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Production and Characterization of Distilled Alcoholic Beverages Obtained by Solid-State Fermentation of Black Mulberry (*Morus nigra* L.) and Black Currant (*Ribes nigrum* L.)

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The present study was conducted to appraise the potential of black mulberry and black currant to be used as fermentation substrates for producing alcoholic beverages obtained by distillation of the fruits previously fermented with *Sacchromyces cerevisiae* IFI83. In the two distillates obtained, the volatile compounds that can pose health hazards are within the limits of acceptability fixed by the European Council (Regulation 110/2008) for fruit spirits. However, the amount of volatile substances in the black currant distillate (121.1 g/hL absolute alcohol (aa)) was lower than the minimum limit (200 g/hL aa) fixed by the aforementioned regulation. The mean volatile composition of both distillates was different from other alcoholic beverages such as four commercial Galician orujo spirits, Portuguese *bagaceiras*, and two distillates obtained from fermented whey and blackberry. The results obtained showed the feasibility for obtaining distillates from fermented black mulberry and black currant, which have their own distinctive characteristics.

KEYWORDS: Black mulberry (*Morus nigra* L.); black currant (*Ribes nigrum* L.); ethanol; solid-state fermentation; volatile compounds

INTRODUCTION

In Galicia, an autonomous region located in northwestern Spain, mountainous areas are economically and demographically depressed zones in comparison with other areas. This is mainly due to the lack of infrastructure and facilities that allow an integral management of the forest and the use of all their resources. In addition, the increasing need of farmers for more parcels to provide enough land to establish a cost-effective and feasible farm has increased monetary and time costs (*1*).

A possible alternative to increase farmer income would be the use of some fruits of the forest grown in Galicia as substrates for producing high-added-value products, such as fruit-based spirits obtained by fermentation and later distillation of the fermented fruits (*2*−*4*). In this sense, the current commercialization of other known alcoholic beverages obtained from different fruits (*3*, *5*−*7*) could facilitate the market penetration of such spirits. Some of these commercialized beverages are very popular, especially those produced from grape marc, such as *grappa* from Italy, *tsipouro* and *tsikoudia* from Greece (*8*, *9*), *bagaceiras* from Portugal (*8*−*10*), the traditional Greek distillate “Mouro” (*4*), black mulberry liquors (*3*), and the Galician orujo spirits from Spain (*5*).

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However, there are two main problems associated with the production of alcoholic beverages from fruits of the forest. On the one hand, in some cases these fruits are not fermented, but simply they are macerated to elaborate liquors, leading to a wide variety of products with different qualities (*11*). On the other hand, the artisanal production of these beverages makes difficult the reproducibility of the fermentation process, which is usually performed by spontaneous or inoculated submerged liquid fermentation (SLF) using fruit juices (*3*). Although SLF requires less complicated control than solid-state fermentation (SSF) of the fruits, generally the latter method yields fermented products with a high aromatic profile, composed by the aromas from the raw material and those produced during the fermentation process.

Studies dealing with the analysis of juices (*12*) and alcoholic beverages (*3*, *4*) obtained, respectively, from black mulberry (*Morus nigra* L.) and black currant (*Ribes nigrum* L.) have been reported before. Both fruits are a good source of vitamins, antioxidants, nonvolatile organic acids, and phenolic acids that contribute to the quality of taste and aroma (*4*, *12*). In addition, these fruits have been reported to have natural therapeutic qualities (*3*, *13*, *14*). However, to our knowledge, reports dealing with the solid-state fermentation of fruits to produce alcoholic beverages are lacking.

For these reasons, the main goal of the present study was to develop a procedure to obtain, in a reproducible way, fruit-based spirits from fermented black mulberry and black currant. First,

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three fermentation strategies were carried out to ferment the fruits: two alcoholic fermentations inoculated with *Saccharomyces cerevisiae* IFI83, using the thermally treated and the nonthermally treated fruits as fermentation substrates, and a spontaneous fermentation, with the native microbiota of the fruits. Second, the fermented fruits obtained from the most adequate fermentation procedure were distilled to determine their average composition with regard to the volatile compounds that have concentration limits fixed by the European legislation 110/2008 (*15*) and other components that are important for quality evaluation. This knowledge not only should lead to a better standardization process and a more uniform quality of these spirits but also must provide data on the safety of these products for consumers without repercussion to their health from methanol levels.

MATERIALS AND METHODS

Yeast Strain. *S. 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 °C 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 °C before their use as inoculum.

Fermentation Substrates. The fruits used in this paper were black mulberry (*M. nigra* L.) and black currant (*R. 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.

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 (3,5-dinitrosalicylic acid reaction (*16*) with glucose (Panreac, Barcelona, Spain) as standard).

