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Production of spirits from dry apple pomace and selected yeasts

*Roberto Rodríguez Madrera**, *Rosa Pando Bedriñana*, *Ana García Hevia*, *Marcos Bueno Arce*, *Belén Suárez Valles*

Área de Tecnología de los Alimentos, Servicio Regional de Investigación y Desarrollo Agroalimentario (SERIDA), E-33300 Villaviciosa (Asturias), Spain

ABSTRACT

Apple pomace spirits were made from dry pomace and selected yeasts strains. Two strains of indigenous cider yeasts (*Saccharomyces cerevisiae* and *Hanseniaspora uvarum*) as well as a commercial enzyme combined with a wine dry yeast were tested. The differences between distillates were studied by means of chemical and sensory analysis. According to the results of this study, treatment with enzymes with pectin methylesterase activity led to excessive levels of distilled methanol, and hence it use is not advisable. In contrast, the indigenous yeasts produced lower concentrations of methanol. Moreover, using different yeast species allows the production of spirits with important differences in their aromatic composition, which is certainly interesting from a commercial point of view. Furthermore, the results showed that drying the apple pomace allows it to be preserved in good condition, thus solving the problem of the seasonality of the raw material for making apple pomace distillates.

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Keywords: Apple pomace; Fermentation; Distillation; Spirits; Selected yeasts; Volatile compounds

1. Introduction

Cider-making is one the main agrifood industries in Asturias (Northern of Spain), apple pomace (the solid residue generated after juice extraction) being its principal waste. Apple pomace is composed of peel, pulp and seeds, and represents about 25% of the processed apple.

Several uses for apple pomace have been proposed, such as the synthesis of pectolytic enzymes (Berovic and Ostroversnik, 1997), as a substrate for the production of fructofuranosidase (Hang and Woodams, 1995) or as a source of antioxidant polyphenols (Diñeiro García et al., 2009) or pectin (May, 1990).

Fruit pomaces are usually employed as the raw material in the production of spirits following traditional methods, which yields quality products of recognized prestige. Fruit marc spirits, as defined by the European Regulation, are drinks with an alcoholic strength higher than 37.5% (v/v), and a minimum quantity of volatile substances of 200 g per hectolitre of pure alcohol (EC 110/2008).

The making of pomace brandies basically consists in a silage stage to ferment the sugars present in the raw material and subsequent distillation. Fermentation is usually brought about spontaneously by indigenous microflora, avoiding contact of the pomace with air in containers with different capacities (40 kg to 4000 qt) and made of different materials (plastic, steel, wood or concrete) (Da Porto, 1998; De Rosa and Castagner, 1994; Silva et al., 2000). Occasionally, inappropriate conditions during fermentation and/or storage may encourage the growth of various microorganisms that give rise to organoleptic defects. Thus, in the case of grape pomace such as *orujo*, *tsipouro*, *marc*, *grappa* and *bagaceira*, all recognized by the European Community, the formation of 2-butanol and increased levels of 1-propanol, acetic acid and short chain fatty acids have been associated with bacterial spoilage (Cortés et al., 2009; Da Porto, 1998; Silva and Malcata, 1998). On the other hand, high levels of ethyl lactate, methyl acetate and ethyl acetate could be as a consequence of deficient and long storage periods under aerobic conditions (Cortés et al.,

* *Corresponding author*. Tel.: +34 985890066; fax: +34 985891854.
E-mail address: rrodriguez@serida.org (R. Rodríguez Madrera).
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2010). To avoid the proliferation of microorganisms that damage grape pomaces and bring about an increase in methanol content by the action of pectin methyl esterases, it has been recommended to avoid long storage periods, not exceeding one month (Cortés et al., 2006).

However, this is a difficult problem to solve due to the seasonality of the raw material (produced during the corresponding harvest season of each fruit), as it requires the availability of facilities with a large capacity for distillation. At the same time, the use of fruit pomace stored under suitable conditions is limited by its limited shelf life.

