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CHEMICAL AND MICROBIAL CHANGES DURING THE NATURAL FERMENTATION OF *ARBUTUS UNEDO* FRUITS

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ABSTRACT

Aguardente de medronho is the name given in Portugal to a spirit made from the fermented fruit of *Arbutus unedo* (strawberry tree), a plant grown in the Mediterranean region. Natural fermentation, with wild microbiota, was carried out during 36 days on the farm, and studied in respect to some physicochemical and microbiological parameters. The microbial parameters analyzed were total viable, lactic acid bacteria, acetic acid bacteria and yeast counts. The physicochemical parameters monitored were sugars, minerals, ethanol, organic acids and pH. Yeasts were the main responsible for the fermentation of the fruits, being the lactic acid bacteria and acetic acid bacteria absent. As fermentation progressed, the sugars increased during the first two days and gradually decreased along the fermentation period. During fermentation period, the mineral content was increasing resulting in a final product in the must, enriched in Ca, Mg, Na and K. Additionally there was an increasing in the content of ethanol resulting from the alcoholic fermentation of the sugars performed by the yeasts. Maintaining the good quality of the product could contribute to the preservation and valorization of traditional resources that are of great importance to prevent their disappearance.

Key words: fruit from *Arbutus unedo*, natural fermentation, sugars, mineral composition

INTRODUCTION

Arbutus unedo L. (strawberry tree) (family: *Ericaceae*) is an evergreen shrub usually smaller than 4 m found in the Mediterranean fringe, being today also cultivated in the Near East and Transcaucasia (Seidemann, 1995). The fruits of strawberry tree are spherical berries of about 1-2 cm in diameter, dark red when mature in autumn. They are tasty and processed into jam, fruit jelly, spirits and liqueurs being rarely eaten as fresh fruit. The fruits and leaves are also well known in folk medicine as antiseptics, diuretics and laxatives in Turkey (Ayat *et al*., 2000; Pabuçcuoglu *et al.,* 2003).

In Portugal strawberry tree has a remarkable implantation in the south (Algarve) where it has been associated with a long tradition in social, cultural and microeconomic aspects. In the Algarve's region the fruits, when fully ripe, are fermented and used in the manufacture of a "traditional product", an alcoholic distillate named Aguardente de medronho (medronho firewater), very appreciated locally for its flavor characteristics. This spirit has been a complement to farmers' income, for generations, and over the last years efforts have been made to improve the quality of the production and the quality of the final product. The preservation and valorization of traditional resources are of great importance to prevent their disappearance (Alarcão e Silva *et al*., 2001; Soufleros *et al.,* 2005).

Fermentations are done on the farm and the results are very variable in quality. There are problems of acidity or lack of flavor or even off-flavors due to uncontrolled fermentations (incomplete fermentations or over fermentations) all of which leading to low crop value for the farmer. It would be desirable to study the process in order to improve the quality of the final product (medronho firewater).

In the Algarve's region, about ten thousand liters of this distilled are produced per year, on a small scale as a home made product. Medronho firewater is made from recently harvested mature fruits mixed with water in closed recipients, where alcoholic fermentation occurs due to wild microbiota, at room temperature, during 4-5 weeks, depending on the weather conditions (temperature). After the fermentation the fermented material is heated by direct fire in a copper still and the condensation of both ethanol and non-ethanolic volatiles are crucial for the flavor and aroma of the final product. The distillate obtained has 40 to 50 % ethanol (v/v) and can be matured in wooden barrels for several years. In general, the fermentation and distillation processes are done between September and January.

The nature of the fermentation with respect to the role played by the microorganisms and their enzymes on the physicochemical changes and on the characteristics of the final product is not known. Therefore, the aim of this work is to study the microbial population dynamics, the changes in sugar composition and some fermentation by-products as ways of monitoring the fermentation evolution.

MATERIALS AND METHODS

Fruit Sampling

Strawberry tree (*Arbutus unedo* L.) fruits used in this study were obtained from trees grown in Caldeirão Saw, Algarve (Portugal), in their normal harvest time. Randomly, three pickings, of fruit at red mature stage (12-23 ºBrix), were made between September and November 2004. The fruits were transported to the farmers in polyethylene bags and kept away from direct sunlight to avoid qualitative losses. Two, 200Kg, samples of fruit were used for the fermentation process. Three, 200g, samples of ground sample were used for the extractions of sugars, ethanol, organic acids and minerals. All determinations were made from these extracts in triplicate. For chemical purposes, the samples were kept in freeze (-20ºC) until analyzed.

