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Major Volatile Composition of Four Distilled Alcoholic Beverages Obtained from Fruits of the Forest

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The aim of the present study was to obtain four distilled alcoholic beverages from red raspberry (RB), arbutus berry (AB), black mulberry (BM) and black currant (BC) by solid-state fermentation with *Sacchromyces cerevisiae* IFI83, and subsequent distillation of the fermented fruits.

The mean concentrations of ethanol in the distillates from RB (41.3 g/hL aa), AB (44.3 g/hL aa), BM (48.3 g/hL aa) and BC (38.5 g/hL aa) were within the limits (from 38.5 to 86.0 g/hL aa) fixed by the European Council (Regulation 110/2008) for fruit distillates. In addition, the mean concentrations of methanol in the four alcoholic beverages were much lower than the maximum levels of acceptability that the aforementioned regulation fixed for RB, AB, BM and BC distillates.

The mean volatile composition of the four distillates was different from other alcoholic beverages such as four commercial Galician orujo spirits, Portuguese bagaceiras, Mexican artisanal Bacanora beverages, and other distillates obtained from fermented blackberry, whey, cherry and black and red currants. These results showed that the pulps from RB, AB, BM and BC can be successfully used as fermentation substrates for producing alcoholic beverages with high quality and with their own distinctive characteristics.

1. Introduction

The fruit industry plays an important role on the economy and employment in many countries. However, a large amount of fruits of the forest is not collected neither to be directly consumed nor to be used as raw materials to produce foods and consequently, these fruits are lost (Alonso et al., 2010). The design of biotechnological processes to valorise these fruits could be an appropriate alternative to produce a variety of new high value-added products.

Although there is information available on the use of fruit of the forests to produce alcoholic beverages (Vulić et al., 2012), the volatile composition of these spirit drinks was found to be dependent on the origin of the fruits (Vulić et al., 2012), the microbial starter (Sorrentino et al., 2012), the methods of fermentation and distillation used (Alonso et al., 2011) and the time and storage system (Soufleros et al., 2004).

Considering the substantial availability of fruit of the forests (including red raspberry, arbutus berry, blackberry and black currant) at very low prices in the mountainous areas of Galicia, an autonomous region located in north-western Spain, the use of these fruits as culture media to produce distilled alcoholic beverages could provide profitable substrates for a low cost production, with advantage of the incorporation of these fruits in a production process (Nazzaro et al., 2012). In addition, the mean volatile composition of distillates produced from these fruits must be characterised and compared with those of other alcoholic beverages to determine the differences and similarities between them.

Therefore, the aim of this study was to determine the effects of the initial pH and the levels of reducing sugars, protein and moisture on ethanol production by *Saccharomyces cerevisiae* IFI83 in solid state fermentations of red raspberry (RB), arbutus berry (AB), black mulberry (BM) and black currant (BC). In addition, the major volatile composition of the four distillates obtained were compared with those of other spirits drinks obtained from wild agave (named Bacanora) (Álvarez et al., 2013), cornelian cherry (named Drenja) (Tešević et al., 2009), red and white grape varieties: Portuguese bagaceiras (Silva et al., 1996) and four commercial Galician orujo spirits (Diéguez et al., 2005), whey (Dragone et al., 2009), blackberry (named Mouro) (Soufleros et al., 2004) and black and red currant cultivars (Vulić et al., 2012).

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2. Materials and methods

2.1. Yeast Strain

Saccharomyces cerevisiae IFI83, a high-ethanol-producing strain, was obtained from the yeast collection of the Institute for Industrial Fermentations (IFI), Spanish National Research Council (CSIC), Madrid, Spain. The yeast strain was grown in a conventional medium composed by (g/L) bacteriological peptone, 20; yeast extract, 15; and glucose, 20; pH, 6.2. Working cultures were maintained as slants at 4 ºC on a medium composed by (g/L) malt extract, 20; yeast extract, 1; and agar, 20; pH, 7.2. These cultures were propagated twice in the same medium at 18 ºC before their use as inoculum.

2.2. Fermentation Substrates

The fruits used in this paper were red raspberry (*Rubus ideaus* L.), arbutus berry (*Arbutus unedo* L.), black mulberry (*Morus nigra* L.) and black currant (*Ribes nigrum* L.), which were collected at their time of production from different plantations of the Galician region, where the fruits are picked. The fruits were manually selected, discarding previously those that were too green or ripe or with bruising.