Total protein, solid residue, moisture content, and ashes were determined in the undiluted pulp. Total protein (N × 6.25) was determined according to the micro-Kjeldahl method, substituting distillation by the spectrophotometric method of Havilah et al. (*17*), with ammonium sulfate (Panreac) as standard. To determine the solid residue, 1 g of pulp was evaporated at 60 °C for 4 h and subsequently at 105 °C until constant weight. Moisture content was determined as the difference between the fresh weight of the undiluted pulp and the weight of the solid residue. Ash content was determined gravimetrically by calcination of 1 g of fruit at 550 °C in a muffle furnace (Hobersal, Barcelona, Spain) to a constant weight. All of the analyses were done in triplicate, thus obtaining the means with their respective standard deviations.

The pulp obtained from black mulberry contained the following mean composition (wet basis): reducing sugars, 14.43 ± 2.12%; total protein (N × 6.25), 4.65 ± 0.12%; moisture content, 83.26 ± 1.24%; ashes, 0.78 ± 0.09%; pH, 4.76 ± 0.03. The mean composition of the black currant pulp was (wet basis) as follows: reducing sugars, 7.17 ± 1.20%; total protein (N × 6.25), 1.30 ± 0.22%; moisture content, 85.51 ± 1.33%; ashes, 1.11 ± 0.15%; pH, 2.87 ± 0.02.

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. The moisture contents after salt supplementation were 84.26% (in black mulberry) and 86.51% (in black currant). Then, three fermentation strategies were used. The first was a spontaneous fermentation of the crushed fruits with their own indigenous microbiota. The second and third cultures were inoculated fermentations with *S. cerevisiae* IFI83 using, respectively, the thermally treated (105 °C/20 min) and the nonthermally treated fruit pulps as the fermentation substrates.

After N and P supplementation, homogenization, and thermal treatment (in the case of the second fermentation), the media were inoculated (in the case of the two inoculated fermentations) 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 moisture contents after inoculation were 85.06% (in black mulberry) and 87.31% (in black currant). The contents of the flasks were mixed thoroughly, and then the cultures were incubated under static conditions at 18 °C.

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. After centrifugation of the mixture (14000 rpm/5 min), the supernatant was used to measure culture pH, reducing sugars concentration (as indicated above), and fermentation products (ethanol, glycerol, and acetic acid). The concentration (on a wet basis) of reducing sugars and fermentation products was expressed in percent (g/100 g of fruit pulp).

Fermentation Product Analysis. Concentrations of fermentation products were quantified by ion exchange 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 °C; RI detection). Patterns were used as solutions of ethanol, glycerol, and acetic acid at a concentration between 1 and 10 g/ L.

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 °C 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 °C and used for volatile compound determination. The last volume of distillate obtained in the temperature interval between 95 and 99 °C was removed as the "tail".

Aromatic Compound Determination. Volatile compounds present in the distilled heart fractions of black mulberry (BMD) and black currant (BCD) samples were determined by gas chromatography on a Hewlett-Packard 5890 series II gas chromatograph equipped with a HP 6890 automatic injector and a flame ionization detector as described by Diéguez et al. (*5*). According to this procedure, the compounds were separated on a Chrompack CP-Wax 57CB (polyethylene glycol stationary phase; 50 m × 0.25 mm i.d. with 0.25 μm film thickness) fused-silica capillary column. The temperatures of the injector and detector were fixed at 250 and 260 °C, respectively. The temperature program of the oven was as follows: 40 °C for 6 min, a first linear ramp from 40 to 80 °C at 1.5 °C/min and a second linear ramp from 80 to 200 °C at 3 °C/min. The carrier and makeup gas used were helium at 1.07 mL/min and nitrogen at 15 mL/min The detector gas flow rates were 40 mL/min for hydrogen and 400 mL/min for air. For volatile compound quantification, 1 mL of an internal standard solution (5 g of 4-methyl-2-pentanol/L of ethanol) was added to a 10 mL sample of distillate. An aliquot (1 μL) was injected directly into the chromatograph and split 1:1.

All of the volatile compounds were identified by comparing gas chromatography retention times and mass spectra data with those of pure standard compounds used as references using an HP 5890 series II coupled to an HP 5989 A mass spectrometer. The detector was set to electronic impact mode (70 eV) with an acquisition made in scanning mode from m/z 10 to m/z 1000 and an acquisition rate of 5 scan/s.

Response factors of 31 reference compounds including alcohols, aldehydes, acetates, and esters were determined. All analyses were done in triplicate.

