



Distillates composition obtained of fermented *Arbutus unedo* L. fruits from different seedlings and clonal plants

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ARTICLE INFO

Keywords:

A. unedo fruits
Clonal plants
Seedlings
Distillates
Volatile composition

ABSTRACT

The fruits of *Arbutus unedo* L., a shrub that grows spontaneously in countries around the Mediterranean basin, are traditionally harvested and used for the production of a distilled spirit. The main objective of this work is to compare the chemical profile of distillates obtained from fermented *A. unedo* fruits. Fifteen distinct distillates were obtained from ten clones and five seedlings, and they were evaluated, based on their physicochemical characteristics. An evident effect of plant propagation type (clone or seeds) on correspondent spirits analytical profile was not clearly achieved. However, the distillate composition variability seems connected to the plants variability. Furthermore, high significant effect of distillation time in all the variables measured was found, corroborating the importance of the splitting of the distillation fractions, as a good manufacturing practice. Regarding methanol, a harmful volatile compound, all the distillates obtained from the different clones or seedling fruits respected the legal limitations. From the principal component analysis, the first component seems to separate the distillates based on the pH and isobutanol, isoamyl alcohols and 2-phenylethanol which are the variables that presented the highest contributes to this component. The differences between the legal requirements and the amounts determined in these distillates could result from differences in the alcoholic fermentation microbiota.

The high analytical quality, that some of the distillates showed, can be used by producers as a criterion to be considered in the future choice of plants to be implemented in new *A. unedo* orchards.

1. Introduction

The *A. unedo*, known as strawberry tree is a shrub that grows spontaneously in countries around the Mediterranean basin. Traditionally the fruits of the wild *A. unedo* trees were harvested and used for the production of a homemade distilled spirit (Galego, 2006; Galego and Almeida, 2007), like other distillates that are homemade from different fruits and plants in other countries (Coldea et al., 2014; Satora and Tuszyński, 2008). That spirit is called “aguardente de medronho” or “medronheira” in Portugal (Cavaco et al., 2007; Galego, 2006) “Corbezzolo” in Italy (Versini et al., 1995), “Koumaro” distillate in Greece (Soufleros et al., 2005) and “aguardiente de madroño” in Spain (Morales, 1995). The production and promotion of traditional alcoholic beverages contributes to the diversification of employment and economic income in rural areas. From the 90's of the 20th century, various

research works started regarding the study of production technology of this beverage (Alarcão-Silva et al., 2001; Cavaco et al., 2007; Galego, 1995, 2006; Santo et al., 2012). The obtained results allowed to increase the quality of that product (Botelho and Galego, 2016) and consequently its greater commercial value and contributed to maintain the rural populations in the Algarve mountain in Portugal (Galego, 2006). In addition, in recent years more attention has been given to this plant, because of its important role in soil regeneration namely following forest fires and due to its drought tolerance (Gomes, 2011; Gomes and Canhoto, 2009; Sánchez-Gómez et al., 2006). On the other side, some authors (Gomes, 2011) enhanced the underuse of this plant that has several other commercial applications such as medicinal, ornamental and pharmaceutical usages. Consequently, in the last years a consistent breeding program was started, adult plants were selected according to fruit production and quality and then micropropagated

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Table 1
Samples codification and analytical results of the fifteen *A. unedo* fruit samples.