2.3. Physicochemical Characterization of the Fruits

Frozen fruit samples were thawed at room temperature and homogenized for 5 min in an Ultraturrax at 9500 rpm. Then, 1 g of pulp from each sample was poured into a 100 mL Erlenmeyer flask and mixed with 50 mL of distilled water. After mixing, the samples were centrifuged (14000 rpm/5 min), and the supernatants were used to determine pH and reducing sugars (Bernfeld, 1951).

Total protein (Havilah et al., 1977), solid residue, moisture content, and ashes were determined in the undiluted pulp. All of the analyses were done in triplicate.

2.4. Solid-State Fermentations of the Fruit Pulps

The fruits were slightly crushed with a mortar and pestle to break all of the berries. Fermentations were conducted in 150 mL Erlenmeyer flasks previously sterilized, containing exactly 50 g of fruit, and covered with cotton plugs. In all cases, the crushed fruits used as fermentation substrates were supplemented with 0.5 mL of a sterile mixture of salts composed of NH_4Cl and KH_2PO_4 to get nitrogen and phosphorus supplements of 200 and 136 mg/kg of fresh pulp, respectively. After homogenisation, the substrates were inoculated with 0.4 mL of a cell suspension of *S. cerevisiae* IFI83 adjusted previously to give an initial concentration of 5×10^5 cells/g of pulp.

The contents of the flasks were mixed thoroughly, and then the cultures were incubated under static conditions at 18 ºC. Samples as whole flasks in triplicate were withdrawn at regular intervals for analytical determinations. These fermentation samples were mixed with 100 mL of distilled water and, after centrifugation of the mixture (14,000 rpm/5 min), the supernatants were used to measure culture pH and the concentrations of reducing sugars and ethanol, which were expressed in g/kg of fresh fruit pulp.

2.5. Fermentation Product Analysis

Ethanol concentration was quantified by ion exclusion chromatography using an ICSep ICE-ION-300 Transgenomic column with a pre-GC-801 Guard ICSep (mobile phase, 8.5 mM H_2SO_4; flow rate, 0.4 mL/min; temperature, 30 ºC; RI detection). Solutions of ethanol at a concentration between 1 and 10 g/L were used as standards.

2.6. Distillation

Fermented pulps were distilled by using a steam drag distillation system equipped with a distilling flask fixed to a rectifying column, which allows the fractional distillation and concentration of volatile compounds on the basis of their volatility. The first volume of distillate corresponding to the beginning of the distillation procedure when the temperature reached 70-85 ºC was removed as “head”. The intermediate fraction called the “heart”, the most important part of the distilling spirits, was obtained in the temperature range from 85 to 95 ºC and used for volatile compound determination. The last volume of distillate obtained in the temperature interval between 95 and 99 ºC was removed as the “tail”.

2.7. Major Volatile Compounds Determination

Volatile compounds present in the distilled heart fractions of the fruit distillates were determined by gas chromatography as described previously (López et al., 2010).

2.8. Statistical analysis

A paired-samples *t*-test was conducted to determine whether significant differences ($P < 0.05$) existed between the means obtained for the pH values, the concentrations of sugars, proteins and moisture in the pulps obtained from red raspberry, arbutus berry, black mulberry and black currant, and the ethanol concentration in the fermented fruits. The data were statistically analysed by using the software package SPSS Statistics 20.0 for Windows (Release 20.0.1; SPSS Inc., Chicago, IL, 2011).

A cluster analysis was used to determine the similarity or dissimilarity between the RB, AB, BM and BC distillates obtained from and other alcoholic beverages including Bacanora samples obtained from four regions of the Sonora state in northwestern México: the Central and Southern Region (BacCS), the Sonora River (BacSR), the High Sierra (BacHS) and the Sierra Baja (BacSB) (Álvarez et al., 2013); four commercial Galician orujo spirits: Albariño (AL), Mencia (ME), Godello (GO) and Treixadura (TR) (Diéguez et al., 2005); Greek blackberry distillate (BBD) (Soufleros et al., 2004); Portuguese whey distillate (WD) (Dragone et al., 2009); Portuguese bagaceiras (BAG) (Silva et al., 1996); distilled alcoholic beverages obtained from black mulberry (BM) (Alonso et al., 2010), black currant (BC) (Alonso et al., 2010), red raspberry (RB) (Alonso et al., 2011) and arbutus berry (AB) (Alonso et al., 2011); distilled alcoholic beverages obtained from black currant cultivars: Malling Juel (BCMJ), Ometa (BCO) and Ben Sarek (BCBS) and red currant cultivars: Rondom (RCR) and Versailles (RCV) grown in Serbia (Vulić et al., 2012); a home-made spirit beverage from cornelian cherry fruits (CCH) (Tešević et al., 2009).
The mean concentrations of major volatile compounds in the different beverages were used as the classification variables. The data were standardized before clustering by using the following formula:

$$z_i = \frac{y_i - \bar{y}}{sd_y} \qquad (1)$$

Where z_i is the z score, y_i is the original value of each variable, $\bar{y}$ is the mean of all values of y, and sd_y is the standard deviation of that mean. With this transformation, each variable has a mean of 0 and a standard deviation of 1. The Euclidean distance was used as the similarity index and the average linkage between groups (in the variant of unweighted pair-group average) was used as the amalgamation (linkage) method. Both the cluster analyses and the dendogram plot were performed using the Cluster Analysis module of the software package SPSS Statistics 20.0 for Windows (Release 20.0.1; SPSS Inc., Chicago, IL, 2011).

2. Results and discussion

3.1. Fermentation of the fruits

Table 1 shows the mean composition as well as the initial pH levels of the pulps from red raspberry (RB), arbutus berry (AB), black mulberry (BM) and black currant (BC), as well as the ethanol concentrations (Et) in the fermented pulps.

Table 1. Chemical composition of fruit pulps and final concentrations of ethanol (g/kg) obtained in the fermentations. Means within the same row, followed by the same letter are not significantly different from each other (P > 0.05)

	Red raspberry	Arbutus berry	Black mulberry	Black currant
Reducing sugars	41.72 ± 2.81a	156.58 ± 12.04b	144.27 ± 21.23b	71.68 ± 12.02a
Total protein	8.71 ± 1.22a	31.79 ± 2.21b	46.52 ± 1.20c	13.04 ± 2.21d
Moisture content	899.00 ± 12.40a	731.03 ± 13.33b	832.60 ± 12.41c	855.08 ± 13.34c
Initial pH	3.36 ± 0.04a	3.50 ± 0.21a	4.76 ± 0.03b	2.87 ± 0.02c
Ethanol	17.82 ± 0.19a	32.02 ± 0.15 b	53.41 ± 0.22c	25.63 ± 0.16d

These results suggest that ethanol production by *S. cerevisiae* IFI83 was affected by the effect of substrate inhibition by high concentrations of the carbon source. In fact, the mean ethanol production increased from 17.82 to 53.41 g/kg with the increase in the reducing sugars (RS) levels from 41.72 to 144.27 g/kg (in case of RB, BC and BM). However, the alcohol production decreased significantly to 32.02 g/kg when the RS level increased to 156.58 g/kg, in case of arbutus berry (Table 1).

3.2. Comparison of the major volatile compounds of the four fruits distillates with those of other spirits.

The concentrations of the major volatile compounds in the heart fractions of the RB, AB (Alonso et al., 2011), BM and BC (Alonso et al., 2010) distillates obtained from the fermented pulps are shown in Table 2.
The concentrations of the major volatile compositions in the RB, AB, BM and BC distillates were compared with those of four commercial Galician (Spain) orujo spirits (Diéguez et al., 2005) and Portuguese bagaceiras (Silva et al., 1996) elaborated in a traditional way from grape marc, with other distillates obtained from fermented Greek blackberry (Soufleros et al., 2004), Portuguese whey (Dragone et al., 2009), Serbian cherry (Tešević et al., 2009), Serbian black currant cultivars (Malling Juel, Ometa and Ben Sarek) and red currant cultivars (Rondom and Versailles) (Vulić et al., 2012), and Mexican artisanal Bacanora beverages collected from four municipalities of the Sonora state (Álvarez et al., 2013).

Since Tešević et al. (2009) and Vulić et al. (2012) reported the individual concentrations of 2-methyl-1-butanol and 3-methyl-1-butanol in a join way, the data concerning the mean concentrations of these two isomers obtained by our research team and other groups were also presented together as 2/3-methyl-1-butanol.
The concentrations of major volatile compounds in our distillates (Table 2) and in the other aforementioned alcoholic beverages showed a high variability, mainly due to the different raw materials, ethanol-producing strains, and the fermentation and distillation technologies used in the production processes. Thus, with a simple visual comparison, it is difficult to establish clear differences or similarities between the different distillates. Therefore, a hierarchical cluster analysis was carried out by using the mean concentrations of their major volatile compounds (previously standardized), as the classification variables.

