Biologically active components of lipid complex in pressed wine yeast

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Abstract. In this work we made an attempt to determine, quantitatively and qualitatively, the isoprenoid including sterol) the composition of the lipid complex of pressed wine yeast from a grape must fermentation. The sterol composition of the lipid complex we obtained was studied using a method for determination of unsaponifiable substances in vegetable oils. Isoprenoid, including sterols, were determined by gas-liquid chromatography. The chromatograph used was an Agilent Technology 6890 with a mass-spectrometry detector and had a HP-1 column. The carrier gas (helium) flow rate was 1 ml/min. The chromatography was effected at a temperature range of 50-300°C. The revealed unsaponifiable fraction of the yeast lipid complex contained diterpenoid and triterpenoid alcohols and sterols. The sterols were campesterol, β-stigmasterol, obtusifoliol, γ-sitosterol, lanosterol, cycloartenol and α-sitosterol, hydrocarbons, α-tocopherol and γ-tocopherol, the total sterols accounting for 22.3 %. These findings clearly indicate the need to investigate, in more detail, the pharmacological and antioxidant effects of the wine yeast lipid complex from a grape must fermentation.

1. Introduction
It is assumed that combined methods to process products of secondary winemaking should result in a number of valuable functional and diet products, which is a concentrate of pressed grape pomace polyphenols and grape seed oil.

Amid products of the secondary winemaking one should emphasize residual wine yeast obtained as the result of grape must fermentation. Currently residual pressed wine yeast is used in agricultural industry as an organic fertilizer. In this work, using the method of gas chromatography, we determined quantitatively and qualitatively the content of isoprenoids, including sterols, derived from the lipid complex of pressed wine yeast, which is the result of grape must fermentation.

Substances of sterol nature can be practically used. Sterol hormones, glycoalkaloids, heart glycosites, bile acid and antifungal drugs are used in medicine [1]. For agricultural needs, sterols are used for the production of Vitamin D group. The most effective are fungisites, anti-rodent and growth stimulators for plants [2, 3]. Liquid crystals based on the cholesterol ester are used in the electronic industry [4]. In most cases these preparations are made of expensive and rare raw materials – cholesterol (animal steroid) and phytosterol (sitosterol, stigmasterol, campesterol). As steroid medications are used in a broad range of industries, it is necessary not just to increase efficiency of the production technology, but also to search for a new type of raw materials.
Lately much attention has been paid to the microorganisms as a steroid source. This work [5] provides combined general data indicating the effectiveness of yeast sterols, used for chemical and biological transformation into Vitamin D group and androstane hormones. Eukaryotic microorganisms, especially yeast are the richest in sterols. Ergosterol is the main component in the sterol yeast fracture. The percentage of ergosterol reaches 60-90% out of all sterols in a cell [5]. A number of yeast cultures show high amount of 24(28)-dehydroergosterol [3, 6]. Besides, some cultures accumulate zimosterol [6]. Other products of sterol biosynthesis can usually be found in trace amounts. Totally up to 20 various sterols can be found in yeast cells. It’s known that the composition of nutrient medium affects the sterol-formation in yeast, i.e. the presence of absorbable forms of carbon and nitrogen.

Detailed studies have shown the effect of nitrogen concentration in nutrient medium. Optimal molar ratio between carbon and nitrogen in medium (C/N=40) provides both the yeast growth and accumulation of sterols in yeast [7]. While the culture is aging the number of sterols is increasing and reaches its maximum in idiophase, when the second metabolism is triggered [14]. Phosphor is one of key factors which limits the triggering of the second metabolism. So phosphor concentration in culture medium will affect the synthesis of sterols [8].

Isoprene [6] is a common structural precursor of sterol synthesis and the synthesis of unsaturated fatty acids and phospholipids. That is why a group of fat-soluble and fat-associated substances (isoprenoids, including sterols) passes into fat-containing components during extraction of lipids from various plant or microbiological materials [22]. Vitamins, and above all, pantothenic acid are the most efficient growth stimulators; the latter enters the CoA and is involved in sterol synthesis [6]. Sterol biosynthesis obeys the “isoprene rule” [6, 9]. Squalene is the final product of the process.

Squalen epoxidizes and turns into lanosterol. Ergosterol is derived from lanosterol through the following transformation: demethylating in positions C 4α, C 4β. C 14α, transmethylating with formation of 24 (28) methylene group, side-chain hydrogenation with formation C22(23) olefinic link, isomerization $\Delta^8 \rightarrow \Delta^7$, dehydrogenation with formation C5(6) olefinic link, reduction of the methylene group to methyl [6,9]. Currently, the sequence of the reactions was determined for the *Saccharomyces cerevisiae* yeast [12].

