Extraction and study of Intracellular compounds of sedimentary wine yeast - waste product of wine production

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Abstract. The present work describes the results of research of a complex of substances obtained from sedimentary wine yeast by extraction with food extractants with the use of ultrasound. The filtrates of alcoholic extracts of yeast and sediments isolated from filtrate were investigated. It has been shown that hydrophobic substances moderately soluble and poorly soluble in alcohol, such as ethyl esters of fatty acids, fatty acids, phospholipids, sterols, squalene, were isolated from sedimentary wine yeast by extraction with alcohol-containing extractants. The mechanical effect of ultrasound provides faster and more complete penetration of the solvent into the intracellular substances. Ultrasound allows one to achieve greater penetration of the solvent into plant tissues and improve mass exchange. Ultrasonic waves, causing cavitation in the liquid, destroy cell walls and promote the release of cell matrix components. Thus, during the US treatment of suspended sedimentary wine yeast a mixture of fatty acid ethyl ester more than 2 times and squalene - 1.5 times, compared to the control, is observed.

1 Introduction

Over recent years, viticulture and winemaking have become the most dynamically developing branches of the Russian agro-industrial complex. In the Russian Federation there is a trend towards increasing the area of vineyards. According to the data of the Federal State Statistics Service (Rosstat), the area of vineyards in agricultural enterprises in 2018 amounted to 74 thous. ha. In 2019, additional 6.7 thousand ha of new vineyards were installed in the country. The annual grape harvest in 2020 increased by 7.5% compared to 2019 and amounted to 490.0 thous. tons. In view of the growth of industrial processing of grapes, the quantity of secondary products of winemaking will also increase accordingly, which amounts to 20 % of the quantity of processed grapes and are a valuable raw material for obtaining a broad range of products. In Table 1 data about composition and quantity of secondary products obtained during processing of grapes are provided.

According to the data of Table 1, grape pomace obtained during industrial processing of grapes at primary winemaking plants by pressing of fresh or fermented pulp is the most widespread waste product, the yield of which is 10-14 kg per 100 kg of grapes. [1].

Nowadays, the implemented innovative projects aimed at obtaining functional products of healthy nutrition for the population from grape pomace are known. One of these functional nutrition products produced in Crimea by RESSFOOD LLC is a grape food concentrate "Enoant", containing from 18 to 20 g/dm^3 of grape polyphenols. The advantage of "Enoant" as a source of polyphenols is that it ensures easy absorption by the human body - in a water-soluble form - of the whole complex of biologically valuable polyphenols and flavonoids of grapes. In addition to "Enoant", since 2017 RESSFOOD LLC has been producing food concentrate "FENOKOR" from grape seeds, containing at least 80.0 g/dm^3 of grape polyphenols, including procyanidins, whose usefulness for healthy diet, treatment and prevention of a wide range of pathological conditions is generally accepted. The authors' work shows the efficiency of using the product of natural origin - grape food concentrate "FENOKOR" exhibiting cardioprotective, hepatoprotective, nephroprotective effects, for prevention of pathophysiological and morphological changes caused by metabolic syndrome [2].

Table 1. Secondary products of winemaking per 100 kg of grapes

Composition	Quantity, kg	
	Range of values	Average value
Stalks	1,8- 8,5	3,5
Sweet pomace	12-17	14,0
Seeds	0,1-6,0	3,0
Pressed yeast deposits, dm^3	0,2-0, 4	4

According to the Strategy for increasing the quality of food products of the Russian Federation until 2030, approved by Decree of the Government of the Russian Federation dated 29.06.2016 No. 1364-з, the priority is