The practice sometimes employed to avoid the inherent problems of storage is to decrease the pH of the mass by adding an acid, which may be accompanied by the use of selected dry wine yeast to improve the chemical composition of the corresponding spirit (Da Porto, 1998; Silva et al., 2000; Silva and Malcata, 1998). Furthermore, the use of indigenous or local selected yeasts is believed to be more effective because these are better acclimated to local environmental conditions (Maqueda et al., 2011). Our group has conducted several research studies on indigenous microflora in cider making, thus facilitating the selection of local yeast strains characterized by their good aptitudes to carry out alcoholic fermentation and interesting enzymatic activities (Pando Bedriñana et al., 2011; Suárez et al., 2000; Suárez Valles et al., 2005, 2007, 2008).

The aim of this study was to make apple pomace spirit from dry pomace. We accordingly tested the ability of 2 strains of indigenous cider yeasts, characterized by their β-glucosidase activity, to bring about the alcoholic fermentation, as well as the use of a commercial enzyme combined with a wine dry yeast. To this end, chemical and sensory analyses were carried out.

2. Materials and methods

2.1. Apple pomace

The apple pomace used in this study came from industrial hydraulic presses with an operating capacity of 15,000 kg belonging to the same cellar in two consecutive harvests (2010 and 2011). Nine batches of 50 kg were taken during each harvest, each of which was dried in an oven with air circulation at 60 °C for 48 h. The different batches of dry pomace were homogenized and placed in 6.8 kg portions, keeping them in sealed bags to preserve them from moisture until the time of fermentation.

2.2. Batch cultivation

Three yeast strains were used in this study. A *Saccharomyces cerevisiae*, referred to as *S.c.* 3′, and a *Hanseniaspora uvarum*, referred to as *H.u.* 283, both belonging to the SERIDA (Asturias, Spain) collection of pure cultures, as well as a commercial wine yeast, *S. cerevisiae*, referred to as Levuline CHP (OenoFrance, France).

The indigenous strains, stored at −80 °C before use, were thawed. The *S.c.* 3′ strain was grown in liquid GPY medium (4% glucose, 0.5% peptone, 0.5% yeast extract) at 30 °C for 24 h. An aliquot of this culture was added at a concentration of 2% to 100 mL of liquid GPY medium and incubated for 16 h at 30 °C to obtain the inoculum used in the bioreactor. The *H.u.* 283 strain was grown on GPY agar and incubated at 30 °C for 48 h. Surface growth from one plate was transferred to 100 mL of liquid GPY medium and incubated for 72 h at 30 °C to obtain the inoculum used in the bioreactor.

To obtain enough biomass to be used in the fermentation of apple pomace, the inocula were transferred to a laboratory bioreactor (Biostat B Bonus, Sartorius) with a working volume of 2 L. Sterile apple juice (4° Brix) and silicone antifoam liquid to remove foaming were used for the batch fermentation. The cultivation conditions in the bioreactor for both yeast strains are shown in Table 1. Two batch tests were carried out for each strain. Yeast creams (10^9 cfu/ml) were stored at 4 °C before use.

The active dry yeast strain (Levuline CHP) was inoculated at 80 g/hL subsequent to rehydration in sterile water during 20 min, as per the supplier's instructions.

2.3. Fermentation

Four apple pomace fermentations were performed for each harvest. Three of them were conducted by inoculating pure cultures of yeast (*S.c.* 3′, *H.u.* 283 and Levuline CHP), while the fourth was carried out with Levuline plus a β-glucosidase enzyme at 10 g/hL (Enovin Varietal, Agrovin, Ciudad Real, Spain).

Each lot of 6.8 kg of dry pomace was rehydrated in 20 L of sterile water, adding the corresponding inoculum to this water.

Fermentations were performed in duplicate for each harvest and took place in 30 L capacity HDPE (high density polyethylene) tanks equipped with an air-lock at 16 ± 2 °C for 4 weeks. After this time, the fermented apple pomace was distilled.