It’s well known that the sterol biosynthesis may change, depending on cultivating conditions. The most important factor for sterol biosynthesis in yeast is oxygen access. It was proved that if yeast cultivates in good aerobic conditions, mainly ergosterol will be synthesized. Various homologs are the result of poor oxygen access [23]. The amount of sterols decreases significantly when yeast cells are being transferred from aerobic into anaerobic conditions. The ratio between various sterol fractions will be violated too. In aerobic conditions the content of bound sterols reaches 85-90% of overall sterol amount. Free sterols make only 10-15 %. In anaerobic conditions the amount of bound sterols decreases to 70-75%. Subsequently the amount of free sterols increases up to 25-30 % [20, 21, 23].

Amid sterols of microbial origin Ergosterol is the most available raw material for synthesis of steroid medications. Many countries produce it on industrial base. Traditionally ergosterol is produced from bread baking yeast biomass. Less frequently beer yeast biomass and mushroom micelle are used as a byproduct of organic acids and antibiotics production [13].

As mentioned above, in yeast sterol formation is affected by the composition of nutrient medium, i.e. by the levels of carbohydrates, nitrogenous substances and minerals as well as by the presence of vitamins: all these found in grape must. As it is known from the literature [14], sugars (up to 32 %), organic acids (of which tartaric acid accounts for 0.4 %) [15], phenolics (0.02-0.07%) [15], [16] and nitrogenous substances (0.04-0.06 %) are its major components [17]. For the major part, grape must contains Group B vitamins (mg): thiamin (0.16-0.45), pantothenic acid (0.5-1.4), pyridoxine (0.16-1.4), biotin (1.5-4.2) [18].
2. The purpose of the study
In this work we made an attempt to determine, quantitatively and qualitatively, the isoprenoid (including sterol) composition of the lipid complex of pressed wine yeast, resulted from a grape must fermentation. Gas chromotography was applied in the study.

3. The object of the study
As an object we used pressed wine yeast, resulted in the process of wine must fermentation, available at the wineries.

4. Materials and methods

4.1 Receiving a lipid complex of pressed wine yeast
Lipid complex was received using low temperature extraction of dry pressed wine yeast till the residual moisture of 8% with Freon. The gas pressure was 0.9-1.0 MPa, the extraction temperature was not more than 18 °С, time of extraction - two hours. Low temperature extraction installation was used for preliminary dried press wine yeast, which was dried till residual moisture of 10% and less. Liquid Freon was flowing under pressure of 5 kgs/sm and temperature 25 °С into extractor. The ratio between dried grape seeds and extragent was 1:2.5. The time of extraction together with saturation lasted three hours. After extraction was over fat-soluble substances together with extragent flowed into evaporator. In evaporator the pressure was decreased to atmospheric one, temperature was increased to 40 °С. Freon was evaporized and directed into the condensator and further into storage for secondary use. Extract, which contained oil and Freon traces, was poured from evaporator. Total removal of Freon from the extract was done in degasser for two hours, at a temperature of 50 °С, at rotation of electric engine of 1,350 times/ min².

4.2 Receiving and identification of unsaponifiable substances of yeast lipid complex by liquid gas chromatography
In order to study sterol and isoprenol composition, derived from lipid complex, the method was used to identify composition of unsaponifiable substances in vegetable oils [19]. Selected samples of lipid complex (5 grams in quantity) was saponified by 2H alcohol solution of potassium hydroxide in a water bath cup fitted with a backflow condenser. Distilled water was added to the cooled solution and transferred quantitatively into a separating funnel.

The unsaponifiable substances were extracted with several portions of petroleum ether. The combined extract was washed with water till it showed a neutral reaction, and the residual moisture was eliminated with anhydrous sodium sulfate. The solvent was distilled, and the unsaponifiable substances were dissolved in 5 ml of petroleum ether. Content of sterols was determined by gas-liquid chromatography. A standard solution of tridecan C₁₃H₂₈ in amount of 1mg/g was injected into lipid complex before the saponification process.

The chromatograph used was an Agilent Technology 6890 with a mass-spectrometry detector, quartz capillary column DB-5. The carrier gas (helium) flow rate was 1 ml/min. The chromatography was effected at a temperature range of 50-300°C.

4.3 Identification of antioxidant capability for lipid complex of pressed wine yeast and its unsaponifiable fraction
A photochem device (made in USA) was used to identify antioxidant activity in alcohol-soluble and fat-contained medium. The method was based on photo- and chemical luminescence. Superoxide anion radicals were formed by irradiation of a photosensitive substance caused by UV-radiation, which led to acceleration of oxidative reactions. The radicals in the measuring cell caused indicator substance to luminescence. The radicals, being under formation in the measuring cell, were partly inhibited by the reaction with antioxidant, present in the sample. It caused photosensitive substance to
reduce its luminescence. Suppression of the luminescence reaction allowed one to identify total content of antioxidants in the sample.

5. Discussion of the results
Table 1 and Figure 1 show the fatty acid composition and chromatogram of the lipid complex of pressed wine yeast.