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the development of technologies for deep processing of agricultural raw materials for obtaining new types of specialized, functional and enriched food products. In an effort to address this problem, the Institute "Magarach" resolves the goal of developing technologies for obtaining functional nutrition products from grapes, saturated with grape polyphenols, in establishing the basic parameters and modes of extraction of grape polyphenols from potential raw materials (pomace, leaves, seeds, vines) to produce experimental samples of functional nutrition products. According to the results of the research work carried out in the Institute "Magarach", the characteristics of grape raw materials (pomace, seeds, stalks) in terms of quantitative and qualitative composition of polyphenols - the main functional ingredients of the biological activity of grape products, have been previously evaluated. The analysis of the obtained data shows that in the aqueous-alcoholic extracts of grape raw materials, the whole spectrum of polyphenols, appropriate for grape wines (flavones, flavan-3-ols, hydroxy-cinnamic, hydroxybenzoic acids, stilbenes, oligomeric and polymeric procyanidins) is presented [3]. Pursuant to the plan of scientific research with respect to the Agreement on provision of subsidies of the Ministry of Education and Science of the Russian Federation with the Institute "Magarach" No. 14.604.21.0077 of June 27, 2014, technologies for obtaining new products from red grapes are Winy beverage "Zdorovie" (Health). They regulate the presence of polyphenols in them at the level of not less than 2.5 g/dm^3, and also the grape polyphenols extract containing at least 20 g / dm^3. Technological methods of the developed technologies are based on the use of the main waste of winemaking production - grape pomace. The novelty of technological solutions is confirmed by the patents of the Russian Federation [4].

Looking at a complex technology of processing of secondary resources of winemaking as a whole, we may conclude that this technology envisages processing of pomace, seeds and stalks with obtaining functional nutrition products, saturated with polyphenols of grapes. As for the processing of sedimentary wine yeast, the yield of which is 4% of the processed grapes, currently, due to a sharp increase in energy prices, the introduction of excise duty on the production of grape spirit, the processing of yeast sediment at the plants of primary winemaking have practically stopped. In the work [5], the possibility of obtaining a lipid complex by low-temperature extraction of dried sedimentary wine yeast to the residual humidity of 8%, by Freon, has been shown. Extraction mode is gas pressure - 0.9-1.0 MPa, extraction temperature - not more than 18 °C, extraction time - 2 hours. The work demonstrates that in the lipid complex of sedimentary wine yeast, unsaturated fatty acids - oleic, linolenic, linoleic acids, dominate, which amounts to 88 %. Synthesis of sterols by yeast in the process of alcoholic fermentation ends in the formation of lanosterol, as the main homologue of ergosterol.

1.1 The purpose of the study

The present work describes the results of scientific research of the complex of biologically active substances obtained by the extraction method using food extractants from sedimentary wine yeast-waste of winemaking production.

2 Materials and methods

The object of research were samples of pressed wine yeast selected at a winery in Crimea, as well as experimental samples of alcoholic extracts of pressed wine yeast. For obtaining alcoholic extracts, sedimentary wine yeast was pre-suspended with an aqueous-alcoholic extractant with the volume fraction of ethyl alcohol of 96% vol. and 70% vol., at a solid phase-liquid ratio of 1:2. Alcoholized yeast extraction mass was subjected to ultrasound treatment (US) (vibration frequency 20-21 kHz), US exposure time - 5 min at a temperature of 20-25 °C.

Experimental and control samples (untreated by US) after infusion were pressed with the formation of press fraction. In order to form a sediment of hydrophobic substances - insoluble or slightly soluble in ethanol - pressed fraction of the extract was treated with cold (processing temperature - not lower than 5 °C, processing time - not less than 48 hours) and filtered. Fatty acids, ethyl esters of fatty acids, unsaponifiable substances were determined in the filtrate and sediment.