Reagents Used as Reference Compounds. Ethanol, of analytical grade, was supplied by Merck (Darmstadt, Germany). 2-Butanol, 1-butanol, 1-propanol, 2-methyl-1-propanol, 4-methyl-2-pentanol, *trans*-2-hexenol, *cis*-2-hexenol, acetaldehyde, 1,1-diethoxyethane, diethyl succinate, and ethyl myristate were purchased from Aldrich (Buchs, Switzerland); methanol, benzyl alcohol, 2-phenylethanol, allyl alcohol, 2-methyl-1-butanol, hexanol, ethyl butyrate, ethyl laurate, hexyl acetate, isoamyl acetate, ethyl acetate, and methyl acetate were purchased from Merck (Barcelona, Spain); ethyl hexanoate, ethyl octanoate, ethyl decanoate, *trans*-3-hexenol, *cis*-3-hexenol, 3-methyl-1-butanol, and furfural were obtained from Fluka (Buchs, Switzerland); and ethyl lactate was purchased from Sigma (Buchs, Switzerland). A stock solution of volatile reference standards was prepared in distilled water containing 40% (v/v) of ethanol. The internal standard, 4-methyl-2-pentanol, was prepared in absolute ethanol.

Statistical Analysis. The data concerning mean concentration of fermentation products and volatile compounds in the distillates obtained from fermented black mulberry and black currant were statistically analyzed by using the software package SPSS Statistics 17.0 for Windows (release 17.0.1; SPSS Inc., Chicago, IL, 2008). A paired-samples *t* test was conducted to determine whether significant differences ($p < 0.05$) existed between the means obtained for the fermentation products concentrations of fermentation products and volatile compounds in the two distillates.

The similarity or dissimilarity between our distillates (BMD and BCD) and other alcoholic beverages (*4*, *5*, *10*, *18*) based on their volatile composition was measured using a cluster analysis. The mean concentrations of each volatile compound detected in the distillates were used as the classification variables. The data were standardized before clustering, to make all characteristics equally contribute to the discrimination process. With this procedure, both the magnitude and variability of all mean concentrations of each volatile compound were homogenized by transforming the original value of each variable into a *z* score. The formula for this transformation is

$$z_i = \frac{x_i - \overline{x}}{\mathrm{sd}_x}$$

where z_i is the *z* score, x_i is the original value of each variable, $\overline{x}$ is the mean of all values of x, and sd is the standard deviation of that mean. With this transformation, each variable, which has a mean of 0 and a standard deviation of 1, contributes equally to the variance in the analysis. The Euclidean distance was used as the distance measure or similarity index, and the average linkage (in the variant of unweighted pair-group average) was used as the amalgamation (linkage) method. Both the statistical analyses and the dendogram plot were performed using the Cluster Analysis module of the Statistica for Windows program, release 5.1 (*19*).

RESULTS AND DISCUSSION

Spontaneous Solid-State Fermentations of the Pulps. Figure 1 shows the behavior of the concentrations of reducing sugars and products (ethanol, glycerol, and acetic acid) and pH variation versus time in the nonthermally treated black mulberry pulp fermented spontaneously. At the end of the incubation, low amounts of glycerol (0.14%), ethanol (0.36%), and acetic acid (0.39%) were obtained, and the ethanol yield from reducing sugars consumed ($Y_{\mathrm{Et/RSc}}$ in g/g) was 0.115.

On the other hand, the spontaneous fermentation on black currant was unsuccessful because no fermentative activity was observed during the first 37 h of incubation, when the culture was stopped due to the undesirable fungal growth observed on the surface of the pulp. These results could be due to the fact that the initial populations of the indigenous microbiota present in the black currant are small compared to the indigenous microbiota present in the black mulberry. In addition, the low pH of this fruit (~3), also reported by other authors (*20*), could explain the preferential proliferation of fungi that are more acid-resistant than yeasts.

Inoculated Solid-State Fermentations of the Thermally Treated Pulps. Because the spontaneous fermentations of black mulberry and black currant were unsuccessful, a new solid-state fermentation was carried out by using the thermally treated pulps as fermentation substrates, which were inoculated with *S. cerevisiae* IFI83, a high-ethanol-producing strain (*2*).

In the black mulberry culture (**Figure 2**), the reducing sugars and glycerol reached final concentrations of 3.12 and 0.47%,

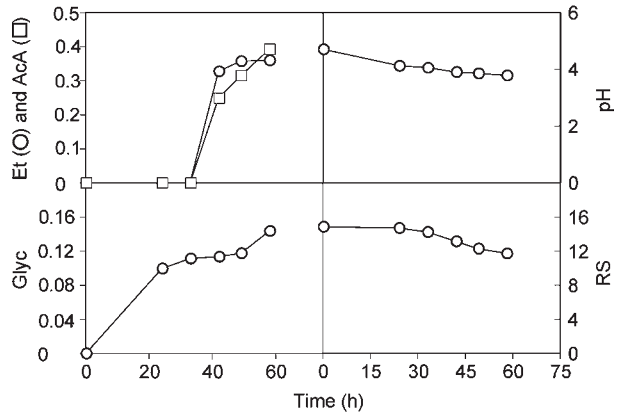

Figure 1. Kinetics of the spontaneous solid-state fermentation of black mulberry pulp. Time courses of ethanol (Et), glycerol (Glyc), acetic acid (AcA), and reducing sugars (RS) are expressed in percent (g/100 g of fermentation medium).