Fermentation conditions

Mature fruits recently harvested, were mixed with water and partially crushed (1 part of water to 10 parts of fruit). Fermentation took place due to indigenous microbiota in closed PVC recipients (200Kg-250Kg), at room temperature, during 36 days. Samples were taken every 3 days during 36 days from the fermentation recipient. Each sample consisted of about 100g of fermented must removed with sterile tongs from different points approximately 30cm from walls and 45cm from upper surface. The samples were aseptically transferred in PVC bags to the laboratory, under refrigerating conditions, and analyzed, for microbial counts in the same day. For chemical purposes samples were kept in freeze (-20ºC) until analyzed.

Determination of pH and total amount of Titratable Acids (TTA)

The pH and the TTA were determined in every sample with a Delta 320 pH meter (Mettler, France). A sample (10g) was mixed with distilled water (90mL) and the mixture was then totated with NaOH (0.1 M) with stirring to pH 8.5. The total titratable acidity was expressed as the amount of Na OH consumed, in milliliters.

Sugars and Ethanol

The mature samples (15g) were homogenized at room temperature, clarified by centrifuging at 4000 rpm (21ºC) for 20 min, and filtered through Sep- Pak C18 (Waters Associates) according to Medlicott and Thompson (1985). The sugars and ethanol content were analyzed by HPLC with a Beckman System Gold Chromatograph (Fullerton, U.S.A.) composed of a 126 gradient pump with a detector UV-Vis Beckman

166 (USA). Sample injection was by means of rheodyne injection valve (Rheodyne, California, USA) equipped with polyspher (OA HY) column the mobile phase was acetonitrile and water (20:80) at a flow rate of 1.0 $mL.min^{-1}$. Individual sugars and ethanol were quantified by comparison of peak areas of samples to those of standard curves of ternary mixture solutions with concentrations of 5 $mg.mL^{-1}$ (sucrose, glucose and fructose) available from Sigma Chemical Co, (St. Louis, MO, U.S.A. 2004) and dried for 12 h at 60ºC under vacuum before being dissolved in water. The standard solutions were stored at -18ºC.

Organic acids

The same extract obtained for sugars and ethanol analysis was used in the quantification of organic acids. The identification of organic acids was performed by HPLC with a detector UV-Vis Beckman 166 (USA). The eluates were detected at 214 nm, on a μBomdapak–C_{18} column (waters Associates) with 0.2M KH_2PO_4 adjusted to pH 2.4 (H_3PO_4) as mobile phase and 1.0 $mL.min^{-1}$.

Mineral Analysis

From each homogenized sample 20g were taken in duplicate for the study of mineral composition. These underwent drying in a thermoregulable swindle at 90º±50ºC overnight. Removal of the organic matter was carried out by means of incineration in a muffle oven at 450º± 250ºC until ash was obtained. Then the ash was dissolved in 2ml of HCL 1:1 (v/v) maintaining it in an ultrasound bath until complete dissolution, later removing the acid by evaporation on a thermoregulable sand bath. The residue was dissolved in deionised water and adjusted to a volume of 50ml and 100ml, for the determinations of micro and macroelements, respectively. The determinations were carried out on the solutions although, in the case of macroelements, it was necessary to dilute them by 1:10. Conditions were carefully controlled to avoid contamination or loss

126 of materials. Ca, K and Na were determined by flame photometry. Intra-assay (n=10) coefficients of variation (CV) were 3.4, and 2.0% for K and Na, respectively. Recovery of added known amounts of standards to samples gave 98-105% of expected values for all minerals. The elements Mg, Fe, Zn, Mn, Pb, Cd and Cu, were measured by atomic absorption spectrophotometer, equipped with hollow cathode lamps. Coefficient of variation of the overall method was 7.7%. Results are the mean of triplicate determinations and expressed as g/ 100g dry matter.