2.4. Distillation

Spirits were obtained by double distillation. In the first distillation, the apple pomace from each experimental unit was distilled in a 50 L pot still in a pre-concentration step until obtaining an intermediate product with an alcoholic strength of 20–22% (v/v). This product was then distilled in a 5 L pot still to obtain a spirit with an approximate alcoholic strength of 60% (v/v).

2.5. Analysis of the apple pomace

2.5.1. Microbiological counting

Samples (10 g) were taken from each tank at the beginning and after 14 days of fermentation. The samples were homogenized with 90 mL of a Ringer serum for 2 min in a Masticator 0410 (IUL Instrument). The quantitative determinations of yeasts

Table 1 – Cultivation conditions in the bioreactor.

	Inoculum (%)	Agitation (rpm)	Temperature (°C)	pH	Air flow (ml/min)	Time (h)
S.c. 3′	2	300	30	5.5	3.5	24
H.u. 283	4	300	25	5.5	3.5	72

and lactic and acetic bacteria were carried out following the methodology described by Cabranes et al. (1996).

2.5.2. *Implantation capacity*

The implantation of the strains in the fermentations was evaluated 14 days after inoculation by analysis of 10 isolated colonies. The isolates were analyzed by mtDNA-RFLP (Querol et al., 1992) for the tanks inoculated with the *Saccharomyces* strains and by RAPD (Bujdosó et al., 2001) for the experimental units inoculated with the *H. uvarum* strain.

2.5.3. *Sugar content and alcoholic strength*

Sugar content and alcoholic strength were determined at 0, 7, 14, 21 and 28 days of fermentation by HPLC analysis (Blanco et al., 1988) and NIR (Alcoholyzer Wine, Anton Paar, GmgH, Graz, Austria), respectively, subsequent to extraction following Pina and Hogg (1999).

2.5.4. *Enzymatic assays*

Samples (50 g) were taken from each tank after 14 days of fermentation and homogenized in a Masticator 0410 (IUL Instrument) for 2 min. Aliquots of the liquid extracts were used to evaluate enzymatic activities according to the following methods.

2.5.4.1. *Pectin methylesterase activity.* Pectin methylesterase activity was evaluated in liquid medium containing 6.7 g/L yeast nitrogen base (Difco), 1% glucose, 1% apple pectin and 50 mM potassium phosphate buffer pH = 5.5. The medium was inoculated to 2% with extracts and incubated at 30 °C for 5 days. The methanol produced was measured by gas chromatography on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and an FFAP semi-capillary column (30 m × 0.53 mm i.d.; phase thickness, 1.0 mm) supplied by Teknokroma (Barcelona, Spain). Injection was carried out in splitless mode (1 min) employing helium as the carrier gas at 10 mL/min. The temperature gradient was as follow: 40 °C isotherm for 5 min, followed by a linear increase of 4 °C/min until 60 °C. This temperature was then raised to 220 °C at a rate of 10 °C/min. Injector and detector temperatures were 240 °C and 275 °C, respectively. The microfiltered samples were directly injected into the chromatograph (1 mL).

2.5.4.2. *β-Glucosidase activity.* β-Glucosidase activity was evaluated in agar slant tubes using arbutin as the substrate following the methodology described by Pando Bedriñana et al. (2011). The basal medium composition was 5 g/L arbutin, 1 g/L yeast extract and 20 g/L agar. Each tube contained 7 mL basal medium and 0.1 mL iron chloride (1%). Tubes were incubated for 15 days at 28 °C. The extracts with this enzymatic activity formed a dark halo around the slant. Pure β-glucosidase enzyme (EC. 3.2.1.21, Sigma, St. Louis, MO) was used as the positive control.