Codes	Origin	Fruits ^a	Fermented masses ^a									
		TSS (°B)	Total reducing sugars (% w/w)		TA (g/L malic acid)		L*	a*	b*	C*	h°	TSS (°B)
A1C	Clonal	32.95 (± 0.1)	13.12 (± 0.3)	8.42 (± 0.2)	31.7 (± 0.2)	33.9 (± 0.1)	9.9 (± 0.1)	36.3	16.1	9.67 (± 0.2)	12.83 (± 0.2)	4.04 (± 0.1)
A2C	Clonal	25.53 (± 0.2)	15.86 (± 0.2)	9.41 (± 0.2)	35.4 (± 0.1)	29.7 (± 0.1)	14.4 (± 0.2)	34.0	25.4	8.90 (± 0.2)	11.59 (± 0.2)	4.03 (± 0.0)
A3C	Clonal	25.59 (± 0.2)	15.43 (± 0.1)	9.28 (± 0.1)	36.4 (± 0.1)	31.5 (± 0.1)	15.5 (± 0.1)	35.8	25.9	8.87 (± 0.2)	12.26 (± 0.2)	3.95 (± 0.0)
A4C	Clonal	25.53 (± 0.0)	15.86 (± 0.2)	9.41 (± 0.2)	35.44 (± 0.1)	29.7 (± 0.1)	14.4 (± 0.1)	34.0	25.4	9.10 (± 0.2)	11.86 (± 0.2)	3.91 (± 0.0)
A5S	Seedling	23.37 (± 0.0)	14.32 (± 0.2)	8.35 (± 0.1)	36.6 (± 0.2)	34.3 (± 0.1)	14.1 (± 0.1)	37.7	22.4	9.00 (± 0.2)	16.75 (± 0.2)	3.93 (± 0.0)
A6C	Clonal	23.69 (± 0.1)	14.66 (± 0.3)	8.70 (± 0.1)	34.2 (± 0.2)	32.3 (± 0.1)	13.0 (± 0.2)	35.7	21.7	9.40 (± 0.2)	13.40 (± 0.2)	3.96 (± 0.0)
A7S	Seedling	23.98 (± 0.1)	12.12 (± 0.3)	8.94 (± 0.1)	36.3 (± 0.1)	30.0 (± 0.1)	15.5 (± 0.1)	35.4	26.5	10.93 (± 0.2)	13.70 (± 0.2)	3.86 (± 0.1)
A8C	Clonal	25.23 (± 0.0)	13.28 (± 0.1)	9.96 (± 0.2)	32.7 (± 0.2)	31.9 (± 0.1)	11.9 (± 0.1)	34.9	19.9	9.83 (± 0.2)	13.47 (± 0.2)	3.86 (± 0.0)
A9C	Clonal	25.23 (± 0.0)	13.28 (± 0.1)	9.96 (± 0.2)	32.7 (± 0.2)	31.9 (± 0.1)	11.9 (± 0.1)	34.9	19.9	9.00 (± 0.2)	13.40 (± 0.2)	3.95 (± 0.0)
A10S	Seedling	25.43 (± 0.1)	11.99 (± 0.2)	10.93 (± 0.2)	34.9 (± 0.1)	35.3 (± 0.1)	13.0 (± 0.1)	38.1	20.5	10.05 (± 0.2)	13.74 (± 0.2)	3.77 (± 0.0)
A11S	Seedling	23.98 (± 0.2)	12.12 (± 0.2)	8.94 (± 0.2)	36.3 (± 0.1)	30.0 (± 0.1)	15.5 (± 0.1)	35.4	26.5	11.10 (± 0.2)	14.81 (± 0.2)	3.87 (± 0.1)
A12S	Seedling	23.78 (± 0.1)	17.30 (± 0.2)	9.31 (± 0.1)	35.5 (± 0.1)	35.1 (± 0.1)	11.7 (± 0.2)	37.3	18.6	13.20 (± 0.2)	16.11 (± 0.2)	3.97 (± 0.1)
A13C	Clonal	23.81 (± 0.0)	15.63 (± 0.1)	8.38 (± 0.1)	35.1 (± 0.1)	36.5 (± 0.2)	12.9 (± 0.1)	39.3	19.6	10.40 (± 0.2)	14.10 (± 0.2)	3.96 (± 0.1)
A14C	Clonal	25.53 (± 0.0)	15.86 (± 0.2)	9.41 (± 0.2)	35.6 (± 0.1)	30.2 (± 0.1)	14.3 (± 0.1)	34.2	25.1	10.50 (± 0.2)	13.94 (± 0.2)	3.92 (± 0.0)
A15C	Clonal	25.73 (± 0.0)	15.73 (± 0.3)	8.94 (± 0.1)	36.7 (± 0.1)	31.5 (± 0.1)	15.8 (± 0.1)	35.9	26.4	9.20 (± 0.2)	11.39 (± 0.2)	3.83 (± 0.0)

TSS - Total Soluble Solids; TA - Titratable Acidity.

^a Average values of three determinations (± standard deviation).

(Gomes, 2011; Gomes and Canhoto, 2009; Gomes et al., 2010). Field trials were established with clonal plants and seedlings, in different environments to compare clonal productivity and quality of seedlings, to evaluate the interaction between the genotype and environment and to identify the elite clones and their clonal site allocation. The results showed that clonal production was significantly higher compared to seedlings (Gomes et al., 2014). Moreover, they suggest that the use of clonal plants appropriately fertilized can lead to a greater fruit productivity and quality (Pato et al., 2014). On the other hand, studies were carried out to evaluate the effect of diverse cultural practices on fruit production and fruit harvesting techniques, leading to the publication of a manual for producers in order to support good cultural practices from planting to harvesting and to expose the different profitable fruit uses (Gomes et al., 2017). This team work allowed clonal plant production for orchards that have been used for the production of spirits (Botelho et al., 2015).

The fermentation process in *A. unedo* fruits was done traditionally without crushing of the fruits and without yeast inoculation. Thus, it is the natural microbiota composed by several yeast species (Santo et al., 2012) that ensure the fermentation process during several weeks (Cavaco et al., 2007). Regarding the distillation process it is usually done by discontinuous process using copper alembics (Galego and Almeida, 2007; Versini et al., 2011) as can be observed in several current distilleries across Portugal (Botelho and Galego, 2016).

So, the main objective of this work is to compare the chemical profile of distillates obtained from seedlings and clonal plants fermentates from *A. unedo* fruits.

2. Material and methods

2.1. Experiment and sampling

2.1.1. Fruit samples

Fifteen samples of *A. unedo* fruits were used for the production of fifteen different distillates being ten from clonal plants and five from seedlings. All fruits were collected (third year of fruit harvest) in an experimental (1920 m² at 4 x 4m²) field, installed in 2007, located in the center of Portugal. About 5 kg of each set of fruits was harvested in autumn 2014.

2.1.2. Alcoholic fermentation

Ripe fresh *A. unedo* fruits were used for the experiment in this study. The fruits were hand harvested and potable cold water (5:1 proportion) was added and poured in 15 dm³ capacity plastic containers without fruit crushing and without yeast inoculation, like the traditional procedure used by the commercial producers. The containers were closed with air locks filled with water, and the spontaneous alcoholic fermentation happened at a room temperature (around 20 °C), in a dark place.