Microbiological parameters

Twenty five gram samples of the must in fermentation, obtained as described above at different time intervals, were mixed with 225 ml of sterile 0.1 % peptone water in a stomacher, during 3 minutes. Sequential decimal dilutions were made with sterile 0.1 % peptone water and aliquots were plated, by surface spreading, in duplicate on the following agar media for the detection and enumeration of microorganisms. The media used was: a) Total viable count: Plate count agar (PCA) (pH 4) incubated at 25ºC for 5 days; b) Lactic acid bacteria: Man, Rogosa and Sharpe agar (MRS) to which cycloheximide (0,05%) was added to inhibit growth of contaminant yeasts and molds, incubated at 25ºC for 5 days; c) Acetic acid bacteria: medium containing yeast extract (10 g), $CaCO_3$ (20 g), ethanol (20 ml), agar (20 g) to a final volume of 1000 ml; d) Yeasts: Malt Extract Agar (MEA) (pH 3,6) incubated at 25ºC for 5 days. All the microorganisms were enumerated and the yeasts were isolated and frozen (-80ºC) for identification in future work.

Statistical analysis

Significant differences between means of experiments were determined by least significant difference. A significance level of 0.05 was chosen (Sokal and Rohlf, 1987).

RESULTS AND DISCUSSION

Fermentation of *Arbutus unedo* fruits took place on the farm at room temperature. External minimum and maximum temperatures and the fermentative recipient temperature are shown in Table I. The maximum external temperature registered was 18,0ºC and the minimum was 9,0ºC. On the other hand, the maximum temperature in the fermentation vessel was 15,9ºC and the minimum was 12,4ºC, in the last day of fermentation.

The changes in hydrogen ion concentration (pH) and ºBrix during the fermentation process are reported in Table 2. The pH increased slightly and ºBrix decreased during the fermentative process probably due to the production of ethanol resulting from consumption of sugars by fermenting microorganisms.

The mineral composition is presented in Table 3. From the results obtained during fermentation period, the amounts of Fe, Zn, Mn, Cu, Pb and Cd (data not show) were underneath the quantification limit for detection. The importance of the Cu, Pb and Cd analysis is due to their toxicity, that most derives from the alembics (Soufleros *et al.,* 2005). During fermentation period, the mineral content (expressed as $mg.100g^{-1}$ dry matter), was increasing resulting in a final product enriched in Ca, Mg, Na and K, where K and Ca are the main elements. At the same time, once phytic acid is well known by chelating and precipitating multivalent cationic minerals with its six anionic phosphate groups, this increase could be explained by the presence of phytate- degrading enzymes present in yeast (Turk et al., 2000) which could release those minerals.

The content of sugars and acids in fruits influences markedly their sensory quality and consequently the quality of the final product. For these reasons, fruit compositional analysis is of interest to food experts. The soluble sugars identified and quantified in the strawberry tree’s fruits and all over the fermentation period were fructose, glucose and sucrose (Fig. 1). The first two sugars were the major sugars while sucrose was in much lesser amounts. One liter of broth of fermentation in the beginning of the process (day 0) contained about 7.3% of fructose, 2.5% of glucose and 4.5% of sucrose at the end of fermentation (day 36) one liter of broth of fermentation contained 2.5% of fructose, 0.2% of glucose and 0.1% of sucrose. Glucose and fructose are generally considered the main sugars in strawberry tree's fruits (Hegnauer, 1986; Ayaz *et al.*, 2000). According to Ayaz *et al.*, (2000) in these types of fruits, the content of sucrose is lower than the amount of fructose and glucose. The explanation may be that the sucrose, synthesized in the leaves, is enzymatically hydrolyzed to glucose and fructose when translocated to the fruit.

During the firsts two days of the fermentation period the levels of fructose and glucose increased reaching their maximum (glucose-66.8 $g.L^{-1}$; fructose-127.978$g.L^{-1}$). This increasing phase was followed by a decline in their contents in the fermentative must accompanied by a rising in the concentration of ethanol that varied from 1.47×10^{-2} l per liter l of fermentation broth in the beginning of the study (day 2) to 7.6×10^{-2} l per liter of fermentation broth in the end of the experiment (day 36).