Obtaining and determination by the GLC method of unsaponifiable substances, ethyl esters of fatty acids in filtrate and sediment

To research the composition of sterols and isoprenoids, ethyl esters of fatty acids in filtrate and sediment, a method of determination of the content of unsaponifiable substances in vegetable oils was used [6]. Saponification of a 5 g of the taken sample of the lipid complex was performed with a 2H alcohol solution of potassium hydroxide in a water bath under reflux. Distilled water was added to the cooled solution and quantitatively transferred to a separation funnel. The unsaponifiable substances were extracted with several portions of petroleum ether. The combined extract was washed with water to neutral reaction, and the remaining moisture was removed with anhydrous sodium sulfate. The solvent was driven off, and the unsaponifiable substances were dissolved in 5 ml of petroleum ether. The sterol content was determined by gas-liquid chromatography. At that, before saponification process, a standard solution of tridecane $C_{13}H_{28}$ in an amount of 1 mg/g was added to the weighed quantity of lipid complex. Chromatography was performed using an Agilent Technology 6890 chromatograph with a mass-spectrometric detector (DB-5 fused silica capillary column, nitrogen as carrier gas), helium as carrier gas, at a flow rate of 1 ml/min, at a temperature of 50-300 °C. Determination of phospholipids was carried out by the photometric method based on the transfer of phosphorus, which is part of the phospholipids of vegetable oils, in the water-

soluble state by cineration and its subsequent determination by the photometric method on the molybdenum blue complex [7].

The qualitative and quantitative composition of amino acids in alcoholic extracts of yeast was determined by HPLC using an Agilent Technologies chromatographic system Agilent Technologies (model 1100). For separation of substances, an ZORBAX-XDB-C18 chromatographic column, 2.1×150 mm 3,5 µm, was used. The chromatography was performed in the gradient mode. Eluent composition includes mobile phase A – 0,05 M of buffer ammonium formate solution in the mixture acetonitrile - 15% and pH - 6,5; mobile phase B of the buffer ammonium formate solution in the mixture acetonitrile - 90%. The eluent flow rate was 0.25 ml/min. The volume of the injection sample was 1 µl. The substances were identified by comparing their spectral characteristics and retention times with similar characteristics of standards.

3 Discussion of the results

An analysis of the filtrate and sediment of yeast extracts obtained as a result of extraction of pressed sedimentary wine yeast with food extractants is presented in Tables 2, 3, 4. Hydrophobic substances moderately soluble and poorly soluble in alcohol, such as ethyl esters of fatty acids, fatty acids, phospholipids, sterols, and squalene, can be isolated from yeast by extraction with alcohol-containing extractants. Owing to the low solubility in alcohol of fatty acids, sterols, these compounds are mainly found in the sediment of the extract. Ethyl esters of fatty acids, alcohol-soluble phospholipids are present in substantial amounts in the filtrate of the extract. The insignificant amount of sterols and hydrophobic compounds, namely squalene and fatty acids, poorly soluble in alcohol, in the filtrate of the extract presupposes insufficient cold treatment of the extract. The concentration of alcohol in the solution affects the extraction of fatty acid ethyl esters, thus, with increasing alcohol concentration up to 96 % vol., the concentration of fatty acid ethyl esters increases (Table 2). During treatment of the suspension of pressed yeast by US, the concentration of esters doubles. This tendency is maintained during extraction of squalene with ethanol of 96,3 % vol.

The concentration of indispensable amino acids in the filtrate of alcoholic extracts of sedimentary wine yeast is from 200 to 300 mg/l, of dispensable - from 400 to 600 mg/l. The increase in concentration of dispensable amino acids by 40%, namely, glutamic acid, in alcoholic yeast extracts (extractant 72% aqueous-alcoholic solution) can be explained by a partial hydrolysis of not only of alcohol-soluble protein fraction - prolamines, but also of water-soluble fraction - albumins (Table 3).