Table 2. Concentration of major volatile compounds (g/HL absolute alcohol) in the four distillates produced from red raspberry (RB), arbutus berry (AB), black mulberry (BM) and black currant (BC). N.D. not detected

Compound	RB	AB	BM	BC
Ethanol (%, v/v)	41.3 ± 1.38	44.3 ± 1.30	48.3 ± 1.73	38.5 ± 1.19
Methanol	113.9 ± 1.44	320.5 ± 3.48	349.6 ± 2.33	167.4 ± 1.32
1-Propanol	36.2 ± 0.15	41.0 ± 0.88	44.7 ± 0.42	38.2 ± 0.52
2-Methyl-1-propanol	33.4 ± 0.74	36.0 ± 1.85	39.2 ± 1.25	17.6 ± 0.53
1-Butanol	0.5 ± 0.07	0.8 ± 0.09	0.9 ± 0.04	0.2 ± 0.03
2/3-Methyl-1-butanol	79.7 ± 2.57	92.5 ± 2.50	100.9 ± 4.39	39.2 ± 0.93
1-Hexanol	1.1 ± 0.04	1.2 ± 0.21	1.3 ± 0.14	0.1 ± 0.01
2-Phenylethanol	N.D.	N.D.	N.D.	0.3 ± 0.02
Ethyl acetate	37.8 ± 0.34	40.7 ± 3.16	144.7 ± 2.34	7.7 ± 0.42
Acetaldehyde	4.4 ± 0.17	32.7 ± 1.88	13.9 ± 1.17	12.8 ± 1.53
Acetals	4.0 ± 0.25	20.5 ± 1.33	N.D.	0.3 ± 0.03

The cluster analysis resulted in three well-defined groups of associations (Figure 1). In the group I (four Bacarona samples), the beverages BacSR and BacSB merge to form subcluster 1 with a distance coefficient (DC) of 0.707. Subsequently, BacHS associated with BacSR to form the subcluster 4 (DC = 1.320) and finally BacCS was grouped to subcluster 4 to form subcluster 9 (DC = 2.098).
In the group II (six samples), subclusters 2 (DC = 1.005) and 5 (DC = 1.364) were respectively formed by Albariño (AL) and Godello (GO) orujo spirits and by the Serbian red currant cultivars RCR and RCV. Mencia orujo spirit (ME) was joined with RCR to form subcluster 7 (DC = 1.848). Subsequently, the subcluster 7 and AL were joined to form subcluster 8 (DC = 1.937), and finally the BM sample was associated to the latter group to form the subcluster 10 (DC = 2.354).
The group III (four samples) was composed by beverages BCMJ and BCO (subcluster 3, DC = 1.234) and BC and AB (subcluster 6, DC = 1.773) and the group formed all them (subcluster 11, DC = 2.438).
Then, the groups I, II and III were joined to form subcluster 13 (DC = 3.162), but group II was previously clustered with group III (subcluster 12, DC = 2.816) and finally, this aggrupation was joined with the first group.
In the last steps, fairly dissimilar subclusters (14-20) are combined, eventhough the beverages Treixadura orujo spirit and the Portuguese Bagaceiras with a DC of 3.695 were joined to form an independent aggrupation (subcluster 15). Thus, the samples BCBS, TR, BAG, BBD, RB, WD and CCH were the most different distilled alcoholic beverages because they have the highest DC values.
From the comparison of the distillates produced by our research team, it can be observed that the samples BC (Alonso et al., 2010) and AB (Alonso et al., 2011) have the nearest major volatile composition, while the samples BM (Alonso et al., 2010) and RB (Alonso et al., 2011) were the most different beverages.
However, when the distillate obtained from the fermented black currant (Alonso et al., 2010) is compared with the fruit spirits produced from Serbian black currant cultivars Malling Juel, Ometa and Ben Sarek (Vulić et al., 2012), it can be noted that there was a similarity between the BC distillate and the BCMJ and BCO beverages, since they are clustered in the group III (Figure 1). The BCBS beverage was the most different beverage, which is only joined with the aggrupation formed by the groups I, II and III.
In fact, when the mean major volatile composition of these four beverages (BC, BCMJ, BCO and BCBS) are compared, it can be observed that the BCBS spirit had concentrations of 1-propanol and 2-methyl-1-propanol considerably higher and concentrations of acetaldehyde and acetals considerably lower than those of the BCO, BCMJ (Vulić et al., 2012) and BC samples (Alonso et al., 2010).