Fig. 1 shows the evolution of soluble sugars (glucose, fructose and sucrose) and yeast population during fermentation of *Arbutus unedo* fruits. The variations in sugars and ethanol contents are related with the activities of the microbial population. In addition, the presence of vestigial concentrations of organic acids is probably related with the inexistence of lactic or acetic acid bacteria.

All over the fermentation period, the number of lactic acid bacteria and acetic acid bacteria detected was inferior to 10 cfu/ml of must and the total viable counts were approximately equal or even less then the yeasts counts. The results presented in this study show that, yeasts were the main responsible for the fermentation of the fruits of

Arbutus unedo in the process monitored. The colonization and subsequent growing of these microorganisms were favored by the initial acidity of the pulp' fruits (pH 3.8). The yeast population in the fermentation must was initially high (1.25×10^5 cfu/ml of must). As can be seen in Fig 1, during the firsts 8 days yeasts showed an exponential phase that lasted about 8 days, reaching a number of 2×10^7 cfu/ml of must. Next, a stationary phase occurred almost all over the period studied. The growing of the yeasts was accompanied by a decreasing of sugars contents in the must and their conversion to ethanol which is a primary activity of yeasts during alcoholic fermentation. The content of glucose and fructose reached their highest value on the second day of fermentation. The initial crushing and mixture with water caused the rupture of the fruits' skin, facilitating the contact between the fruits' flesh and the microorganisms. During this period the indigenous flora produce hydrolytic enzymes which break down cell components and complex carbohydrates, producing simple molecules, including fructose and glucose, which content increased during the initial phase of the fermentation. In the exponential growth phase of the yeasts, glucose was the first sugar to be fermented followed by fructose which degradation began in the stationary phase. The conversion of sucrose, glucose and fructose to ethanol during the fermentation process is the primary activity of the fermentative yeasts and the ethanol produced, showed in Fig. 2, is, probably, a contribution for the beginning of the decline phase. In fact, the yeasts' growth inhibition can be attributed either to toxicity of fermentation products including ethanol, sugar transport inefficiency or other nutritional limitations (Bisson, 1999).

Although yeasts were not yet identified, it could be observed that a succession of species took place as the fermentation proceeded. The earlier fermentation samples contained a rich mixture of species. As fermentation progressed, the species diversity was reduced considerably and *Saccharomyces cerevisiae* (preliminary results) became the dominant yeast at the end. Yeasts successions are known in numerous fermentation processes as the fermentation of grape juice into wine (Fleet *et al*., 1984; Querol *et al*., 1990), the fermentation of sugars to produce tequila (Lachance, 1995) and in the production of rum (Fahrasmane and Ganou-Parfait, 1998).

CONCLUSION

The purpose of this paper is to contribute to the understanding of this traditional spirit, in order to help optimized and standardize the fermentation process and consequently to obtain a more uniform composition and quality of this spirit. From the results obtained in natural fermentation, with wild microbiota, of *Arbutus unedo* fruit it can be concluded that the sugars increased during the first two days and gradually decreased along the fermentation period. At the same time, the mineral content was increasing resulting in a final product, in the must, enriched in Ca, Mg, Na and K. Additionally there was an increasing in the content of ethanol resulting from the alcoholic fermentation of the sugars performed by the yeasts. Although yeasts were not yet identified, it could be observed that a succession of species took place as the fermentation proceeded. *Aguardente de medronho* is a traditionally made distillate, generally of good quality, produced on a small scale from local distillers of the south region of Portugal. Maintaining the good quality of the product could contribute to the preservation and valorization of traditional resources that are of great importance to prevent their disappearance. Moreover arbutus berry show to be an appreciable source of sucrose and minerals. Further studies are necessary to investigate and identify the microbiological quality.

REFERENCES

Alarcão-e-Silva, M. L.C. M. M., Leitão, A. E. B., Azinheira, H. G. and Leitão, M C. A.. 2001. The Arbutus Berry: Studies on its Color and Chemical Characteristics at Two Mature Stages. J. Food Compos. Anal., 14: 27-35.

Ayaz,F. A., Kucukislamoglu, M. and Reunanen, M.. 2000. Sugar, Non volatile and Phenolic Acid Composition of Strawberry Tree (*Arbutus unedo* L. var. *ellipsoidea*) Fruits. J. Food Compos. Anal. 13: 171-177.