2.1.3. Distillation description

After the end of the alcoholic fermentation, each fermented mass was individually distilled in a small size copper alembic (16 dm³ capacity), applying the cutting, which means, the separation of the different fractions of the distillation namely heads, heart and tails. These fractions were sampled in order to evaluate their volatile composition. The first part with approximately 5% (above 70% vol., with a strong, pungent and unpleasant flavour) of the distillates was collected as head fraction. The heart fractions obtained by single distillation, were collected when the ethanol concentration varied from 70 to 35% v/v; finally, the tail fractions were obtained when the alcoholic content decreased below 35% v/v, as previously described (Botelho et al., 2015).

2.2. Physicochemical analyses

2.2.1. Fruit analysis

A set of each fruit sample was evaluated in order to collect analytical information about their total soluble solids content (TSS, Brix degree) using a portable digital device (ATAGO, model FG-113), titratable acidity (adapted from NP 2139, 1987), reducing sugars (% w/w) and CIEL*a*b* colour system colour data (Zhejiang Top Instrument Co. Ltd., model HP-2132), using a red standard for calibration (CR-A47 x = 480; y = 15.3; z = 313). Chromatic characteristics of the fruits were measured using CIEL*a*b* space (CIE, 1986). The determined parameters involved clarity (or luminosity, L*), red/green colour component (a*) and yellow/blue colour component (b*), from which the psychochemical parameters correlated with the perception of colour are obtained, including C* (chroma or saturation) and h° (hue angle). Table 1 shows codes and general information of studied fruit samples. The meaning of the samples' names are: A – *A. unedo*; # 1 to 15 – the different samples; C – clonal plants origin; and, finally, S – seedlings origin. The analyses were performed in triplicate.

2.2.2. Fermentate analysis

The alcoholic fermentation of *A. unedo* fruits was monitored through physical and chemical analysis, namely, total soluble solids (TSS, °B), titratable acidity and pH (Crison, microPH 2002 model). The alcoholic fermentations finished when the TSS decreased below 13.50 and these TSS values were stable during at least seven days.

2.2.3. Distillates analysis

In order to evaluate the distillation process of the fifteen samples, various analytical determinations were done on the distillation fractions (head, heart and tail): alcoholic strength, pH, methanol, acetaldehyde, ethyl acetate, fusel alcohols, acetic acid, ethyl lactate and 2-phenylethanol amounts.

Alcoholic strength (v/v), expressed as Total Alcohol by Volume (TAV) - was assessed by electronic densimetry (OIV, 2014).

pH - the pH was evaluated by potentiometry (OIV, 2014).

Volatile quantification - methanol, ethyl acetate, acetaldehyde and fusel alcohols of the distillation fractions were quantified at a gas chromatograph attached to a flame ionization detector (GC-FID), using the equipment Agilent 6890 GC (Agilent Technologies, Wilmington, DE, USA), according to the method validated previously (Luís et al., 2011). The quantification was done by analyzing the standard compounds in the same chromatographic conditions.

It was also quantified the ethyl lactate, acetic acid and 2-phenylethanol by GC-FID. For these compounds the quantification was carried out by the internal standard method, assuming a response factor arbitrarily fixed at 1.0 and the results were expressed as g of internal standard by dm³.

Volatile identification - the identification of volatile compounds was carried out by gas chromatography connected to mass spectrometry (GC-MS). It was employed an Agilent 7890A gas chromatograph equipped with a DB-WAX capillary column (30 m × 0.25 mm × 0.25 µm; J&W, Folsom, CA), connected to an Agilent 5973 mass selective detector (Agilent, CA, USA). The oven program was analogous to that used for GC-FID. The volume injected was 0.8 µL. The injector temperature was kept at 250 °C, the transfer line at 250 °C and the average velocity of carrier gas (helium) was 30 cm s⁻¹. Mass spectra were obtained in the electron impact (EI) mode (ionization energy, 70 eV; source temperature, 230 °C), by full-scan mode (mass range *m/z* 20–450) under autotune conditions. The identification was done by comparing their mass with the NIST library and by analyzing the mass spectra of standards.

2.3. Standards and chemicals

Ethanol and methanol were purchased from Merck (Darmstadt,

Germany).

GC-FID standards: Ethyl acetate (CAS N° 141-78-6; purity ≥ 99.8%) and acetic acid (CAS 64-19-7; purity 99.8%) were purchased from Riedel-de-Haen (Seelze, Germany), methanol (CAS N° 67-56-1; purity ≥ 99.9%) was purchased from Merck (Darmstadt, Germany). 2-methylbutan-1-ol (CAS N° 137-32-6; purity ≥ 98%) 3-methylbutan-1-ol (CAS N° 123-51-3; purity ≥ 98.5%), butan-1-ol (CAS N° 71-36-3; purity ≥ 99.5%), 2-methylpropan-1-ol (CAS N° 78-83-1; purity ≥ 99.5%), propan-1-ol (CAS N° 71-23-8; purity ≥ 99.5%), 2-propen-1-ol (CAS N° 107-18-6; purity ≥ 98%), butan-2-ol (CAS N° 78-92-2; purity ≥ 99.5%), 4-methylpentan-2-ol (CAS N° 108-11-2; purity ≥ 98%), acetaldehyde (CAS N° 75-07-0; purity ≥ 99.5%), ethyl-l-lactate (CAS N° 687-47-8; purity ≥ 99%), 2-phenylethanol (CAS N° 60-12-8; purity ≥ 99%) were purchased from Fluka (Buchs, Switzerland).

