee honey with the appropriate amount of water or fruit juice. Depending on the proportion to which honey is diluted, different types of mead are obtained; the finest at 1:0.5 (honey: water), or at 1:1, 1:2 and 1:3. Worts that contain the most sugar (the 1:0.5 and 1:1 types) are prepared by successively adding the appropriate portions of honey so as not to stop the fermentation, due to an excessive osmotic pressure. Bee honey is a raw material characterised by low acidity. To ensure the optimum pH of mead wort, sometimes citric, tartaric or lactic acid is added (Gogol & Tuszyński, 1996). Organic acids also form as the by-products of ethanol fermentation. A quick increase in acidity is observed in the first hours of the process and sometimes combines with the low buffer capacity of mead wort to cause a rapid fall of pH causing the fermentation to stop. This phenomenon, caused mainly by the formation of succinic acid, is strongly dependent on the strain of yeast and the presence of nitrogen compounds (Fleet, 1994).

The production of mead, as an alcoholic beverage, has been known since ancient times. Poland has many centuries of tradition in mead making, but the production of mead has suffered in recent years, partially due to the lack of scientific progress in this field. The relatively long time needed for wort fermentation and mead maturing, ranging from several months (meads made from wort 1:3) to several years (meads made from worts 1:0.5 and 1:1), requires producers to use large capacity vessels and also to spend significant sums on raw material purchases (Aleksandrowicz, 1988). Certain process difficulties also result from the high acidity and the shortage of substances necessary for yeast development. For these reasons, research is conducted to optimise the production process of these beverages.

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Frequently, during fermentation, the appropriate alcohol content is not reached within the appropriate time. The few studies published on this subject dealt with the characteristics of mead (Kime, McLellan, & Lee, 1991), the breeding of yeast adapted to high sugar concentrations (Sawicka, 1970), thermal processing (Wintersteen, Andrae, & Engeseth, 2005), and methods of preparing worts as well as the processes of their fermentation and maturing (Wzorek & Lisak, 1980; Wzorek, Konieczna, Szymańska, & Bugajewska, 1983).

Experience with grape wine production indicates that changes in the concentration of organic acids can cause the fermentation to halt. Non-dissociated particles of the octanoic, decanoic and acetic acids are antiseptic, and in greater concentrations can completely stop yeast development (Brul & Coote, 1999; Cabral, Viegas, & Sá-Correia, 2001). Fatty acids can also cause an increased activity of certain enzymes, like ATP-ase. Higher concentrations of octanoic acid activate that enzyme and at the same time stop cell growth (Arneborg, Jespersen, & Jakobsen, 2000). The antiseptic effect of medium-chain fatty acids usually prolongs the lag phase and reduces the growth and the cell weight output, in some cases also contributing to cell death. Octanoic acid retards the biosynthesis of acetic and succinic acids by yeast cells (Viegas & Sá-Correia, 1995). Decanoic acid intensifies the outflow of amino acids from the cell, improves the passive flow of protons through the cell membrane, and can even cause it to be broken leading to outflow of cytoplasm (Stadford & Anslow, 1996). It should be mentioned that the reduction of the yeast biomass growth by acetic acid also has a partially beneficial impact on the capacity of ethanol production (Taherzadeh, Niklasson, & Liden, 1997).

The biochemical transformations of organic acids have been relatively well researched and described for the fermentation of grape must (Fleet, 1994). However, mead worts are characterised by a lower pH and a different combination of acids originating from bee honey. So far, no research has been done on the transformations of organic acids during mead wort fermentation. Due to the high sugar content, the fermentation process progresses very slowly, requires the right pH, temperature, and specific yeast breeds and biocatalysts growth. The identification and elimination of factors which restrict cell activity in these conditions could shorten the process and thus, cut costs.

The purpose of this study was to identify the quantity of carboxylic acids in mead worts and the changes that occur in a group of these compounds during the fermentation.

2. Materials and methods

2.1. Biological material

Free cells of *Saccharomyces cerevisiae* yeast, Johannisberg–Riesling (JR) breed, obtained from the Collection of Pure Industrial Microorganism Cultures of the Institute of Fermentation Technology and Microbiology, Technical University of Lodz (collection no. ŁOCK 105) were used.