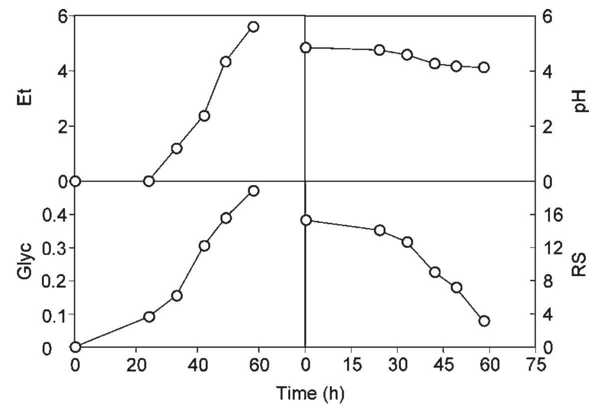

Figure 2. Kinetics of the solid-state fermentation of the thermally treated black mulberry pulp inoculated with *S. cerevisiae* IFI83. Time courses of ethanol (Et), glycerol (Glyc), and reducing sugars (RS) are expressed in percent (g/100 g of fermentation medium).

respectively. However, the ethanol concentration at the end of the incubation was 5.60%, which was 15 times higher than that observed in the spontaneous fermentation of the same fruit ($p < 0.05$). Consequently, the $Y_{\mathrm{Et/RSc}}$ value of 0.459 obtained in this culture was 90% of the theoretical ethanol yield from glucose (0.511 g/g). From the detailed observation of the culture, it can be noted that the culture pH was maintained at values above 4.0 during the incubation period, which are not very harmful for the growing yeast (*2*).

Although ethanol production would probably be increased by extending the incubation period until the exhaustion of the carbon source, previous results showed that prolonged submerged fermentation of chestnut pulp by *S. cerevisiae* IFI83 led to the production of compounds with a strong and disagreeable sulfur odor (*2*). The accumulation of these sulfur compounds at the end of fermentative processes appears to be associated with the decline in the availability of the carbon or nitrogen sources. This stimulates the activity of proteases, the action of which on proteins causes release of sulfur amino acids, which could be used as a carbon and/or nitrogen source by the growing yeast (*21*, *22*).

However, in the case of black currant fermentation (**Figure 3**), the reducing sugars were almost completely consumed and reached a final concentration of 0.73% after 65 h of incubation, without sulfur odor detection. This fact constitutes an interesting operational advantage and suggests that detention of the

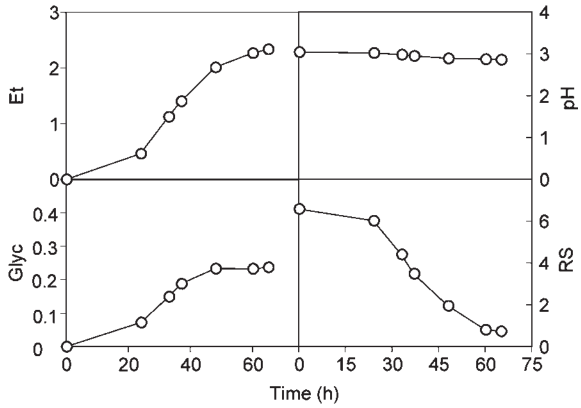

Figure 3. Kinetics of the solid-state fermentation of the thermally treated black currant pulp inoculated with *S. cerevisiae* IFI83. Time courses of ethanol (Et), glycerol (Glyc), and reducing sugars (RS) are expressed in percent (g/100 g of fermentation medium).

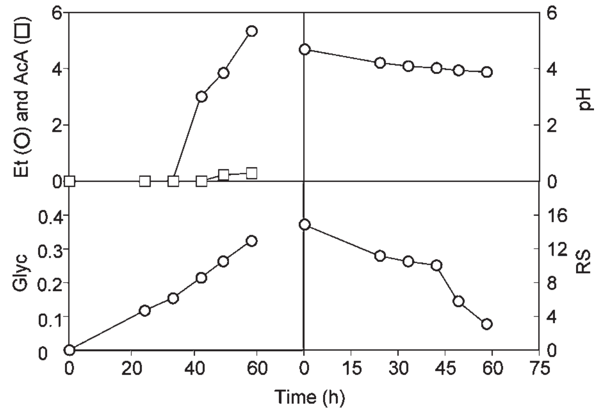

Figure 4. Kinetics of the solid-state fermentation of the nonthermally treated black mulberry pulp inoculated with *S. cerevisiae* IFI83. Time courses of ethanol (Et), glycerol (Glyc), acetic acid (AcA), and reducing sugars (RS) are expressed in percent (g/100 g of fermentation medium).

fermentation before the total depletion of reducing sugars is not always a prerequisite to prevent the formation of sulfur-containing off-odor compounds.