Table 2. Qualitative and quantitative analysis of components of alcohol filtrate of sedimentary wine yeast

Concentration of filtrate components, mg/kg	Volume fraction of ethyl alcohol, 96,3 % vol.		Volume fraction of ethyl alcohol, 70% vol.	
	Control	Experiment (US)	Control	Experiment (US)
Fatty acids:				
capric	155	511	135	95
lauric	146	-	-	140
stearic	-	362	125	-
caprylic	-	-	-	66
palmitic	-	-	-	281
Sum	301	873	260	582
Ethyl esters of fatty acids:				
ethyl caprilate	75	365	55	56
monoethyl succinate	102	-	185	28
ethylcaprinate	936	1824	1278	774
ethyl laurate	1140	2958	1720	744
ethyl myristate	466	835	428	217
ethyl pentadecanoate	78	136	62	29
ethyl palmitoleate	507	957	573	260
vinyl palmitate	223	1927	857	586
ethyl palmitate	8966	19271	7833	3857
ethyl heptadecanoate	121	210	72	32
ethyl linoleate	5552	12487	6148	2944
ethyl oleate	967	1657	317	448
ethyl linolenate	1892	5173	3025	1257
ethyl stearate	3442	7473	2131	947
isoamyl caproate	82	196	-	
ethyl eicosanoate	319	568	118	48
ethyl docosanoate	692	1017	123	53
ethyl tricosanoate	124	173	-	-
ethyl tetracosanoate	178	189	-	-
Sum	25862	57416	24980	12280
Squalene (Acyclic polyunsaturated hydrocarbon)	3585	5605	505	185
Sterols				
stigmasta-3,5-diene	344	298	123	49
stigmast-4-ene-3-one	82	81	-	-
Sum	426	379	123	185

Hydrophobic compounds, such as fatty acids, phospholipids, and sterols, were found in the sediment of alcohol extracts of pressed yeast (Table 4). The alcohol concentration has a negligible effect on the extraction of fatty acids from the yeast cell. An increase in fatty acid concentration by 40-50% is observed with

US treatment of the yeast suspension. The concentration of sterols in yeast extracts reaches 4 g/kg. US treatment of suspension of pressed yeast allows one to increase the concentration of sterols by 31%.

Table 3. Amino acid composition of filtrate of alcoholic extracts of sedimentary wine yeast

Name of amino acids	Content of amino acids, mg/dm^3			
	Volume fraction of ethyl alcohol, 96,3 % vol.		Volume fraction of ethyl alcohol, 70 % vol.	
	Control	Experiment (US)	Control	Experiment (US)
Glutamine	0,0	0,0	10,1	4,9
Asparagine	34,7	25,3	51,2	53,8
Oxyproline	1,6	1,9	3,2	6,1
Aspartic acid	41,1	34,2	65,5	66,5
Serine	38,7	38,6	63,1	58,7
Glutamic acid	85,8	82,0	114,0	111,6
Threonine	0,0	0,0	0,0	0,0
Arginine	62,0	54,9	83,0	83,6
Glycine	13,7	14,6	30,3	30,3
Cysteine	0,0	0,0	0,0	0,0
Alanine	83,2	84,4	102,3	106,4
Tyrosine	20,0	29,2	33,9	35,4
Proline	64,4	91,4	101,5	109,6
Monoethanol amine	3,2	4,1	5,4	5,7
Methionine	15,0	15,2	12,3	12,9
Valine	81,3	69,7	80,1	79,2
Phenylalanine	54,8	53,8	35,6	40,7
Isoleucine	14,9	13,7	12,5	11,8
Leucine	90,0	81,5	69,6	72,3
Histidine	0,0	0,0	0,0	0,0
Lysine	21,2	38,6	50,1	34,1
Sum of dispensable amino acids	428,4	431,9	629,6	638,0
Sum of indispensable amino acids	297,2	301,0	294,1	285,7
Sum of amino acids	725,6	732,9	923,7	923,7

As the alcohol concentration decreases, the concentration of phosphorus-containing substances in the filtrate of the alcohol extract of sedimentary wine yeast increases. Analysis of the constituents of alcohol extracts leads to the conclusion on destruction of yeast cell wall and recovery, by means of extraction, of classes of biological membrane compounds such as phospholipids, glycolipids, and steroids. Phospholipids are subdivided into glycerophospholipids (derivatives of phosphatidic acid -phosphatidicholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol)and phingophospholipids (ceramide derivatives, sphingomyelins).