2.2. Mead wort preparation and fermentation

Buckwheat honey (Bartnik Sadecki, Poland) was mixed with potable water (66.1 mg/l Ca^{2+}, 9.0 mg/l Mg^{2+}) in the following proportions: 1:2 (v/v) and 1:3 (v/v), heated and gently boiled for 10 min, then topped up with diammonium hydrogen phosphate(V) (0.4 g/l) and citric acid (0.25 g/l); the extract was checked after mixing.

The hot wort was poured into conical flasks (1.5 l of wort into 3 l flasks) stopped with sterile fermentation trap tubes. After the wort was cooled down to approximately 30 °C, a precisely defined amount of starter yeast was added (0.5 g/l calculated for the dry substance); this yeast was prepared in a three-stage culture (agar medium, 9% brewer's wort, still culture (10 ml) and shaken culture (100 ml)). The fermentation progressed at room temperature (20 °C to 22 °C) until two subsequent weights of samples measured at an interval of 4 days did not differ by more than 1 g. All fermentation experiments were conducted twice.

2.3. Analytical methods

2.3.1. General

The ethanol content, pH, the total and volatile acidity were determined using official methods (O.I.V., 1990).

2.3.2. Acetic and formic acid determination

2.3.2.1. General. The analysis was performed by gas chromatography after first esterifying the analysed acids with benzyl bromide and extracting them using headspace solid-phase microextraction (HS-SPME) (Wittman, Van Langenhove, & Dewulf, 2000).

2.3.2.2. Sample preparation. A 15 ml vial was filled with 1.5 ml of Clark–Labes buffer (pH 7), 0.2 ml of the sample to be analysed and 0.02 ml of a 50% benzyl bromide solution in benzyl alcohol. The vial was sealed with a screw cap with a Teflon liner and heated at 50 °C for 4 h, during which time it was mixed using a magnetic stirrer. Then, the sample was cooled in an ice bath, 0.99 g of NaCl was added, and the sample was placed in an incubator (30 °C, magnetic stirrer). After 30 min, an SPME (PDMS, 100 μm, Supelco) fibre was placed in the headspace for another 30 min. The analytes absorbed were desorbed for a time of 3 min in the sample injector of a gas chromatograph.

2.3.2.3. Chromatographic separation conditions. The chromatographic analysis was performed using an HP 5890, series II gas chromatograph, with a flame ionization detector (FID) and an HP5 capillary column (dimensions: 30 m × 0.53 mm × 2.65 μm). The temperature of the injector and detector was set to 250 °C. A programmed increase

of the column temperature to 40 °C (5 min), and to 250 °C (7 °C/minute) and 1 min of final heating were applied. The carrier gas was helium at 20 ml/min. Signals were identified by comparing their retention times with formic and acetic acid esters. The recovery of formic and acetic acids was relatively high, at 96.4% and 95.0%, respectively.

2.3.3. Fatty acid determination

2.3.3.1. General. Fatty acids were analysed as their volatile methyl esters (Gallart, Francioli, Viu-Marco, Lopez-Tamames, & Buxaderas, 1997).

2.3.3.2. Determination of non-volatile carboxylic acids. Non-volatile organic acids (hydroxy- and multi-carboxylic derivatives) were determined by gas chromatography in the form of trimethylsilyl derivatives (TMS) after preliminary purification on ion-exchanger resin. The samples for determination were prepared using a modified J.T. Baker procedure (Phillipsburg, NJ) (Application Notes).

2.3.3.3. Sample preparation. A BAKER SPE column processor and SPE-SAX 3 ml columns (Supelco) filled with 500 mg of ion-exchange resin (quaternary amines) were used for the preparation. Prior to the analysis, the columns were conditioned by rinsing them with the following solutions: methanol–water 1:1, 2 ml, 0.3 ml/min; NaOH 1 M, 2 ml, 0.3 ml/min; 30 ml of redistilled water, 3.0 ml/min; CH_3COOH solution (pH 2.05), 5 ml, 0.5 ml/min; CH_3COOH solution (pH 4.5), 45 ml, 3.0 ml/min. The sample (0.5 ml) supplemented with 0.2 ml of internal standard (malonic acid 4576 mg/l) was fed into the prepared column at 0.3 ml/min, the column was then rinsed with 3 ml of distilled water, dried for 5 min and eluted with 5 M formic acid. The eluant was gathered into 4 ml glass vials, water was added to 4 ml and the contents were mixed. One millilitre of the resulting solution was measured, transferred to a dry 4 ml vial, dried at 45 °C, dissolved in 0.5 ml of freshly-distilled pyridine (dried over KOH), after which 0.1 ml of BSTFA (N,O-bis-(trimethylsilyl) trifluoroacetamide) was added. After 10 min, the sample was separated on a gas chromatograph.