2.5.4.3. *β-Xylosidase activity.* β-Xylosidase activity was evaluated on agar plates containing 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate (Difco), 5 g/L ammonium sulphate, 5 g/L D-xylose and 20 g/L agar (pH = 5.5) (Manzanares et al., 1999). Three hundred microliters of 4-methylumbelliferyl-b-Dxyloside (Sigma, USA) were spread onto the surface of the agar plates, after which the yeasts were inoculated and the plates were incubated at 25 °C for 24 h. Enzymatic activity was visualized under UV illumination as fluorescent halos surrounding yeast growth. Two *H. uvarum* strains obtained from the Spanish Type Culture Collection (CECT 11105 and CECT 11106) were used as the positive control.

2.5.4.4. *α-L-Arabinofuranosidase activity.* α-L-Arabinofuranosidase activity was assayed according to the method described by Nurcholis et al. (2012) based on the hydrolysis of *p*-nitrophenyl-α-L-arabinofuranoside (Aldrich, USA). The assay contained 175 μL of sample, 175 μL of 50 mM citric acid-$NaHPO_4$ buffer (pH 6.0) containing 1 mM of pNP-A. Mixtures were incubated at 40 °C for 20 min and the reaction was stopped by the addition of 700 μL of 1 M Na_2CO_3. The presence of yellow colouring indicates the release of p-nitrophenol, confirming α-L-arabinofuranosidase activity.

2.6. *Analysis of spirits*

2.6.1. *Alcoholic strength*

Alcoholic strength was determined by direct measurement in an Anton Paar DMA 500 Density Meter (Graz, Austria).

2.6.2. *Volatile acidity and pH*

Volatile acidity and pH were determined following the methodology described in the Official Analytical Methods of the AOAC (1984).

2.6.3. *Volatile compounds*

Analyses were carried out on the same equipment: an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detection system (FID), a 7673 autosampler, a split/splitless injector and GC Chemstation software (version A.09.03). Separations were carried out on a Meta.WAX capillary column (100% polyethylenglycol, 30 m × 0.25 mm; phase thickness 0.5 μm) supplied by Teknokroma (Barcelona, Spain).

The spirits were analyzed for major and minor volatile compounds according to the method previously validated by our group for the analysis of volatile compounds in spirits (Rodríguez Madrera and Suárez Valles, 2007). Prior to GC analysis, samples were filtered through a 0.22 μm PVDF (polyvinylidene difluoride) membrane filter supplied by Teknokroma (Barcelona, Spain).

2.6.3.1. *Chromatographic conditions for major volatiles.* Oven temperature: initial isotherm at 60 °C (10 min), raised to 70 °C at a rate of 8 °C/min and finally raised to 220 °C at a rate of 15 °C/min, and final isotherm of 220 °C (15 min). Flow conditions: initial flow 0.6 mL/min (5.2 min) raised to 1.5 mL/min at a rate of 3 mL/min each min, this flow was maintained during 10 min and finally raised to 2.5 mL/min at a rate of 1 mL/min each min. Injector temperature: 260 °C; detector temperature: 275 °C; injection volume: 1 μL; split ratio: 1/20.

2.6.3.2. *Chromatographic conditions for minor volatiles.* Oven temperature: initial isotherm at 35 °C (5 min), raised to 60 °C at a rate of 5 °C/min, raised to 90 °C at a rate of 10 °C/min and finally raised to 220 °C at a rate of 8 °C/min, and final isotherm of 220 °C (10 min). Flow conditions: 0.8 mL/min (constant flow). Injector temperature: 260 °C; detector temperature: 275 °C; injection volume: 1 μL; split ratio: 1/3.

All analyses in apple pomace and spirits were carried out in duplicate.

2.7. *Sensory analysis*

A panel of 40 consumers in 2010 (22 males and 18 females with ages ranging between 22 and 62 years) and 41 in 2011 (24 males and 17 females with ages ranging between 24 and 58 years) participated disinterestedly in this experiment. The tasters worked individually in separate booths. All the spirits evaluated by the consumers were the mixture of the two duplicate distillates under study. Each spirit was evaluated at 20 °C, previously diluted to 40% (v/v) alcoholic strength, employing grappa-type glasses (small tulip shaped glasses with a long straight body) covered with watch-glasses.