On the other hand, it could be pointed out that, despite the low values of pH, the thermal treatment of the black currant pulp and the inoculation of the yeast at a high concentration resulted in a successful fermentation. However, the final concentrations of ethanol and glycerol (2.33 and 0.24%, respectively) were lower than those observed in the culture on black mulberry pulp ($p < 0.05$), probably as a consequence of the lower sugars content in black currant pulp in comparison to black mulberry pulp. Also, the $Y_{Et/RSc}$ value (0.399) was lower, which suggests that black currant has a low potential as a substrate for ethanol production, probably because its pulp had a more acidic initial pH and a higher viscosity in comparison with the black mulberry pulp. The high viscosity of the black currant pulps, probably due to a high content in pectins, could interfere with the mass transfer and reduce the water activity level, thus limiting the growth of the ethanol-producing strain and, consequently, ethanol production (*23*).

Inoculated Solid-State Fermentations of the Nonthermally Treated Pulps. The results obtained in the previous cultures showed that the thermally treated pulps can be fermented by *S. cerevisiae* IFI83 for ethanol production. However, odors of thermally treated pulps were characterized as nondisagreeable cooked and less fruity. For this reason, the following solid-state cultures with *S. cerevisiae* IFI83 were carried out by using the nonthermally treated pulps as fermentation substrates.

The profiles described by the time courses of reducing sugars and pH as well as the productions of ethanol and glycerol in the black mulberry culture are shown in **Figure 4**. During the incubation, the reducing sugars concentration dropped from 14.43 to 3.08% and low amounts of glycerol (0.32%) and acetic acid (0.28%) accumulated in the medium. On the other hand, the final ethanol concentration (5.34%) as well as the $Y_{Et/RSc}$ value (0.452) were comparable to those levels (5.60% and 0.459, respectively) obtained in the culture on the thermally treated black mulberry pulp ($p < 0.05$).

The results obtained in the fermentation of thermally treated black currant pulp are depicted in **Figure 5**. It is again noteworthy that the addition of a concentrated yeast inoculum was enough to avoid fungus proliferation, thus allowing a successful fermentation without the need of a previous thermal treatment. In this fermentation, acetic acid was not produced and the time courses of reducing sugars, pH, and ethanol production were similar to

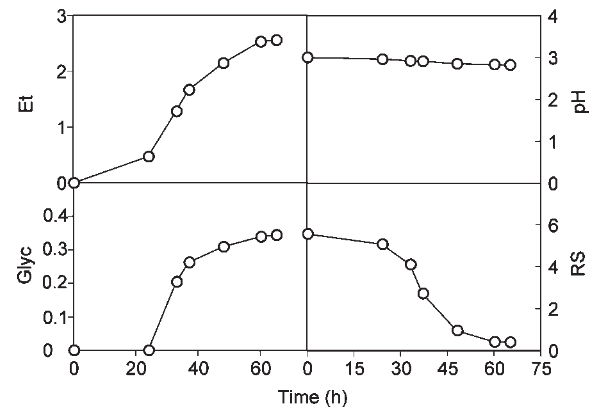

Figure 5. Kinetics of the solid-state fermentation of nonthermally treated black currant pulp inoculated with *S. cerevisiae* IFI83. Time courses of ethanol (Et), glycerol (Glyc), and reducing sugars (RS) are expressed in percent (g/100 g of fermentation medium).

those observed in the culture on thermally treated black currant pulp (**Figure 3**). The final concentrations of ethanol (2.56%) and glycerol (0.34%) as well as the $Y_{Et/RSc}$ value (0.414) obtained were slightly higher than those (2.33%, 0.24%, and 0.399, respectively) observed in the culture on the untreated black currant pulp ($p < 0.05$).

The results obtained in the fermentations with both fruit pulps either with or without thermal treatment showed that ethanol production and ethanol yield were practically unaffected. As these fruits contain sugars, vitamins, minerals, and amino acids (*3*, *24*, *25*), the composition of their pulps either with or without thermal treatment provides sufficient amounts of nutrients to satisfy the nutritional requirements of *S. cerevisiae* IFI83. According to these results, the aroma compounds identification was carried out by using the fermented samples obtained by solid-state fermentation of the nonthermally treated black mulberry and black currant pulps inoculated with *S. cerevisiae* IFI83 to avoid the formation of cooked odors, as was commented before.