Bisson, L.. 1999. Stuck and Sluggish Fermentations. Am. J. Enol. Vitic. 50, 107-119.

Fahrasmane, L. and Ganou-Parfait. B.. 1998. Microbial flora of rum fermentation media. J. Appl. Microbiol. 84: 921-928.

Fleet, G., Lafon-Lafourcade, H. and Ribereau-Gayon, S..1984. P. Evolution of Yeasts and Lactic Acid Bacteria During Fermentation and Storage of Bordeaux Wines. Appl. Environ. Microbiol. 48: 1034-1038.

Hegnauer, R.. 1986. Chemotaxonomiee der Pflanzen. Dictyledonae-Lythraceae. Vol. 4, pp.S. 67-69, 74, 85, 87-88; Vol. 8 (Machtrag), pp. 418-420, Birkhauser Verlag, Basel, Stuttgart.

Lachance, M. A.. 1995. Yeasts communities in a natural tequila fermentation. Antonie van Leeuwenhoek. 68: 151-160.

Medlicott, A. P. and Thompson, A. K.. 1985. Analysis of sugars and organic acids in ripening mango fruits (Mangifera indica L. var. Keitt) by high performance liquid chromatography. J. Sci. Food Agric. 36: 561-566.

Pabuçcuoglu, A., Kivçak, B. Bas, M. and Mert T.. 2003. Antioxidant activity of Arbutus unedo leaves. Fitoterapia 74: 597-599.

Querol, A., Jiménez, M., and Huerta, T.. 1990. Microbiological and Enological Parameters during Fermentation of Musts from Poor and Normal Grape-Harvests in the Region of Alicante (Spain). Journal of Food Science. 55: 1603-1606.

Seidemann, J.. 1995. ZurKenntnis von wenig bekannten exotischen Fruchten.5. Mitt.: Baumerdbeere (Arbutus unedo L.). Dtsch. Lebensmittel- Rdsch. 91. 110-113.

Sokal, R R and Rohlf, F. J.. 1987. Introduction to biostatistics (Second edition) New York: W H Freeman Company.

Soufleros, E. H., Mygdalia, S. A. and Natskoulis, P.. 2005. Production process and characterization of the traditional Greek fruit distillate “Koumaro” by aromatic and mineral composition. J. Food Compos. Anal. 18: 699-716.

Turk, M., Sandberg, A.S., Carlsson,N. G., and Andlid, T.. 2000. Inositol hexaphosphate hydrolysis by baker´s yeast. Capacity, kinetics and degradation products. J. Agric. Food Chem., 8: 100-104.

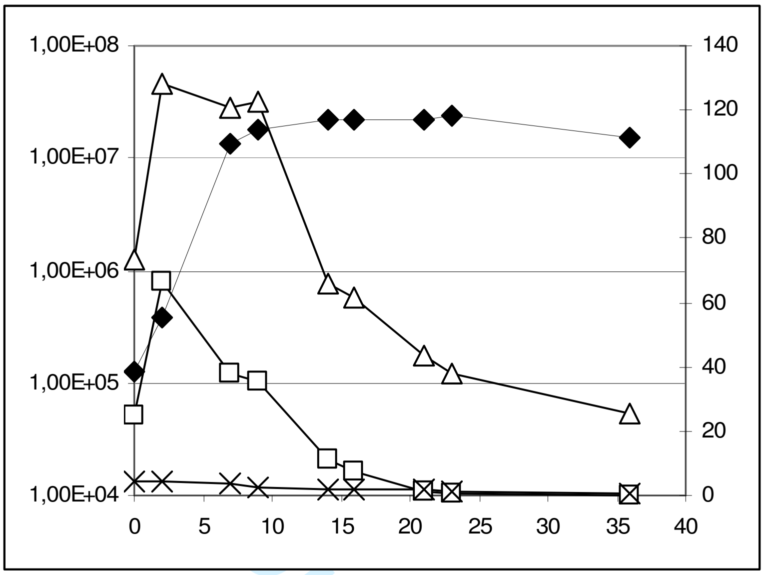

Fig. 1- Changes in consumption of sugars (glucose ( ), fructose (Δ), sucrose (×)) and yeast growth (♦) cycle during fermentation of *Arbutus unedo'* fruits.