2.3.3.4. Chromatographic separation conditions. The chromatographic analysis was conducted using the apparatus described above. This time the column was heated from 55 °C (6 min) to 260 °C (at 8 °C/min), with 5 min of final heating.

Signals were identified by comparing the retention time of trimethylsilyl-derivatives (TMS) of succinic, tartaric, lactic and citric acids.

2.4. Statistics

The calculated average deviations were plotted on graphs. For selected series, a one-factor analysis of variance (ANOVA) was calculated using StatSoft Statistica 6.1 software (significance level $\alpha = 0.05$). The differences between the averages were verified using the Duncan test ($p < 0.05$).

3. Results and discussion

3.1. General

The process of mead fermentation is particularly difficult because of the high sugar concentration of the wort and the resultant high osmotic pressure. In the first week, the 1:3 mead wort fermented almost twice as fast as the 1:2 mead wort (Fig. 1). The rate of the process probably depended on the osmotic pressure (1:2 worts with approx-

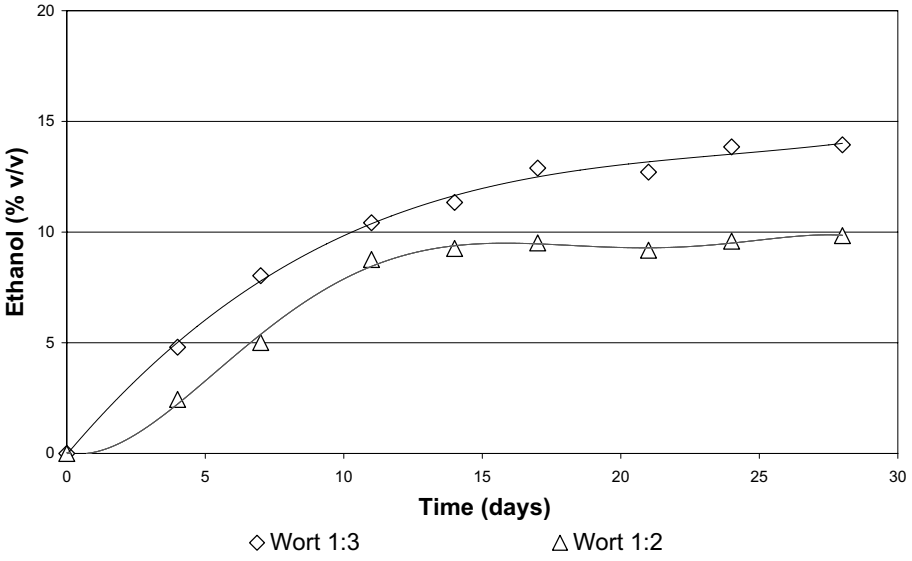

Fig. 1. Changes in the contents of ethanol during the fermentation of mead worts.

imately 35% of extract) and the longer time over which yeast cells could adapt to the specific environmental conditions.

In the first week of fermentation, total acidity increased markedly, up to 4.5 g/l (Fig. 2). This increase in acidity was caused mainly by the yeast cells synthesising acetic acid (0.7–1.0 g/l) and the succinic acid (0.1–0.3 g/l) (Figs. 3 and 4). This changed concentration of organic acids reduced pH to 3.0–3.2, which remained practically unchanged until the fermentation end. The quick pH drop in the fermenting worts is beneficial, as it stops the development of foreign microflora.

In the second week of fermentation, acidity dropped by between 8% and 22%, and in the following weeks changed only insignificantly. The total acidity of 1:3 samples was usually 0.1–0.7 g/l lower (Fig. 2).

3.2. Acetic acid

Volatile acidity increased during fermentation, mainly as a result of acetic acid synthesis, whose concentration in the 1:2 samples reached a maximum (1.3 g/l) on the 24th day of fermentation (Fig. 2). The acetic acid content of 1:3 worts (less concentrated) was 60% (0.44 g/l) lower in the second week.