In each session, the consumers evaluated the 4 samples from each harvest. The samples were evaluated on a 9-point hedonic scale (1: very deficient; 5: correct; 9: excellent).

2.8. *Statistical methods*

A one-way analysis of variance (ANOVA) was performed to detect significant differences for volatile compounds and consumer preferences depending on the inoculated yeast strain. Duncan's test was carried out to detect significant differences for chemical variables depending on the inoculated yeast strain. Principal components analysis (PCA) was used to reduce the dimensionality of the data matrix and to establish relationships between the spirits and their chemical composition. The program used was SPSS version 11.5.

3. Results and discussion

3.1. *Apple pomace fermentation*

The sugar content of the apple pomaces ranged between 36.6 g/L for the first year and 42.6 for the second, with similar ethanol yields at the end of alcoholic fermentation in all cases, presenting mean values of 2.8% (v/w) (Fig. 1). Microbiological analysis showed yeast concentrations above 10^6 cfu/ml and a degree of implantation of the inoculated strains equal to or higher than 90% in all cases (data not shown). Although all the strains showed similar fermentation kinetics (Fig. 1), it is worth to note that the fermentation with Levuline and the enzyme took place before 14 days, which could be relevant from the industrial perspective. Moreover, it was observed that under the conditions employed in our study, in which ethanol production is not high, the *H.u.* 283 strain was able to complete the consumption of sugars, with a production of ethanol equivalent to those detected in the experimental units fermented with *Saccharomyces* strains. On the other hand, the concentrations of lactic and acetic bacteria were lower than 10^3 cfu/ml in all cases.

Table 2 – Enzymatic activities detected in the apple pomace fermentations.

	β-Glu	PME	α-ARAF	β-Xyl
Levuline	–	–	–	+
Levuline + enzyme	+	+	+	+
H.u. 283	+	–	–	–
S.c. 3′	+	–	–	–
Enzyme ENOVIN	+	+	+	–

β-Glu: β-glucosidase; PME: pectin methylesterase; α-ARAF: α-L-arabinofuranosidase; β-Xyl: β-xylosidase; +: activity; –: not activity.

Table 2 summarizes the enzymatic activities detected. The commercial enzyme not only showed β-glucosidase activity, as expected, but also pectin methylesterase and α-L-arabinofuranosidase activities. In this regard, Wightman et al. (1997) have pointed out that the use of oenological enzymes of fungal origin may lead to the unexpected appearance of