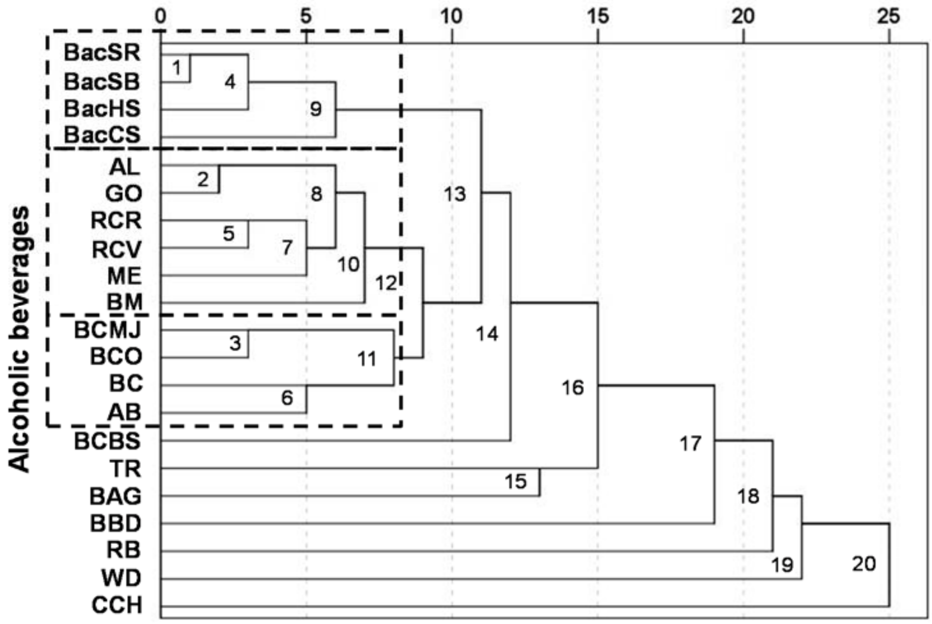

Figure 1. Dendrogram showing the cluster analysis for the 21 alcoholic beverages

These differences could be related to the different origin of the fruits used as substrates and the different fermentation and distillation procedures used to obtain the alcoholic beverages.

Thus, although the three Serbian cultivars (Ben Sarek, Malling Juel and Ometa) were harvested in Belgrade, they were respectively developed in Scotland, England and Switzerland, meanwhile, the fruits used to obtain the BC distillate were cultivated and harvested in the Galician region, Spain.

In addition, the Serbian black currant cultivars Malling Juel, Ometa and Ben Sarek were spontaneously fermented with the autochthonous microflora under identical conditions over a period of 15 days, being the distillation system used to obtain the spirits, a simple laboratory copper pot still (Vulić et al., 2012).

In contrast, the Galician BC distillate was fermented after inoculation of the fruits with *S. cerevisiae* IFI83, a high-ethanol-producing strain, during 65 h, and the fermented fruit was distilled by using a steam drag distillation system equipped with a distilling flask fixed to a rectifying column (Alonso et al., 2010).

A similar comparison was done between the Galician BM distillate and the Greek BBD (Figure 1), both obtained from the fruits of the mulberry tree. However, in this case, the BBD form a dissimilar cluster due to its large DC value (5.242), indicating that the major volatile compositions of both alcoholic beverages were very different. Also, the production of both distillates was carried out with fruits of different origin and by using different procedures of fermentation and distillation. However, the storage system and the storage time are other aspects that must be taken into account to explain the differences observed between the different alcoholic beverages (Soufleros et al., 2004).

On the other hand, the distillates RB, AB, BM and BC have methanol concentrations (Table 2) much lower than the maximum permitted by the standards of the Regulating Commission (Regulation 110/2008) of 1000 g/hL aa, for AB and BM spirits, 1200 g/hL aa, for a RB spirit or 1350 g/hL aa, for a BC spirit. The ethanol levels in the four distillates (Table 2) were within the limits of acceptability (from 37.5 to 86.0 % (v/v) given by the aforementioned regulation.

4. Conclusions

According to the results presented in this work, we can conclude that the four fruits of the forests (red raspberry, arbutus berry, black mulberry and black currant) can be used to produce high-quality distillates with a major volatile composition different to those of currently commercialised alcoholic beverages including four Galician orujo spirits (Mencia, Godello, Treixadura and Albariño), the Mexican Bacanora, the Greek Mouro, the Portuguese bagaceiras and others produced from whey, cherry and black and red currants. The production of distillates from RB, AB, BM and BC could also contribute to the valorisation of these fruits.

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