The acetate quantity depends mainly on the concentration of carbohydrates and the source of nitrogen as well as the pH (Fleet, 1994). Acetic acid content increases signif-

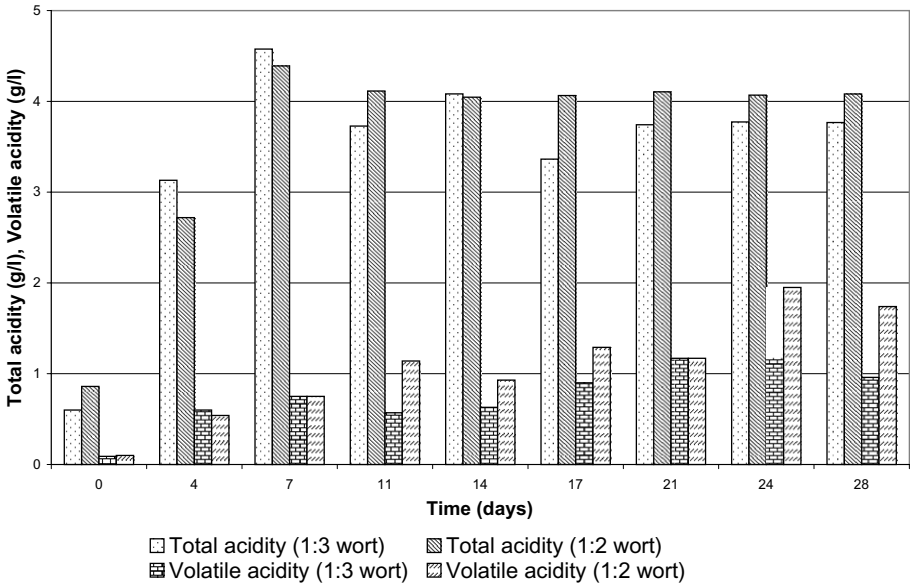

Fig. 2. Changes in total and volatile acidity during the fermentation of mead worts.

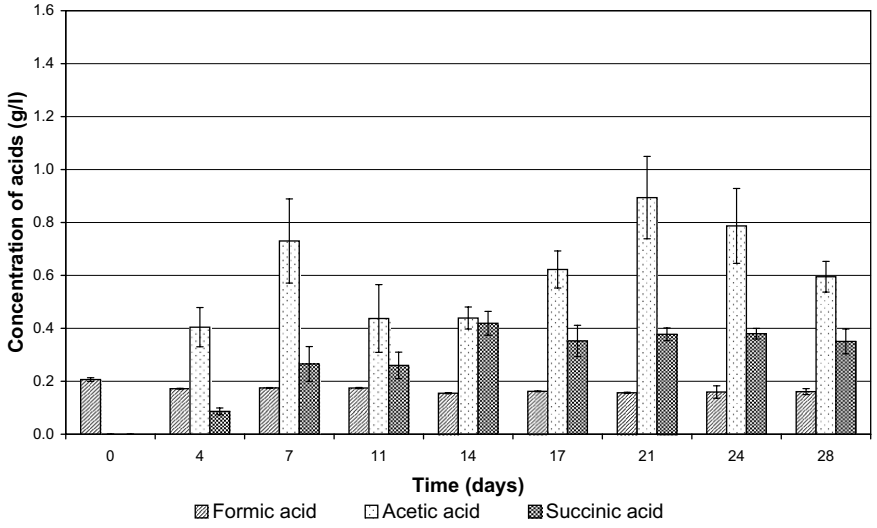

Fig. 3. Changes in the contents of formic, acetic and succinic acids during the fermentation of 1:3 mead worts.