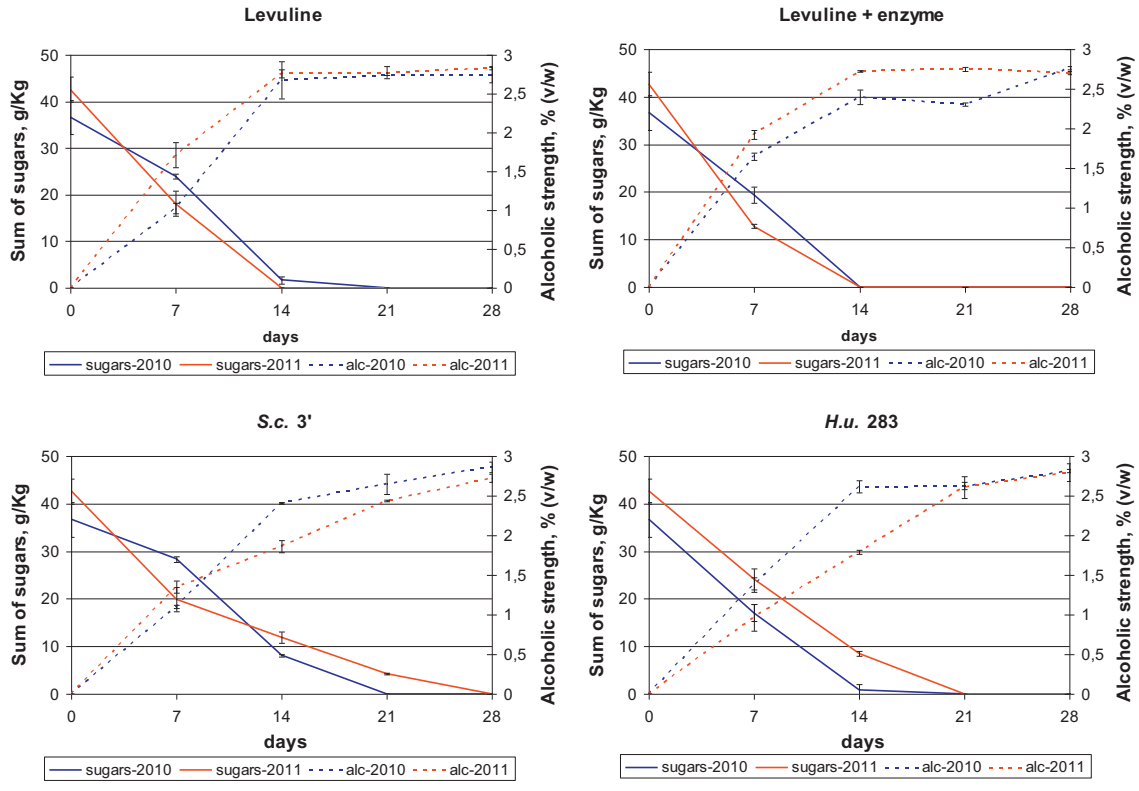

Fig. 1 – Evolution of the content in sugars (sum of sucrose, glucose and fructose) and alcoholic strength during fermentation of apple pomaces (mean values of two experimental units and standard deviations).

Table 3 – Volatile compounds (mean ± SD) in apple pomace spirits. Values in harvests 2010 and 2011 (expressed in g/hL of absolute alcohol) are the mean of two experimental units.