Characterization of Volatile Compounds Present in Spirits Obtained by Distillation of Fermented Black Mulberry and Black Currant Pulps. The concentrations of the main volatile compounds in the heart fractions of the black mulberry and black currant distillates (BMD and BCD, respectively) are shown in **Table 1**. Because there are no legal restrictions concerning fruit distilled beverages in Spain, our results were also compared with

Table 1. Mean Concentrations (Grams per Hectaliter Absolute Alcohol) of Volatile Compounds Present in the "Heart" Fractions of Black Mulberry and Black Currant Distillates Obtained after Fermented Fruit Pulps Distillation[a]

no.	compound	BMD	BCD
1	ethanol (% v/v)	48.3 ± 1.73 a	38.5 ± 1.19 b
2	methanol	349.6 ± 2.33 a	167.4 ± 1.32 b
3	2-butanol	nd	0.1 ± 0.003 b
4	1-propanol	44.7 ± 0.42 a	38.2 ± 0.52 b
5	1-butanol	0.9 ± 0.04 a	0.2 ± 0.03 b
6	2-methyl-1-propanol (isobutyl alcohol)	39.2 ± 1.25 a	17.6 ± 0.53 b
7	2-methyl-1-butanol	15.0 ± 2.30 a	5.6 ± 0.29 b
8	3-methyl-1-butanol	85.9 ± 2.09 a	33.5 ± 1.22 b
	total alcohols (3−8)	185.8 ± 2.69 a	95.3 ± 1.62 b
9	allyl alcohol	nd	0.9 ± 0.06 b
10	1-hexanol	1.3 ± 0.14 a	0.1 ± 0.01 b
11	benzyl alcohol	1.5 ± 0.13 a	3.7 ± 0.15 b
12	2-phenylethanol	nd	0.3 ± 0.02 b
13	ethyl acetate	144.7 ± 2.34 a	7.7 ± 0.42 b
14	ethyl lactate (ethyl 2-hydroxypropanoate)	0.3 ± 0.06 a	nd
15	acetaldehyde	13.9 ± 1.17 a	12.8 ± 1.53 a
16	acetal	nd	0.3 ± 0.03 b
	total volatile substances (3−16)	347.6 ± 3.47 a	121.1 ± 2.19 b

[a] Means within the same row followed by the same letter are not significantly different at 95% confidence. BMD and BCD are the black mulberry and the black currant distillates. nd, not detected.

the maximum and minimum concentrations fixed by the European Council (Regulation 110/2008) for fruit distillates (*15*).

Alcohols. **Table 1** shows that the alcoholic contents of BMD (48.3%) and BCD (38.5%) were within the limits of acceptability given by the European Council (Regulation 110/2008) for fruit distillates (from 37.5 to 86.0% (v/v)).

Methanol, the inhalation or ingestion of which can cause blindness and eventually death (*10*, *26*, *27*), is generated by pectinolytic enzymes that split the methoxyl group from pectin present in crushed fruits (*4*, *10*). Thus, the mean methanol levels present in BMD (349.6 g/hL aa) and BCD (167.4 g/hL aa) were found to be much lower than the maximum permitted by the standards of the Regulating Commission (*15*) of 1000 g/hL aa, for a black mulberry spirit, or 1350 g/hL aa, for a black currant spirit. This indicates that both the black mulberry and black currant pulps were adequately manipulated and that their distillates were obtained by using an adequate distillation procedure (*4*, *10*).

The concentration of higher alcohols (2-butanol to 3-methyl-1-butanol) has a great influence in the quality of a distillate (*4*,*5*,*18*). In this way, total concentrations of higher alcohols of 350 g/hL aa or higher in distilled beverages indicate a poor quality (*28*). According to this criterion, the BMD and BCD samples, with concentrations of higher alcohols of 185.8 and 95.3 g/hL aa, respectively, will have a good quality.

The presence of 2-butanol in distillates at concentrations higher than 30 g/hL aa is deleterious to the quality of the product mainly due to the off-flavor generated by it (*5*, *10*, *26*). This compound was not detected or detected in a low concentration (0.09 g/hL aa) in the BMD and BCD samples, respectively. Thus, the upper permissible value was not violated in these two distillates.

The concentrations of 1-propanol were higher ($p < 0.05$) than those of 1-butanol in BMD and BCD (**Table 1**). Both compounds are considered to be strong odor compounds. However, the concentrations of 1-propanol and 1-butanol in BMD (215.9 and 4.3 mg/L, respectively) and BCD (147.0 and 0.8 mg/L, respectively) did not surpass the perception thresholds of 800 and 450 mg/L for both compounds (*29*). These results suggest that the fermentations of black mulberry and black currant pulps and their storage were properly controlled (*18*).

On the other hand, the concentrations of 2-methyl-1-propanol in both the BMD (39.2 g/hL aa, equivalent to 189.3 mg/L) and BCD (17.6 g /hL aa, equivalent to 67.8 mg/L) were lower than the perception threshold of 200 mg/L for this compound (*29*). Nevertheless, this would not affect the quality of both distillates because the Galician orujo spirit from Godello (*5*), an alcoholic beverage with a recognized high taste and bouquet, also had a 2-methyl-1-propanol content (195 mg/L) lower than 200 mg/L.