Table 1. Fatty acid composition of lipid complex in the pressed wine yeast

| №  | Peak retention time for fatty acids | Fatty acids | Percentage |
|----|-----------------------------------|------------|------------|
| 1  | 20.00                             | Palmetic   | 5.84       |
| 2  | 22.89                             | Liniletic  | 18.44      |
| 3  | 22.95                             | Linoleic   | 1.60       |
| 4  | 23.26                             | Linoleic   | 46.50      |
| 5  | 23.35                             | Oleic      | 20.99      |
| 6  | 23.41                             | Elaidic    | 0.62       |
| 7  | 23.75                             | Stearic    | 2.90       |
| 8  | 26.30                             | Eicosatrienic | 0.88    |
| 9  | 26.39                             | Eicosadiene | 0.36     |
| 10 | 26.69                             | Eicosadiene | 0.50     |
| 11 | 26.77                             | Eicosene   | 1.03       |
| 12 | 27.20                             | Eicosan    | 0.29       |

Figure 1. Chromatogramme of the lipid complex for pressed wine yeast

Table 2 shows the composition of isoprenols and sterol components, which are found in unsaponifiable fraction of lipid yeast complex. As shown in Table 2, unsaponifiable fraction of lipid yeast complex includes diterpenic acohol, triterpenic alcohol, sterols (campesterol, stigmasterol, obtusifoliol, γ-Sitosterol, Lanosterol, Cycloartenol, α-Sitosterol), fatty alcohols,
hydrocarbons, α-Tocopherol, γ- Tocopherol, Total amount of sterols in unsaponifiables of yeast lipid complex is 22.3%.

We expressed our interest in the research of yeast lipid complex and its unsaponifiables for a reason. The works [24], [25] provide convincing information that mainly sterols in unsaponifiable part are responsible for biological activity of sea buckthorn oil. The work [26] proved that lanosterol, cycloartenol, obtusifoliol perform antiblastic activity. Antiblastic activity expresses itself in inducing tumor cell apoptosis [10], which leads to the death of tumor cells. Also it shows the decrease of tumor cell size, with chromatine condensation and fragmentation, compaction of outer and cytoplasmic membranes, without the release of the cell content into the environment [11].

Table 2. Unsaponifiable fraction of the yeast lipid complex

| Component                                | Amount, mg % |
|-------------------------------------------|--------------|
| Standard C13H28                           | 100.0        |
| Nerolidol                                | 13.0         |
| Dodecene-1                                | 25.0         |
| 2,3-Dihydrofarnesol                       | 27.0         |
| Farnesol                                  | 38.6         |
| Hexahydrofarnesil acetone                 | 36.0         |
| Hexadecanol                               | 145.3        |
| Octadecanol                               | 281.0        |
| Phytol                                    | 366.0        |
| Hexadeca-2,6, 10,14-tetraen-1-ol          | 147.1        |
| Octadecene-1                              | 147.0        |
| Docosene-1                                | 46.0         |
| Pentacosane                               | 43.1         |
| Mixture of sterols                        | 206.0        |
| Squalene                                  | 614.0        |
| Mixture of sterols                        | 179.1        |
| Mixture of sterols                        | 30.1         |
| γ- Tocopherol                             | 86.1         |
| Geranyl linalool                          | 290.0        |
| Mixture of sterols                        | 39.9         |
| α-Tocopherol                              | 67.0         |
| Campesterol                               | 300.0        |
| Stigmasterol                              | 50.0         |
| Obtusifoliol                              | 81.6         |
| γ-Sitosterol                              | 2217.6       |
| Lanosterol                                | 80.0         |
| Stigmast-7-en-3-ol                        | 125.0        |
| Cycloartenol                              | 204.0        |
| Lanosterol 2                              | 74.0         |
| Mixture of sterols                        | 66.0         |
| 24-Methylene-9,19-cycloannostal-3-ol      | 1040.0       |
| α-Sitosterol                              | 838.0        |

Table 3 shows antioxidant ability of pressed yeast lipid complex and its unsaponifiable fraction. It’s shown that antioxidant ability was found in both yeast lipid complex, which mainly consists of unsaturated fatty acids, as well as unsaponifiable fraction of lipid yeast complex, containing sterols and tocopherols.
Table 3. Antioxidant capacity of the components under research

| Components under research                      | Expressed as standard antioxidant, trolox, mg/100 ml | Expressed as α-Tocopherol mg/100 ml |
|------------------------------------------------|-----------------------------------------------------|-------------------------------------|
| Lipid complex of pressed wine yeast            | 340.1                                               | 585.4                               |
| Unsaponifiable fraction of lipid complex in pressed wine yeast | 195.5                                               | 336.4                               |

6. Conclusion
As the work has shown, unsaturated fatty acids -- oleic, linolenic, linoleic, contribute up to 88% in the lipid complex of pressed wine yeast. The synthesis of sterols by the wine yeast during the alcoholic fermentation ends in the formation of lanosterol as a main homologue of ergosterol. Also it was found that the unsaponifiable fraction of the wine yeast lipid complex contains a considerable amount of sterols and tocopherols, both known for their antioxidant capability.

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