2.4. Statistical data analysis

In this experiment, the plant origin was a blocking variable because it was intended to study the influence of distillation time but we need to take into account the variability due to the plants. So the analytical results obtained for distillation fractions were evaluated under the ANOVA two-way, with distillation time as a fixed factor and the plant factor as block. A statistically significant level of 5% (*p* < 0.05) was chosen and the LSD test (*p* < 0.05) was applied for multiple means comparisons when a significant effect was detected. All the calculations were performed using Statgraphics from Statsoft (vs 7.09; Tulsa, OK, USA).

The analytical results of heart fractions (corresponding to the *A. unedo* distillate) were also submitted to a multivariate analysis (Principal Component Analysis and Clustering Analyses) to compare the similarity/dissimilarity between the samples proceeding from different clones and seedlings. All the calculations were performed using NTSYS-*pc* package, version 2.1q.

3. Results and discussion

The samples codification and the analytical results of the fifteen *A. unedo* fruits and fermentates evaluated under the present study are summarized in Table 1.

The total soluble solids of the fifteen *A. unedo* fruit samples ranged from 23.37 °B in sample A5S to 32.95 °B in sample A1C. For reducing sugars, the sample A10S showed the lowest value, 11.99% against the sample A12S presenting the highest value of 17.30%. On the other side, the titratable acidity (expressed as g/L of malic acid) ranged in an interval between 8.38 g/L for A13C sample and a maximum average value of 10.93 g/L for A10S. The luminosity (L*) values varied from 31.7 of A1C sample to 36.7 of A15C sample. Sample A13C presented the highest value of a* coordinate, 36.5, followed by samples A10S (35.3) and A12S (35.1), being these three samples the most red ones. For the Chroma (C*) and hue angle (h°) that were calculated from a* and b*, also a consistent result was obtained from samples for colour saturation being the last mentioned samples the most saturate whereas the hue angle was accordingly low. After the alcoholic fermentation the resulting masses showed a total soluble solids interval between 8.87 °B for sample A3C and 13.20 for sample A12S.

Previously, Botelho and Galego (2016) had already indicated that values less than around 10.0 °B are an indicator of the end of alcoholic fermentation in *A. unedo* masses. The titratable acidity values and the pH values varied in an opposite way as expected, ranging the pH values from 3.77 (sample A10S) until 4.04 (sample A1C). In the present work we decided to not add any organic acid in order to reduce the initial pH of the masses with the purpose of respecting the traditional management of fermentation. Eventually, the high values of samples pH could promote the growth of some microorganism species that probably led to undesirable volatile compounds, usually associated with off-flavours of final distillates.

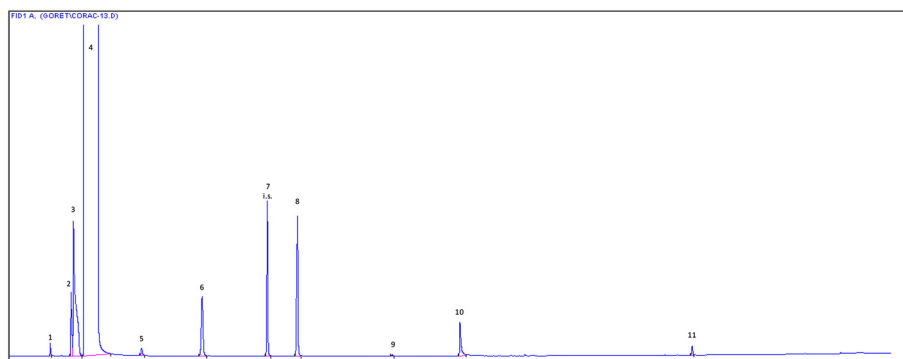


Fig. 1. GC-FID chromatogram of the heart fraction from distillate A1C obtained at an INNOWax column. Compounds identification: 1: acetaldehyde, 2: ethyl acetate, 3: methanol, 4: ethanol, 5: 1-propanol, 6: isobutanol, 7: 4-methyl-2-pentanol (internal standard), 8: 2 + 3-methyl-1-butanol, 9: ethyl lactate, 10: acetic acid, 11: 2-phenylethanol.

3.1. Effect of distillation time on volatile composition

Fig. 1 presents the chromatogram of a GC-FID analysis with the volatiles compounds identified and quantified in the studied distillates.

The major volatiles identified comprised the acetaldehyde, ethyl acetate, methanol and fusel alcohols. Acetic acid, ethyl lactate and 2-phenylethanol were also identified and quantified. All these compounds were also identified in *A. unedo* distillates by other researchers (Galego, 1995; González et al., 2011; Soufleros et al., 2005; Versini et al., 1995, 2011).

The ANOVA results of analytical determinations on the distillates are presented in the Table 2. Nevertheless only the heart fractions were used for the production of the *A. unedo* distillate, as consequence the means values of heart fractions were recalculated (Table 3) and expressed as g/hL of pure alcohol (using the ethanol content results) in order to verify the compliance with regulatory requirements (EC N° 110/2008; Portuguese Decree-Law 238/2000) of the distillates obtained from the different fruits.