The concentrations of 2-methyl-1-butanol and 3-methyl-1-butanol were higher ($p < 0.05$) in the BMD (72.4 and 414.9 mg/L) than in BCD (21.6 and 129.0 mg/L). Thus, only the 2-methyl-1-butanol concentration in the BCD was lower than the perception threshold of 65 mg/L reported for both compounds (*29*). Because low concentrations of amyl alcohols (2-methyl-1-butanol and 3-methyl-1-butanol) indicate orujo spirits with very little body (*10*), the BMD has a better body than the BCD according to this criterion.

Allyl alcohol was not detected in the BMD, but it was detected in the BCD at a concentration of 0.9 g/hL aa. The presence of this compound and 2-butanol is related with a deficient storage of the raw material during ensilage (*30*).

1-Hexanol is not an alcoholic fermentation product, and its concentration in the distillates depends on the raw material employed in the distillation (*31*, *32*). Its presence is considered to be favorable at concentrations between 0.5 and 10 g/hL aa, but at concentrations higher than 10 g/hL aa, this compound will contribute to a grassy flavor, affecting both the aroma and taste of the distillates (*33*). The concentration of 1-hexanol in BMD (1.3 g/hL aa) was higher than that in BCD (0.1 g/hL aa). Thus, the BMD will have a more pleasant herbaceous aroma in comparison to BCD.

Benzyl alcohol and 2-phenylethanol have earlier been described as contributors to the odor of alcoholic beverages (*34*,*35*). The presence of these compounds contributed to flowery and sweet-like odors, which could be considered as a positive characteristic for our two distillates (BMD and BCD). Benzyl alcohol was detected in the BMD and BCD samples at concentrations of 1.5 and 3.7 g/hL aa, respectively. The concentration of 2-phenylethanol in the BCD (0.3 g/hL aa) was very low; meanwhile, in the BMD this compound was not detected. The presence of 2-phenylethanol in distillates has been associated with the fruit variety, its content in L-phenylalanine, and the extraction time (*8*, *9*). Because this substance is a marked tail component, only low amounts of it should be present in the heart fraction of the distillates (*32*, *36*).

Acetates and Esters. High concentrations of methyl and ethyl acetate indicate aerobiosis in the raw material during the fermentation process or the result of an incorrect separation of the head fraction during distillation (*5*). In the BMD and BCD samples, methyl acetate was not detected, but the concentration of ethyl acetate was significantly higher ($p < 0.05$) in the BMD (144.7 g/hL aa, equivalent to 698.9 mg/L) than in BCD (7.7 g/hL aa, equivalent to 29.6 mg/L) (**Table 1**). This last compound is the major ester present in distillates from alcoholic beverages (*5*), although when its concentration exceeds the perception threshold of 33 mg/L, it will contribute nuances of glue and dissolvent to the distillates (*29*).

The concentration of ethyl lactate increases considerably in distillates from fruits that have been stored or fermented in inadequate conditions or ensilaged for a long time (*5*). The presence of ethyl lactate in distillates is related with the metabolic activity of lactic acid bacteria (*37*). Thus, its absence in the BCD and its presence at a low concentration in the BMD (0.3 g/hL aa) suggest that both the black mulberry and black currant pulps were fermented under favorable conditions and without intervention of unwanted lactic acid bacteria.

Acetaldehyde and Acetal. The presence of acetaldehyde in distillates is considered to be due to the yeast metabolism in alcoholic fermentation, because this compound is produced by decarboxylation of pyruvate, which is the final product of glycolysis (*38*). Its concentration increases during the distillation and aging of spirits (*8*). The mean concentrations of acetaldehyde detected in our samples (13.9 g/hL aa for BMD and 12.8 g/hL aa, for BCD) were not significantly different ($p < 0.05$). The presence of acetaldehyde at low concentrations in both distillates means that the nonthermally treated black mulberry and black currant pulps were fermented and distilled under favorable conditions and without intervention of unwanted bacteria (*8*, *10*). In addition, a low amount of acetal (0.3 g/hL aa) was detected in BCD; meanwhile, this compound was not detected in BMD (**Table 1**).

The total volatile compounds content in the BMD (347.6 g/hL aa) was higher than the minimum level of 200 g/hL aa fixed by the European Council Regulation 110/2008 (*15*) for fruit distillates. However, the black currant distillate had a total amount of volatile compounds (121.1 g/hL aa) lower than 200 g/hL aa. This last inconvenience suggests the need of adjusting the time for removal of head and tail fractions to achieve the desirable concentrations of volatile compounds in the heart fraction of the black currant distillate, without surpassing the maximum limits that legislation permits for the volatile compounds that can pose health hazards.