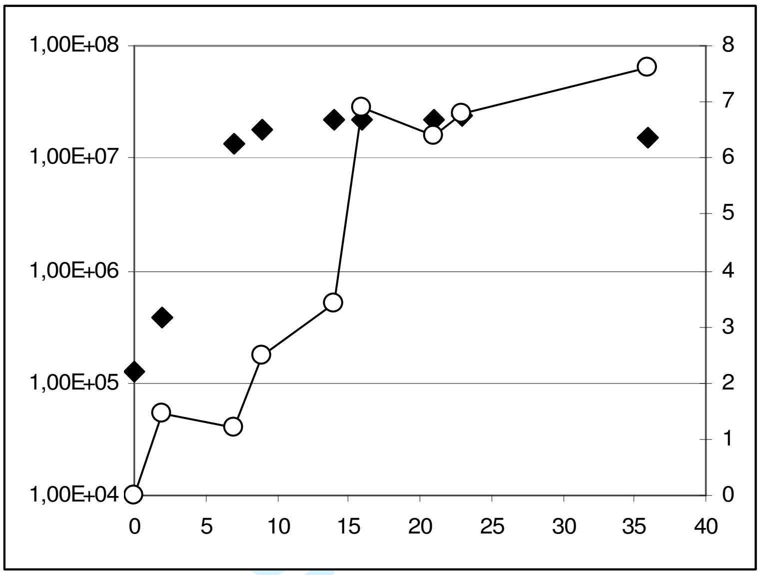

Fig. 2.- Production of ethanol (o) and yeast growth cycle(♦) during fermentation of *Arbutus unedo'* fruits.

Table 1. Changes in external and internal temperature of the bottle during the *Arbutus unedo*'s fruit fermentation.

	External Temperature (ºC)		Internal Temperature (ºC)
Fermentation period (day)	Máx.	Min.	
0	16.0	13.0	14.6
2	16.2	14.0	14.8
7	16.0	12.5	15.8
9	18.0	12.0	15.9
14	16.5	10.0	15.5
16	18.0	11.0	15.4
21	11.0	10.0	14.6
23	14.0	9.0	13.1
36	11.0	10.0	12.4

Table 2. Changes in pH and ºBrix during the *Arbutus unedo*'s fruit fermentation.

Fermentation period (day)	**pH**	**º Brix**
0	3.28±0.1	23.50±0.1
2	3.28±0.1	23.00±0.1
7	4.03±0.1	22.00±0.1
9	4.43±0.1	-
14	4.62±0.1	17.00±0.1
16	4.94±0.1	15.80±0.1
21	4.74±0.1	14.00±0.1
23	5.06±0.1	13.50±0.1
36	4.82±0.1	12.00±0.1

Table 3. Mineral composition (mg/100g dry matter of seeds, n = 3) during the *Arbutus unedo*'s fruit fermentation.

Fermentation period (day)	Ca	Mg	Na	K
0	1.665[a] ±0.09	0.327[a] ±0.091	1.021[a] ±0.00	1.241[a] ±0.01
2	1.629[a] ±0.07	0.443[a] ±0.01	1.021[a] ±0.01	1.080[a] ±0.00
7	1.933[a] ±0.05	0.461[a] ±0.01	1.153[a] ±0.01	1.206[a] ±0.00
9	2.258[b] ±0.14	0.554[a] ±0.01	1.123[a] ±0.01	1.595[a] ±0.00
14	1.905[b] ±0.06	0.415[a] ±0.02	0.923[a] ±0.02	1.242[a] ±0.01
16	2.163[b] ±0.07	0.542[b] ±0.02	1.059[a] ±0.04	1.407[a] ±0.02
21	2.606[c] ±0.06	0.678[b] ±0.02	1.257[a] ±0.03	1.966[b] ±0.02
23	3.368[c] ±0.10	0.781[b] ±0.01	1.209[a] ±0.03	2.051[b] ±0.01
36	3.036[c] ±0.19	0.720[b] ±0.03	1.138[a] ±0.03	1.783[b] ±0.01

Means (± s.d.) in the same column with different letters are significantly ($P<0.05$) different.