The results presented in Table 2 revealed a high significant effect of distillation time in all the variables measured, corroborating the importance of the splitting of the fractions, as a good manufacturing practice which is recommended during the traditional alembic distillation (Botelho and Galego, 2016; Galego, 1995). In fact a decrease of pH was verified from an average value of 5.04 in the heads until 3.56 in the tail fractions. A similar behaviour was reported in previous work dealing with *A. unedo* distillation (Botelho et al., 2015; Galego, 1995, 2006). The pH values of heart fractions, ranging from 3.97 until 4.30 (Table 3), are similar to those of previous works in Portugal (Botelho et al., 2015; Galego, 1995) but lower than the values reported for the Greek *A. unedo* distillates (Soufleros et al., 2005). However, those distillates are obtained by double distillation and the ethanol content is lowered after the distillation with water addition (Soufleros et al., 2005), which could explain the high values of pH.

As expected the ethanol content also decreased during the

distillation (Table 2), as verified in other works (Botelho et al., 2015; Caldeira et al., 2018; Galego, 1995, 2006). All the values determined in the heart fractions (Table 3) meet the minimum of regulatory requirements.

The ethyl acetate amounts are significantly influenced by the distillation time (Table 3), in spite of the variability across the samples. These results are in accordance with previous works with *A. unedo* distillate (Botelho et al., 2015; Caldeira et al., 2018; Galego, 1995, 2006) and with other distillates (Spaho, 2017). In fact, the ethyl acetate which presents high volatility and high solubility in ethanol and as consequence its high amounts are detected in heads and the beginning of heart fractions (Spaho, 2017). The ethyl acetate is the major ester of several distillates (Christoph and Bauer-Christoph, 2007) and also of *A. unedo* distillate (Galego, 1995, 2006; González et al., 2011; Soufleros et al., 2005; Versini et al., 1995, 2011). Its presence is not only due to the microbial metabolism during the fermentation process (Christoph and Bauer-Christoph, 2007) but also result from esterification's reactions that are promoted and favored by the presence of oxygen (Reazin et al., 1976). The problem of the high values of ethyl acetate reported by Versini et al. (1995) in Portuguese *A. unedo* distillate, are solved according to Galego et al. (2014) by reducing the contact with air during the fermentation process, by decreasing the time between the fermentation and distillation and by applying a correct cutting process during the distillation. This compound must present an amount below 300 g/hL of pure alcohol according to EU regulatory requirements, but in this work, only the distillates obtained from samples A1C, A12S, A13C and A15C comply the legislation. The differences between the samples could suggest differences in the microbial metabolism.

Regarding the acetaldehyde content in the distillates (Table 3), its amount was not affected by the distillation time (Table 2). These results are in disagreement with other works about *A. unedo* distillate (Botelho et al., 2015; Caldeira et al., 2018; Galego, 1995). Only Galego (1995) found a similar behaviour for one *A. unedo* harvesting. Like ethyl acetate, the presence of acetaldehyde in the distillates is related to

Table 2

Average values ($\pm$ standard deviation) from ANOVA and summary of ANOVA effects on the analytical determination of the distillates.

	Heads	Heart	Tails	Distillation time effect	Block effect
pH	5.04 ($\pm$ 0.2)	4.03 ($\pm$ 0.1)	3.56 ($\pm$ 0.1)	***	**
Ethanol (% v/v)	64.77 ($\pm$ 0.2)	49.62 ($\pm$ 2.3)	33.99 ($\pm$ 2.8)	***	ns
Acetaldehyde (g·dm ⁻³)	0.20 ($\pm$ 0.1)	0.20 ($\pm$ 0.1)	0.16 ($\pm$ 0.0)	ns	ns
Ethyl acetate (g·dm ⁻³)	4.59 ($\pm$ 2.9)	2.63 ($\pm$ 1.4)	1.04 ($\pm$ 0.7)	***	*
Methanol (g·dm ⁻³)	3.87 ($\pm$ 0.9)	3.27 ($\pm$ 0.6)	2.58 ($\pm$ 0.5)	***	***
1-Propanol (g·dm ⁻³)	0.10 ($\pm$ 0.0)	0.08 ($\pm$ 0.0)	0.05 ($\pm$ 0.0)	***	***
Isobutanol (g·dm ⁻³)	0.45 ($\pm$ 0.1)	0.31 ($\pm$ 0.1)	0.19 ($\pm$ 0.1)	***	***
2 + 3-Methyl-1-butanol (g·dm ⁻³)	0.82 ($\pm$ 0.2)	0.61 ($\pm$ 0.1)	0.37 ($\pm$ 0.1)	***	***
Ethyl lactate (mg·dm ⁻³) ^ψ	1.57 ($\pm$ 2.7)	2.60 ($\pm$ 4.2)	2.38 ($\pm$ 4.0)	ns	***
Acetic acid (mg·dm ⁻³) ^ψ	30.81 ($\pm$ 28.2)	74.66 ($\pm$ 51.0)	122.73 ($\pm$ 72.2)	***	***
2-Phenylethanol (mg·dm ⁻³) ^ψ	12.73 ($\pm$ 3.3)	20.45 ($\pm$ 5.7)	28.36 ($\pm$ 7.6)	***	***

ANOVA - Analysis of variance; ^ψ - expressed as mg of internal standard. ns - no significant difference, $p > 0.05$; *0.01 $< p < 0.05$; **0.001 $< p < 0.01$; *** $p < 0.001$.