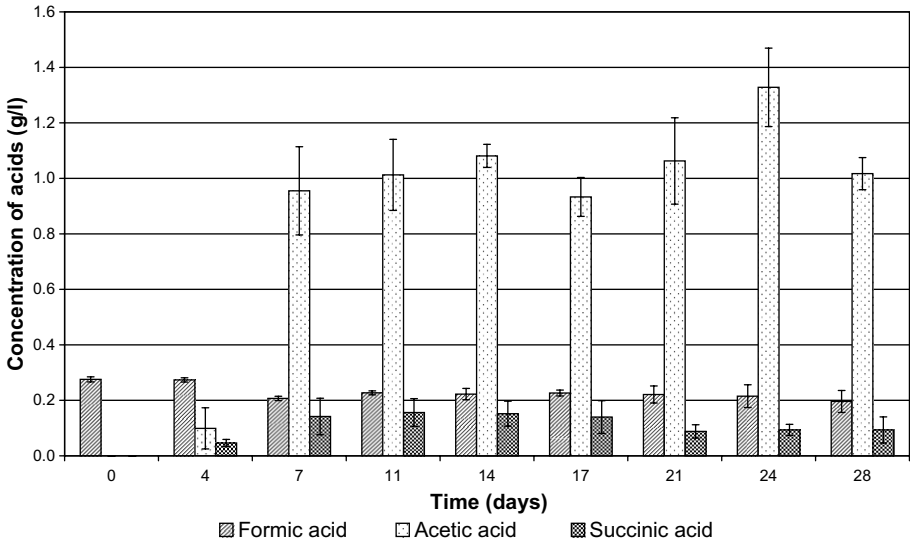

Fig. 4. Changes in the contents of formic, acetic and succinic acids during the fermentation of 1:2 mead worts.

icantly during alcoholic fermentation if the sugar concentration is high. If yeast is subjected to osmotic stress, the reduction of acetaldehyde to ethanol is frequently observed to slow down, as a result of which more acetic acid is synthesised (Erasmus, Merwe, & Vuuren, 2003). A similar effect was noted if the wort had a low pH (Shimazu & Watanabe, 1981; Monk & Cowley, 1984). The acetic acid in the fermenting worts changes yeast metabolism, reducing the glycerol synthesis and the biomass output. Yeast cells use more ATP to transform acetic ions into acetyl-CoA and remove the protons coming from the acid dissociation, which leads to a slight increase in ethanol production (Taherzadeh et al., 1997).

3.3. Formic acid

Another component which had a degree of impact on the volatile acidity was formic acid. The formate contained in the wort, originating from honey, was partially metabolised by yeast, so its content in the 1:2 mead wort dropped from 0.28 to 0.20 g/l (Fig. 4). However, in 1:3 mead wort, the concentration dropped only by some 0.03 g/l, reaching 0.16 g/l in the fourth week of fermentation (Fig. 3). Like acetic acid, formic acid also significantly increases volatile acidity, which in the case of mead should not exceed 1.4 g/l recalculated for acetic acid (Dziennik Ustaw, 2004). These compounds also slow down fermentation rate (Arneborg et al., 2000).

3.4. Medium-chain fatty acids

Of the medium-molecule fatty acids determined in mead worts, comparatively high concentrations were detected for decanoic (13–42 mg/l), dodecanoic (7–31 mg/l) and octanoic acids (5–26 mg/l) (Figs. 5 and 6). Tetradecanoic acid was present in lower quantities (8–15 mg/l). During the first two weeks of 1:2 mead wort fermentation, the concentrations of octanoic (42%) and decanoic (51%) acids dipped significantly, but went up again in the third and fourth weeks (Fig. 6). The contents of dodecanoic and tetradecanoic acids also fell in the first week of the process (by 28% and 60%, respectively), but they did not change much at later fermentation stages. In 1:3 mead worts, only the dodecanoic acid concentration shrunk markedly (Fig. 5). The decreasing concentrations of fatty acids during the first two weeks of fermentation results from their metabolisation by yeast cells (Fleet, 1994). It is much more difficult to explain the increase of their concentrations in the third week of the process, the probable cause being the solution of the fatty acids in the ethanol created. The determined concentrations of medium-chain fatty acids were almost ten times greater than those so far determined in fermenting grape musts (Ancin, Ayestaran, & Garcia, 1998). These compounds probably slow mead wort fermentation down. The octanoic and decanoic acids are present in the fermenting wort mainly in their non-dissociated form. Their molecules, which have an amphipathic structure, are easily adsorbed on yeast surfaces and penetrate into the cells. Non-dissociated fatty acids can also adsorb on the insides of fermentation vessels and on matter suspended in mead worts. The increasing concentration of medium-chain fatty acids in cell membranes reduces their hydrophobic characteristics, impacts the operation of enzymes and reduces the cell's resistance to ethanol. Octanoic acid is very toxic to yeast cells at concentrations as low as 16 mg/l (Viegas & Sá-Correia, 1995). The octanoic as well as other lipophilic weak acids, particularly at low pH, kill microorganisms by making their cytoplasm acidic and accumulating toxic anions inside the cells. Fat-soluble acids have a significant impact on the spatial organisation of cell membranes, causing the energy necessary to transport protons to be exhausted and as a result bring about a drop of the pH inside the cell (Cabral et al., 2001). Other acids, like dodecanoic (lauric) and tetradecanoic acids, which were