	Harvest 2010				Harvest 2011				Mean*			
	Levuline	Levuline + enzyme	*S.c.* 3′	*H.u.* 283	Levuline	Levuline + enzyme	*S.c.* 3′	*H.u.* 283	Levuline	Levuline + enzyme	*S.c.* 3′	*H.u.* 283
Alcohols												
Metanol	1346.49 ± 76.28	2898.14 ± 1.33	727.59 ± 5.03	766.53 ± 50.97	818.45 ± 36.95	2244.99 ± 65.91	774.07 ± 1.34	773.98 ± 10.81	1082.47b	2571.56a	750.83c	770.26c
2-Butanol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Propanol1	45.91 ± 4.86	47.59 ± 1.79	17.05 ± 1.82	33.23 ± 0.06	29.26 ± 0.14	28.47 ± 0.97	18.56 ± 0.27	31.25 ± 1.14	37.58a	38.03a	17.81c	32.24b
2-Methyl-1-propanol1	70.83 ± 14.80	63.21 ± 1.52	63.45 ± 0.90	36.22 ± 0.93	43.39 ± ± 1.18	41.07 ± 2.64	61.80 ± 0.57	36.53 ± 2.16	57.11ab	52.46b	62.62a	36.37c
2-Propen-1-ol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-Butanol1	1.34 ± 0.26	1.83 ± 0.06	1.48 ± 0.01	1.11 ± 0.02	0.88 ± 0.01	1.28 ± 0.04	1.24 ± 0.04	0.86 ± 0.01	1.11c	1.56a	1.36b	0.99c
2-Methyl-1-butanol1	85.15 ± ± 17.65	78.17 ± 1.70	54.54 ± 0.04	38.94 ± 1.43	52.22 ± ± 2.03	51.45 ± 2.99	50.99 ± 0.10	35.26 ± 2.20	68.69a	64.81a	52.76b	37.1c
3-Methyl-1-butanol1	233.88 ± 47.33	201.48 ± 5.01	368.9 ± 11.88	192.29 ± 6.44	190.45 ± 7.32	180.26 ± 10.25	345.36 ± 3.29	154.67 ± 8.7	212.16b	190.87bc	357.13a	173.48c
1-Hexanol1	3.03 ± 0.65	3.55 ± 0.05	2.72 ± 0.10	2.80 ± 0.17	2.03 ± 0.06	2.63 ± 0.14	2.07 ± 0.02	1.95 ± 0.09	2.53b	3.09a	2.40b	2.37b
2-Phenylethanol1	0.52 ± 0.03	0.56 ± 0.02	1.03 ± 0.11	0.69 ± 0.10	0.79 ± 0.16	0.73 ± 0.03	1.29 ± 0.02	0.65 ± 0.18	0.65	0.64	1.16a	0.67
1-Pentanol	0.52 ± 0.10	0.51 ± 0.02	0.55 ± 0.03	0.56 ± 0.04	0.52 ± 0.02	0.55 ± 0.02	0.59 ± 0.01	0.56 ± 0.04	0.52	0.53	0.57	0.56
3-Methyl-1-pentanol	0.08 ± 0.01	0.08 ± 0.01	0.58 ± 0.03	n.d.	n.d.	0.12 ± 0.00	0.52 ± 0.01	n.d.	0.04c	0.10b	0.55a	n.d.
1-Heptanol	0.33 ± 0.08	0.30 ± 0.01	0.29 ± 0.02	0.30 ± 0.02	0.29 ± 0.01	0.44 ± 0.21	0.27 ± 0.01	0.27 ± 0.02	0.31	0.37	0.28	0.29
1-Octanol	0.38 ± 0.04	0.35 ± 0.00	0.36 ± 0.00	0.36 ± 0.01	0.36 ± 0.00	0.39 ± 0.00	0.37 ± 0.01	0.39 ± 0.00	0.37	0.37	0.37	0.37
6-Methyl-5-heptanol	0.14 ± 0.00	0.08 ± 0.01	0.16 ± 0.00	0.32 ± 0.02	0.07 ± 0.00	0.06 ± 0.02	0.08 ± 0.01	0.23 ± 0.02	0.11b	0.07c	0.12b	0.28a
1-nonanol	0.64 ± 0.11	0.61 ± 0.00	0.48 ± 0.06	0.72 ± 0.04	0.25 ± 0.01	0.47 ± 0.04	0.18 ± 0.00	0.20 ± 0.02	0.45a	0.54a	0.33c	0.46ab
Benzol	0.05 ± 0.03	0.06 ± 0.01	0.03 ± 0.02	0.12 ± 0.05	0.13 ± 0.04	0.16 ± 0.01	0.11 ± 0.00	0.17 ± 0.04	0.09b	0.11ab	0.07b	0.15a