Table 3

Average values, expressed in g/hL of pure alcohol (PA), determined in heart fractions proceeding from the 15 different samples (A1 until A15) and the legal requirements.

	pH	TAV (% v/ v)	Methanol	Acetaldehyde	Ethyl acetate	2-Butanol	1-Propanol	Isobutanol	1-Butanol	2 + 3-Methyl-1-butanol	Total of fusel alcohols	Isobutanol/ propanol
A1C	4.30	42	674	18	117	0	13	95	0	165	273	7.4
A2C	4.22	51	647	41	577	0	14	84	0	141	239	5.9
A3C	4.10	50	644	42	504	0	13	57	0	158	228	4.6
A4C	4.07	52	673	58	660	0	14	97	0	155	266	6.9
A5S	3.97	50	898	45	914	0	16	55	0	117	187	3.5
A6C	4.02	49	847	48	867	0	14	40	0	105	160	2.8
A7S	3.92	50	572	45	1049	0	13	64	0	101	177	4.8
A8C	3.99	50	538	52	366	0	16	57	0	103	176	3.5
A9C	3.87	49	580	38	455	0	20	56	0	94	169	2.8
A10S	3.99	52	487	42	815	0	15	44	0	97	156	3.0
A11S	3.90	48	687	36	458	0	18	39	0	99	156	2.2
A12S	3.99	51	763	40	259	0	25	62	0	109	196	2.5
A13C	4.07	51	694	24	189	0	11	60	0	142	213	5.6
A14C	3.97	50	627	36	371	0	14	62	0	124	199	4.6
A15C	4.07	49	569	30	287	0	12	76	0	126	214	6.1
Legal minimum		42	500	5			10	30		80	130	1.5
Legal maximum			1000	40	300	2	40	70	3	185	300	4

Underlined values mean average values outside the legal limits. TAV – Total Alcohol by Volume.

microbial metabolism of the fruit fermentation (Christoph and Bauer-Christoph, 2007) but is also favored by the oxidation of ethanol (Reazin et al., 1976) and it is influenced by the maturity of the fruits (Botelho et al., 2015). Taking into account the legal limitation only the distillates produced from clonal origin A1C, A9C, A13C, A14C, A15C and A11S, A12S from seedling origin, fulfilled the requirements (Table 3). Considering the influence of fermentation conditions and microbiota (Liu and Pilone, 2000; Santo et al., 2012) on the acetaldehyde amounts, the differences among samples could result from differences in the microbiota.

The methanol, which was originated from enzymatic degradation of fruit pectins (Micheli, 2001) exhibit a well-known toxicity (Siu et al., 2013) and consequently its amount in *A. unedo* distillate have to be below 1000 g/hL AE (absolute ethanol) regarding the health consumers (Table 3). In this study the methanol content is significantly influenced by the distillation time despite the high variability from the samples (Table 2). These results are in agreement with previous ones (Caldeira et al., 2018) in *A. unedo* distillate and also in other distillates (Spaho, 2017). When the methanol results are expressed in g/hL of absolute ethanol and because the ethanol decrease over the distillation was more pronounced, it was noted an increase in the methanol contents as reported by some authors (Botelho et al., 2015; Galego, 1995, 2006). Regarding the legal requirements, it is very interesting to point out that all the distillates proceeding from the different clones or seedling fruits respected the legal limitations. In addition, it should be emphasized that the results obtained with distillates from three clones A8C, A9C, A15C, as well as, from seedling origin, A7S and A10S, presented the lowest amounts of methanol (around the minimum of legal limit).

The fusel alcohols included 1-propanol, isobutanol and 2 + 3-methyl-1-butanol. It was noticed the absence of 2-butanol and 1-butanol (Table 3), respecting the legal requirement for all heart samples (Table 3), in accordance with our previous work (Botelho et al., 2015) but in disagreement with other results in *A. unedo* distillates (Galego, 1995; González et al., 2011; Versini et al., 1995, 2011). The 2-butanol in the wine distillates was related to the presence of lactic bacteria metabolism (du Plessis et al., 2004) and high concentrations are normally considered indicative of low quality of the distillate (Diéguez et al., 2005). Regarding the quantified fusel alcohols, a significant influence of the distillation time was verified (Table 2), with a decreasing of their amounts from the heads until the tails, regardless of origin influence. These results, about the distillation time effect, were in accordance with other research works. (Botelho et al., 2015; Caldeira

et al., 2018; Galego, 1995). These alcohols are quantitatively the major group of flavour compounds in alcoholic beverages and are resulting from yeast metabolism during the fermentation (Christoph and Bauer-Christoph, 2007). Taking into account the maximum and minimum allowed for the legislation, all the distillates proceeding from the different clones or seedlings comply the requirements regarding the 1-propanol, the 2 + 3-methyl-butanol and the total fusel alcohols amounts (Table 3). However, the isobutanol amount of the distillates proceeding from clone origin A1C, A2C, A4C and A15C are higher than the maximum allowed (Table 3) and the ratio isobutanol/propanol do not comply the requirements for the distillates proceeding from samples A1C, A2C, A3C, A4C, A7S, A13C, A14C and A15C. Given the knowledge about the several factors that can affect the formation of higher alcohols during the grape wine fermentation, including yeast species and strain, initial sugar, fermentation temperature, pH and composition of grape juice, assimilable nitrogen, aeration, level of solids, grape variety and skin contact time (Ugliano and Henschke, 2009), further studies are needed in order to understand the formation of higher alcohols during the fermentation of *A. unedo* fruits and to establish the influence of fruits composition namely its nitrogen and sugar composition.