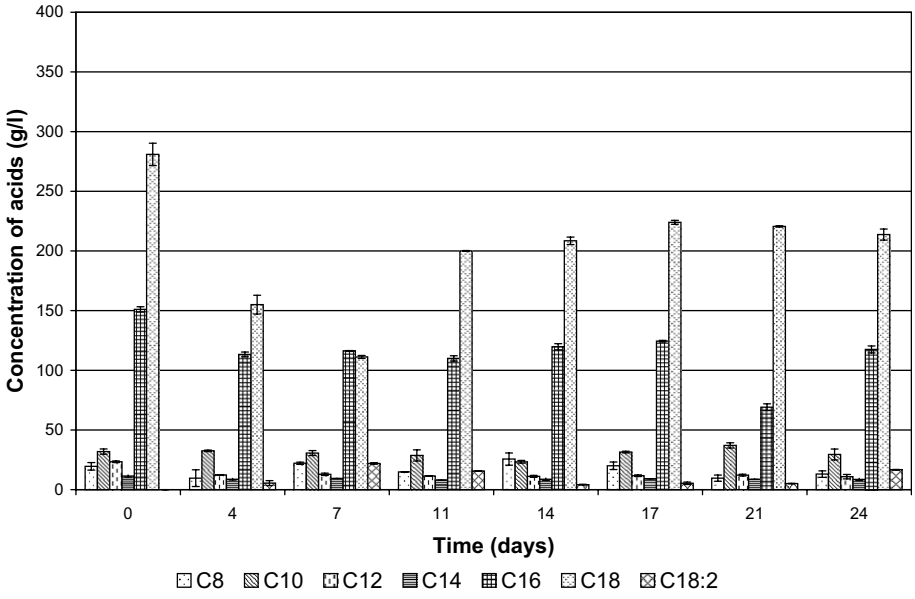

Fig. 5. Changes in the contents of octanoic (C8), decanoic (C10), dodecanoic (C12), tetradecanoic (C14), hexadecanoic (C16), octadecanoic (C18) and octadecadienoic acids (C18:2) during the fermentation of 1:3 mead worts.

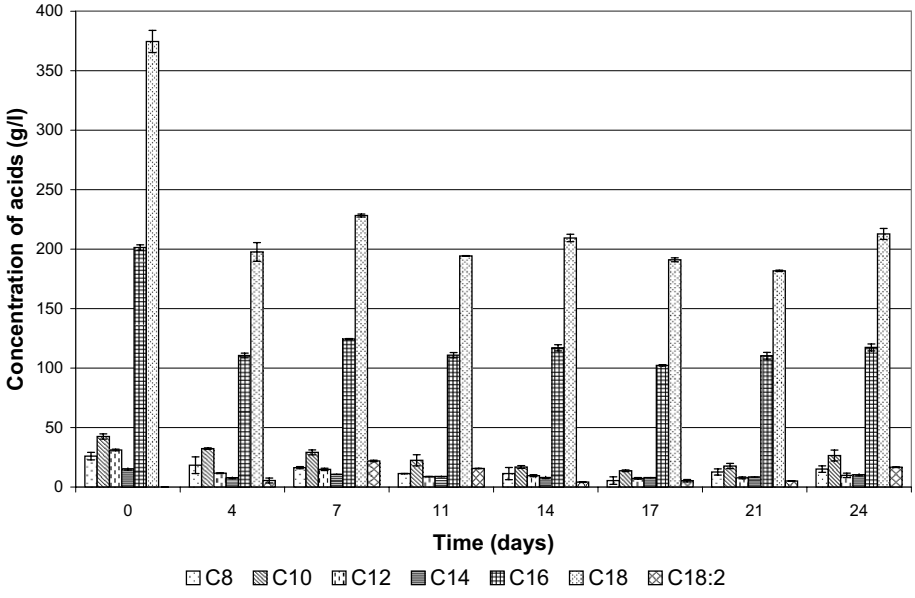

Fig. 6. Changes in the contents of octanoic (C8), decanoic (C10), dodecanoic (C12), tetradecanoic (C14), hexadecanoic (C16), octadecanoic (C18) and octadecadienoic acids (C18:2) during the fermentation of 1:2 mead worts.

present in relatively high concentrations (Figs. 5 and 6), probably have a similar influence. The high osmotic pressure of mead worts may increase the toxicity of these acids to yeast cells and thus, slow the fermentation down.

