**Rapid Communication**

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**Changes in the antioxidative properties of honeys during their fermentation**

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**Abstract:** The aim of this preliminary study was to investigate whether the fermentation process affects the antioxidative properties of honeys. Therefore, the total antioxidant potential (TAP) of eleven meads was examined. TAPs were estimated using DPPH, hydroxyl radicals, and amperometric measurements in the flow-through HPLC system. The results were correlated with the total content of polyphenols and anthocyanins. Additionally, the concentrations of ethanol, sugars, and hydrogen peroxide were measured. The influence of side reactions and the presence of hydrogen peroxide, glucose, ethanol, and anthocyanins on the free radicals generation were also tested. The use of HPLC electrochemical detection for TAP measurements is particularly preferred. Depending on the potential used, different antioxidants can be measured. It turned out that fruit and species meads are characterized by much higher TAPs measured at high potential of the working electrode than at lower potential due to the high concentration of weak antioxidants which do not provide signals at low potentials. It was found that dark honeys (buckwheat, honeydew) are characterized by much higher TAP values than light ones (acacia, linden, multi-floral). The concentration of anthocyanins decreased during fermentation. They are removed together with the sediment.

**Keywords:** mead, honey, total antioxidant potential, high-performance liquid chromatography, free radicals, Fenton reaction

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1 Introduction

Metabolic processes in every living organism are carried out to produce a sufficient amount of energy for the body, to grow and function properly. Most of the metabolic processes are sustained by oxygen [1]. Unfortunately, this leads to the formation of reactive oxygen species (ROS), among them free radicals (FR) [2]. Radicals non-specifically react with all cell components and compounds by oxidizing them and cause cell apoptosis [3]. The human body itself can eliminate the free radicals by producing endogenous free radicals scavengers and/or antioxidants. Antioxidants can also be administered by food, drink, cosmetics, or injections [4].

In the literature, various total antioxidant potential (TAP) assays are described [5,7,14]. The classical photometric or fluorimetric assays use various stable radicals or their generators. The sample oxidation is measured using different analytical assays (photometric, fluorimetric, luminescence measurements, MS, HPLC, GC, thermogravimetric, etc.). The TAP measure is in this case the inhibition time, induced by the sample. It seems that natural methods of examination of oxidation process should involve electroanalytical techniques. As a matter of fact, we have elaborated such voltammetric assay [16]. The use of voltametry in HPLC detection significantly decreases the detection limit due to the high convection currents and the lack of capacitive currents.

The aim and the novelty of the paper are to examine the influence of the fermentation on changes in the antioxidative properties of honeys/meads.

2 Materials and methods

2.1 Reagents

Methanol (pure for chromatography), buffered saline tablets pH 7.4, DPPH (2,2-diphenyl-1-picrylhydrazyl), iron(ii) sulfate(vi), and 4-hydroxybenzoic acid were
obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Other reagents were at least of analytical-reagent grade and were used without further purification.

2.2 Instrumentation

HPLC measurements were performed using a chromatograph (Knauer, Berlin, Germany) equipped with a Cosmosil 5 microns, 4.6 × 150 mm, 5C18-MS-II column. Photometric measurements were performed using Helios Epsilon spectrophotometer (Thermo Fisher Scientific, Rochester, USA).

2.3 Materials

The antioxidative properties of eleven samples of meads (five fruit, one herbal, and five species) were investigated. Honeys were purchased from apiaries situated in two localities of Siedlce vicinity, Mazowieckie Voivodeship, Poland. They were prepared as mead trójniak, made using two units of water for one unit of honey. Fruit and herbal meads were prepared on the basis of linden honey.

The honey wort was boiled for 60 min, and then foam was removed from the surface. The wort was then transferred to the fermentation balloon. Mother yeast, 4 g of citric acid, and 0.5 g of diammonium phosphate were added. The balloon was closed with a distillation tube, into which a tablespoon of water was poured and set aside at room temperature (about 22°C). After six days, a baker’s yeast was added. On 41 day, sediments were collected. Samples were collected at various stages of production on day 0, 6, 13, 20, 27, 34, 41, 57, 69, 85, and 120.

2.4 Methods

Determination of the TAP related to the hydroxyl radical, TAP$^{OH}$, is based on the generation of hydroxyl radicals in the Fenton reaction as it was described in [5]. The oxidation of p-HBA by Fe(III) ions in the presence of glucose and ethanol was also investigated [6]. TAP assay based on HPLC-ED measurements, TAP$^{ED}$, and related to DPPH radicals, TAP$^{DPPH}$, was described by us earlier [7]. The total polyphenol content, PF, was determined by the colorimetric method with Folin-Ciocalteu (FC) reagent [8]. Determination of anthocyanins was carried out using two analytical assays, photometric and HPLC [9]. Ethyl alcohol was determined by pycnometric measurement of distillate density according to PN-A-79120/04 [10]. Determination of sugar content has been performed by the refractometric method according to PN-A-74252 [11].

Ethical approval: Not applicable, the conducted research is not related to either human or animal use.

3 Results and discussion

The changes of averaged TAP$^{OH}$, TAP$^{DPPH}$, and PF relative (100% refers to the highest value measured with a given assay) values of overall tested honeys during their fermentation are presented in Figure 1. For most of honeys, the TAPs decreased during the first 6 days. After this time, the fermentation accelerated. Similar changes were observed for TAP$^{DPPH}$ and PF. Because OH radicals react nearly with all chemical compounds (among them with the alcohols and sugars), the TAP$^{OH}$ for all meads are very similar (Figure 2).

The measurements of TAP$^{ED}$ were performed at the potential range 0.2 to 1 V. The oxidation potential defines the antioxidant strength, while the electric current – the antioxidant concentration. The fruit meads (elderberry, raspberry, bilberry) are characterized by high antioxidant potential measured at 1 V (Figure 2). However, only one of them (melissa) contains strong antioxidant (big peaks at 0.4 V). Species honey is characterized by much bigger total surface area at 1 V potential than at lower potentials as the high concentration of weak antioxidants does not provide signals at low potentials. It turned out that dark

![Figure 1: Changes of relative TAP$^{OH}$ (●), PF (▲), and TAP$^{DPPH}$ (■) [%] values during fermentation of honeys/meads.](image)
hones (buckwheat, honeydew) are characterized by much higher TAP values than light honeys (acacia, linden, multi-floral) [12].

The concentration of anthocyanins in fruit honeys ranged from 0.17 mg/100 g for bilberry up to 2.5 mg/100 g for blueberry honey. It turned out that their concentration decreased 2 times during fermentation. The sediments collected from honey had an intense red-blue color, characteristic of anthocyanins. It means that most of the anthocyanins are removed together with the sediment, and only a small percentage of them remain in the meads.

During the honey fermentation, sugar is converted into alcohol. The highest decrease of sugar concentration during fermentation (conversion from honey to mead) has been observed for a blueberry, the smallest for the chokeberry honey/mead. The level of alcohol concentration in the obtained meads varied from 8 to 13%.

Honeys contain hydrogen peroxide that explains their antibacterial properties [13]. The original method of its determination was elaborated. It was based on the Fenton reaction (honey was used instead of H₂O₂) and was characterized by the detection limit of 1.2 × 10⁻⁵%, for S/N = 3. It turned out that the hydrogen peroxide in investigated honeys and meads was below the detection limit.

The TAP is equal to the sum of products from the concentrations of all antioxidants in the sample and their antioxidant power [14]. It turned out that TAP⁰H values are not additive. Hydroxyl radical reacts with sugars and alcohol. On the other side, it was found that glucose and ethanol may participate in the formation of hydroxyl radicals [15].

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