As we hypothesized in previous works (Botelho et al., 2015), the differences between the legal requirements and the amounts determined in these distillates could result from differences in the microbiota, since it is well-known the significant yeast role on the fusel alcohols contents (Lilly et al., 2006; Satora and Tuszyński, 2010) and it was also established the association between the production regions and the fermentation microbiota (Bokulich et al., 2016). In fact, the actual Portuguese legislation is based on several experimental assays done in Algarve (Galego, 1995, 2006; Galego and Almeida, 2007) and with the studied microbiota flora (Cavaco et al., 2007; Santo et al., 2012). Therefore, further research is needed about the autochthonous microbiota characterization of *A. unedo* fermentation at different regions.

Concerning the acetic acid, ethyl lactate and 2-phenylethanol amounts during the distillation, it was verified a significant influence of distillation time on the acetic acid and 2-phenylethanol amounts (Table 2), which increase over the distillation with the highest levels on tail fractions. In the same accordance, a similar behaviour in other distillations was verified (Spaho, 2017).

The acetic acid accounts for > 90% (v/v) of the total acidity in spirits, because it is the major volatile acid in the fruit fermentates, as secondary product derived from pyruvic acid on fermentation by

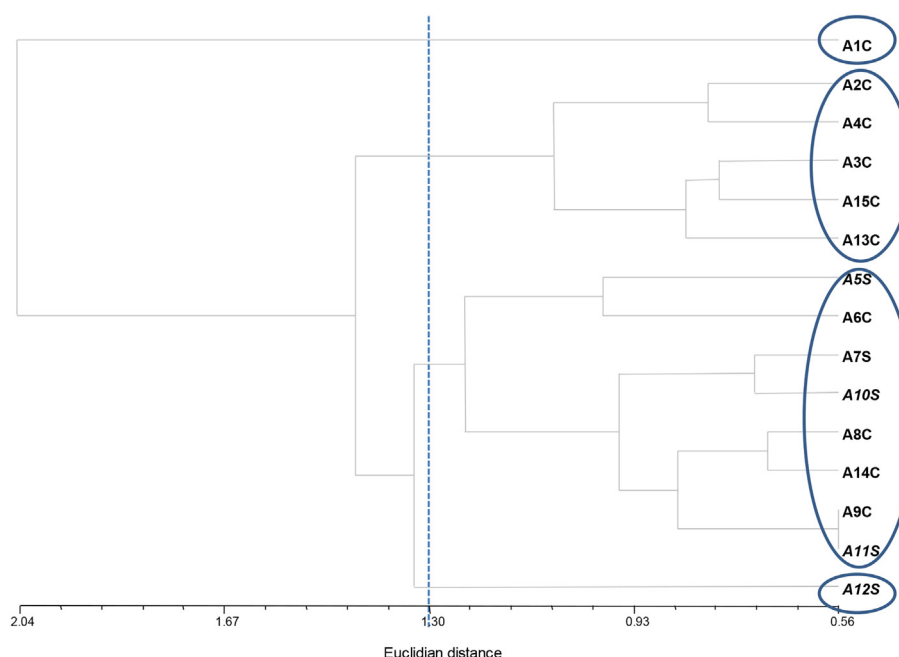


Fig. 2. Phenogram of UPGMA (unweighted pair group method with arithmetic mean) clustering of all the analysed heart fractions according to analytical determinations. The initial matrix was composed by 15 heart fractions by 11 variables.

Saccharomyces cerevisiae (Spaho, 2017). According to the Portuguese legislation (Portuguese Decree-Law 238/2000) the total acidity of *A. unedo* spirit must be below 2000 mg/L AE (absolute ethanol), meaning that the values of acetic acid determined in the heart fractions (Table 2) are very acceptable.

As expected and taking into account the variability introduced by the *A. unedo* sample origin, in the distillate composition, translated in significant effect of the block (Table 2), multivariate analysis was applied to heart fractions results, in order to check whether the measured variables could help to distinguish among the fifteen distillate samples. All the variables of the heart fractions were submitted to clustering and principal component analysis. Fig. 2 presents the phenogram of distances for the distillates from the 15 samples, which presented a co-phonetic correlation coefficient of 0.85.

The results at Fig. 2 show that distillate from A1C and A12S form individual clusters, well separated from the other distillates. It is interesting to point out that A12S distillate is the only one that comply all the legal requirements. Other cluster joins together the distillates from clones A2C, A4C, A3C, A15C and A13C while another cluster seems to connect the distillates from the clones or seedling origin: A5S, A6C, A7S, A8C, A10S, A14C, A9C and A11S.