It should be emphasised that short-chain fatty acids improve the taste of mead and also form esters, which give mead its bouquet. If these components are removed using sorbents, the sensory characteristics of the product may change (Bugajewska et al., 1998). Processing wine with ion-exchange resins does allow all anions of the undesirable acids to be removed, but the process is illegal in Poland (Dziennik Ustaw, 2004).

3.5. Long-chain fatty acids

The concentration of octadecanoic (stearic) acid fell during the first week of fermentation, to rise again in the following two (in a 1:3 mead wort) up to 224 mg/l (Figs. 5 and 6) The hexadecanoic (palmitic) acid concentration was lower (69–151 mg/l), and decreased slightly during the process. The decreasing amount of fatty acids can be explained by their being metabolised by yeast cells. The increased stearic acid content in the second week of fermentation may have been the result of the increasing content of ethanol, in which waxes originating from honey

Table 1
Concentration of non-volatile acids in fermenting and fermented worts

Compound	Mead worts		Fermented mead worts	
	1:3	1:2	1:3	1:2
Succinic acid (mg/dm^3)	nd	nd	351 ± 10	93 ± 20
Citric acid (mg/dm^3)	223 ± 34	250 ± 9	216 ± 11	194 ± 30
Lactic acid (mg/dm^3)	720 ± 20	1070 ± 100	620 ± 20	1130 ± 110
Tartaric acid (mg/dm^3)	43 ± 12	45 ± 12	51 ± 8	40 ± 3

nd, not detected.

would dissolve. The octadecanoic acid represented about 50%, and the hexadecanoic acid about 30% of all medium- and long-chain fatty acids found in fermented mead worts.

In fermenting worts, the level of unsaturated higher fatty acids containing two and three unsaturated bonds (Figs. 5 and 6) was also monitored. The linoleic acid concentration changed during the fermentation of both the 1:3 and 1:2 mead worts. After the first week, the amount of this acid dropped to some 4 mg/l, but in the third and fourth weeks increased again (8.0–16.7 mg/l).

3.6. Non-volatile acids

The acidification of mead worts is necessary for the correct progress of their fermentation (Gogol & Tuszyński, 1996). In the first days of fermentation, the acidity of mead is insufficient to ensure the optimum pH values for a microbiological purity.

During the fermentation, succinic acid also formed, reaching a concentration four times greater in the 1:3 mead wort (0.10–0.42 g/l, Fig. 3) than in the 1:2 wort (Fig. 4). The contents of other acids, like lactic (approx. 1 g/l), citric (0.25 g/l) and tartaric acids (40–50 mg/l) did not change significantly during the fermentation (Table 1), while the differences in their concentrations were mainly the result of differences in the dilution of honey performed to prepare 1:2 and 1:3 worts.

The acetic and succinic acids, formed during fermentation, reduce the wort pH. The increased total acidity caused the pH of the wort to drop, simultaneously reducing the dissociation of fatty acids present in the wort. The above factors, combined with the relatively high concentration of medium-chain fatty acids, may cause the fermentation to slow down, or stop.

The results obtained show that during the production of mead particular attention should be paid to the contents of the above acids in the processed honey. There is a need for further research conducted in industrial-scale conditions.

4. Conclusions

- Total acidity rises mainly in the first week of mead wort fermentation. The dominant compounds determining the change in the acidity of fermenting wort are acetic and succinic acids.
- Mead worts contain relatively large amounts of medium-chain fatty acids, which are believed to inhibit fermentation. The main compounds in this group were the following acids: decanoic (42 mg/l), dodecanoic (31 mg/l) and octanoic (26 mg/l).
- A higher extract content and the increase in general acidity of worts cause the yeast to synthesise more acetic acid, leading to an increased content of non-dissociated fatty acids, which slow down ethanol fermentation.

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