Many distillates from fruits of the forest are artisanally produced from local distillers by spontaneous or inoculated liquid fermentation of the fruit juices. This makes difficult the reproducibility of the fermentation process and, consequently, alcoholic beverages with different volatile compositions and qualities are produced. The results obtained in this paper showed the feasibility for obtaining, in a reproducible way, two distillates (BMD and BCD) that have their own distinctive characteristics and concentrations of volatile compounds with health hazards that are within the limits of acceptability fixed by the European Council (Regulation 110/2008) for fruit spirits. Thus, the better standardization of the process of production of distillates, including both the fermentation and distillation procedures, gives a more uniform quality for the two alcoholic beverages. However, the present study on the production and characterization of two fruit distillates is a first approach on a subject that requires further investigation.

Comparison of the Volatile Composition between the BMD and BCD Samples with Those of Other Spirits. To put our results in perspective, the volatile compositions of BMD and BCD were compared with those of four commercial Galician orujo spirits (*5*), Portuguese bagaceiras (*10*), which were elaborated in a traditional way from grape marc, and other two distillates obtained from fermented whey (*18*) and blackberry (*4*).

From a direct comparison, it is clear that our distillates and the other aforementioned alcoholic beverages (*4*, *5*, *10*, *18*) have different volatile compositions, probably due to the different raw materials, fermentation techniques, ethanol-producing strains, and distillation technologies used to obtain the different distillates. However, it is difficult to establish clear differences or similarities between the compositions of all these alcoholic beverages with a direct comparison, due to the high variability in the number and concentrations of volatile compounds present in them. Therefore, the data were processed by cluster analysis using the mean concentrations of their volatile compounds previously standardized as the classification variables. This approach allows grouping the above-mentioned alcoholic beverages with the most similar volatile compositions together.

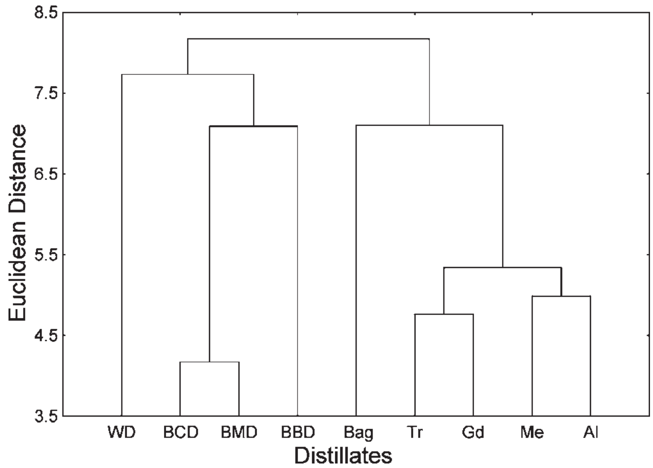

Figure 6. Average linkage cluster analysis of nine alcoholic beverages: four commercial Galician orujo spirits [Albariño (Al), Mencia (Me), Godello (Gd) and Treixadura (Tr)], Portuguese bagaceiras (Bag), and the distillates obtained from whey (WD), blackberry (BBD), black mulberry (BMD), and black currant (BCD). The standardized mean concentrations of the volatile compounds of each sample were used as classification variables.

The cluster analysis resulted in three well-defined groups of associations; the first group was formed by the BCD and BMD samples with the lowest Euclidean distance, a second group was formed by the Treixadura and Godello orujo spirits, and the third group includes the Mencia and Albariño orujo spirits (**Figure 6**). Then, the second and third groups merge to form a new cluster in which the four commercial Galician orujo spirits (*5*) are included. Subsequently, the bagaceiras (*10*) and the blackberry fruit distillate (BBD) were associated, respectively, with the group formed by the four orujo spirits and with the cluster formed by our two distillates, although with higher Euclidean distances (**Figure 6**). Finally, the whey distillate (WD) was grouped with the cluster formed by the fruit distillates BMD, BCD, and BBD.

Thus, the cluster analysis suggests that the raw material is the main factor to form the groups in comparison with the other factors, because the alcoholic beverages obtained from grape marc and those obtained from fruits (BMD, BCD, and BBD) formed two clearly separate clusters; meanwhile, the whey distillate was the most different. However, other aspects such as the storage system, storage time, and fermentation and distillation procedures must be taken into account to explain the intrinsic differences observed in each cluster formed.

ABBREVIATIONS USED

aa, absolute alcohol; RI, refractive index; $Y_{Et/RSc}$, ethanol yield from reducing sugars consumed; BMD, black mulberry distillate; BCD, black currant distillate; Al, Albariño; Me, Mencia; Gd, Godello; Tr, Treixadura; Bag, bagaceiras; WD, whey distillate; BBD, blackberry distillate.

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