The principal component analysis, for the 15 distillate heart fractions, was performed (Fig. 3). The first three principal components, which accounted 71% of the total variance, seems to separate the distillates from different origin, which is related with the clusters established in the phenogram. The first two components, which explained 38% and 19% respectively of the total variance, creates a separation between the distillates proceeding from the different origin. The first component seems to separate the distillates based on the pH and isobutanol, isoamyl alcohols and 2-phenylethanol which are the variables that presented the highest contributes to this component. The second component seems to separate the distillates based on ethanol and ethanal contents which are the variables with highest loads for this component. Therefore in the plane formed by the first components it is possible to observe the complete separation of A1C distillate, related to high levels of pH and isobutanol, isoamyl alcohols and 2-phenylethanol and very low levels of ethanol and ethanal. In fact, this distillate formed an individual cluster in Fig. 2 and does not comply the legal requirements concerning the fusel alcohols amounts (Table 3). Located in the

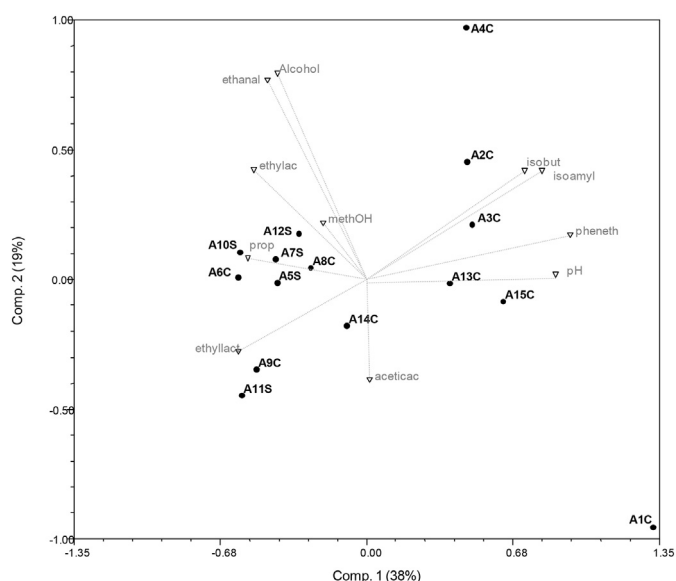


Fig. 3. Projection of distillate samples and variables in the space defined by the first and second components. Variable identification: alcohol: alcohol content, ethanal: acetaldehyde, ethylac: ethyl acetate, metOH: methanol, prop: 1-propanol, ethylact: ethyl lactate, aceticac: acetic acid, pH: pH, pheneth: 2-phenylethanol, isobut: isobutanol, isoamyl: 2 + 3-methyl-1-butanol.

same plane are the samples A13C and A15C also related with high levels of pH, isobutanol, isoamyl alcohols and 2-phenylethanol but also with moderate amounts of ethanol and acetaldehyde. In other side are located A3C, A2C and A4C distillates related to high amounts of pH, isobutanol, isoamyl alcohols and 2-phenylethanol but also high levels of acetaldehyde and ethanol. In negative side of component 1 and 2 are located the samples A14C, A9C and A11S linked to the high amounts of ethyl lactate.

Taking into account all the results achieved during this work, it is not possible to know clearly if the differences observed can be attributed to differences among the plants or perhaps to differences in the microbiota present in each fermentation. In fact, some sensory negative

compounds present in high or moderated concentrations in the distillates are associated to some species of bacteria or spoilage yeasts. Although there is no evident effect of the type of propagation of the plant (seed or clone) in the composition of the spirits obtained, there are plants that originate distillates with very different profiles and that deserve further studies.

Despite this, this research has the potential of providing valuable information aiming at the standardization of the fermentation and distillation methods, as well as, supporting the choice among seedlings or clonal plants (and among clonal plants). This would result in better quality products, adding value to the beverages and helping to develop the economy of the region where they are produced.

4. Conclusions

Under the experimental conditions of this study, the results obtained suggest that the main differences found among the fifteen *A. unedo* fruits distillates can result from the alcoholic fermentation process being the autochthonous microbiota more important for the volatile profile of each sample than the clonal or seedling origin. However some plants originated distillates with a quite different profile, and further studies are needed to deepen these results. Taking into account the results achieved further research on the microbiota characterization, before, during and at the end of *A. unedo* fruits alcoholic fermentation, is needed, either in clones and seedlings fruits from the same production region.

Moreover, the present work demonstrated and reinforces the great importance of following good manufacturing practices in the *A. unedo* spirit production, namely, the positive effect that a correct separation of the different fractions of distillate, head, heart and tail has to obtain a high quality final product, which comply with the current legislation. In fact, better distillates production practices and critical choice of the vegetal material with the greatest quality potential will certainly contribute to the creation of non-agricultural livelihoods in rural areas and an increase in income of agricultural producers.

Acknowledgements

The authors thank the technical support of Amélia Soares and Otilia Cerveira (INIAV - Dois Portos), on the physicochemical analyses carried out, and the grant student Celso Santos (ESAC-IPC) for his distillation process support. ICAAM and CERNAS are both R&I Centres funded by National Funds through FCT - Foundation for Science and Technology under the Project UID/AGR/00115/2019 and UID/AMB/00681/2019, respectively. This work was mainly financial supported by the FCT project PTDC/AGR-FOR/3746/2012 and ProDeR project n° 43748 - medida 4.1.

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