Table 4. Qualitative and quantitative analysis of components of sediment of alcohol filtrate of sedimentary wine yeast

Concentration of sediment components, mg/kg	**Volume fraction of ethyl alcohol, 96,3 % vol.**		**Volume fraction of ethyl alcohol,70 % vol.**	
	Control	Experiment (US)	Control	Experiment (US)
capric	-	180	244	2022
lauric	374	884	1451	2130
myristic	536	932	1009	1527
pentadecylic	145	219	234	287
palmitoleic	1211	1766	1658	2426
palmitic	25273	33340	26898	36589
heptadecanoic	307	422	223	335
linoleic	14852	21583	17904	19632
oleic	2705	3458	686	5173
linolenic	9211	10323	7716	17453
stearic	12707	16188	6100	6711
eicosanoic	1059	1435	254	301
heneicosanoic	341	480	-	-
docosanoic	2303	3053	358	438
tricosanoic	440	640	-	-
tetracosane	667	1422	1556	510
tetracosanoic	558	1003	-	
Sum of saturated fatty acids	44610	60198	38417	50850
Sum of unsaturated fatty acids*	27979	37130	27278	44684
Sterols stigmasta-3,5-diene	4249	5585	1493	2046

Membrane steroids built on the sterane skeleton in the yeast cell are represented mainly by stigmasterols (Table 4). Since all phospholipids are products of phosphatidic acid metabolism, it is the fatty acid composition of its molecules that will determine the type of phospholipid. So for the fatty acid composition of phosphatidylcholines, palmitoleic acid, and for phosphatidylethanolamine - arachidonic acid are typical. In our case, the fatty acid composition of lipids is represented by mono- and saturated fatty acids - myristic, stearic, palmitic, arachidic, behenic, lignoceric, as well as by mono- and polyunsaturated fatty acids - palmitoleic, oleic, linoleic, linolenic.

Table 5. Component composition of filtrate and sediment of it, obtained from alcoholic extracts of sedimentary wine yeast

Name of components	Filtrate of alcoholic yeast extract		Filtrate sediment	
	alcohol, 96,3 % vol.		alcohol, 96,3 % vol.	
	Control	Experiment (US)	Control	Experiment (US)
Sum of fatty acids, g/kg	0,3	0,87	62,6	97,3
Sum of fatty acid ethyl esters, g/kg	25,9	57,4	not found	
Mass concentration of phosphorus-containing substances, %	5,2	5,3	6,76	4,50
Sterols, g/kg	0,40	0,37	4,2	5,6
Squalene, g/kg	3,6	5,6	not found	
Sum of amino acids, g/dm^3	0,7	0,7		

4 Conclusion

Thus, the results of experimental studies have shown (Table 5) that it is possible to isolate from sedimentary wine yeast by extraction with alcohol-containing extractants, hydrophobic substances moderately soluble and poorly soluble in alcohol, such as ethyl esters of fatty acids, fatty acids, phospholipids, sterols, squalene – an acyclic polyunsaturated hydrocarbon, which is a powerful antioxidant and natural antibiotic.

The mechanical effect of ultrasound provides faster and more complete penetration of the solvent into the intracellular substances. Ultrasound allows one to achieve greater penetration of the solvent into plant tissues and improve mass exchange. Ultrasonic waves, causing cavitation in the liquid, destroy cell walls and promote the release of cell matrix components. Thus, during the US treatment of suspended sedimentary wine yeast, an increase in the yield of enanthic ester – a mixture of fatty acid ethyl ester more than 2 times and squalene - 1.5 times, compared to the control, is observed. Due to the low solubility in alcohol of fatty acids, sterols, these compounds are mainly found in the sediment of the filtrate of the alcohol extract of sedimentary wine yeast.

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