Sum of higher alcohols	440.66	396.39	509.17	305.28	319.25	306.74	481.56	261.43	379.96	351.56	495.37	283.35
Aldehydes,ketones and acetals												
Acetaldehyde	58.37 ± 0.73	62.18 ± 3.78	95.00 ± 10.04	68.74 ± 7.88	173.26 ± 46.72	125.42 ± 32.8	132.71 ± 24.82	81.48 ± 49.18	115.81	93.80	113.86	75.11
Acetal	4.53 ± 0.97	4.57 ± 0.18	7.91 ± 0.22	5.48 ± 0.33	16.21 ± 1.14	8.88 ± 2.17	27.94 ± 14.44	11.95 ± 5.14	10.37	6.72	17.93	8.72
Furfural	14.98 ± 2.52	35.08 ± 1.60	7.57 ± 0.40	5.55 ± 1.34	14.86 ± 2.48	24.83 ± 5.23	9.30 ± 0.77	10.14 ± 1.77	14.92b	29.95a	8.43c	7.85c
Benzaldehyde	11.57 ± 0.23	10.23 ± 0.97	9.90 ± 0.19	12.24 ± 0.64	10.99 ± 0.97	10.98 ± 1.52	13.04 ± 0.88	13.13 ± 0.57	11.28	10.61	11.47	12.69
2-Heptenal	0.28 ± 0.05	0.25 ± 0.01	0.27 ± 0.01	0.24 ± 0.02	0.25 ± 0.01	0.26 ± 0.02	0.31 ± 0.01	0.26 ± 0.01	0.26	0.25	0.29	0.25
6-Methyl-5-heptenone	0.50 ± 0.11	0.50 ± 0.01	0.38 ± 0.01	0.23 ± 0.01	0.34 ± 0.02	0.36 ± 0.01	0.37 ± 0.01	0.21 ± 0.02	0.42	0.43	0.38	0.22
Hexanal	0.94 ± 0.11	0.85 ± 0.09	0.90 ± 0.07	0.86 ± 0.05	0.81 ± 0.05	0.88 ± 0.02	0.91 ± 0.02	0.88 ± 0.03	0.87	0.86	0.91	0.87
Esters												
Methyl acetate	4.10 ± 0.39	7.94 ± 0.17	3.23 ± 0.24	10.03 ± 0.63	5.07 ± 0.03	8.19 ± 1.36	6.60 ± 0.75	14.59 ± 6.04	4.59b	8.06b	4.92b	12.31a
Ethyl acetate	52.62 ± 10.2	49.87 ± 2.31	72.14 ± 29.76	372.41 ± 33.97	32.49 ± 3.68	32.90 ± 0.99	109.77 ± 3.05	426.76 ± 112.48	42.56b	41.38b	90.95b	399.58a
3-Methyl-1-butyl acetate	0.41 ± 0.11	0.42 ± 0.00	0.70 ± 0.13	1.05 ± 0.15	0.26 ± 0.00	0.24 ± 0.00	0.98 ± 0.22	1.03 ± 0.05	0.33c	0.33c	0.84b	1.04a
2-Phenylethyl acetate	0.03 ± 0.00	0.03 ± 0.00	0.08 ± 0.02	0.12 ± 0.00	0.09 ± 0.03	0.03 ± 0.00	0.51 ± 0.20	0.45 ± 0.10	0.06b	0.03b	0.30a	0.29a
Ethyl 2-methylbutyrate	0.34 ± 0.11	0.29 ± 0.01	0.39 ± 0.04	0.53 ± 0.14	0.30 ± 0.02	0.22 ± 0.01	0.22 ± 0.02	0.28 ± 0.05	0.32ab	0.25b	0.30ab	0.40a
Ethyl hexanoate[a]	0.85 ± 0.24	0.83 ± 0.06	1.67 ± 0.02	0.34 ± 0.06	0.66 ± 0.04	0.61 ± 0.01	1.53 ± 0.08	0.25 ± 0.01	0.75b	0.72b	1.60a	0.30c
Ethyl octanoate[a]	4.76 ± 1.11	5.13 ± 0.15	7.98 ± 0.14	0.55 ± 0.04	3.15 ± 0.13	3.46 ± 0.04	6.81 ± 0.17	0.54 ± 0.04	3.96b	4.30b	7.39a	0.54c
Ethyl decanonate[a]	3.78 ± 0.64	4.47 ± 0.36	6.50 ± 0.29	0.65 ± 0.19	2.99 ± 0.22	3.48 ± 0.01	3.59 ± 0.62	0.43 ± 0.04	3.38b	3.97b	5.04a	0.54c
Ethyl dodecanoate[a]	0.83 ± 0.12	1.28 ± 0.18	1.30 ± 0.30	0.80 ± 0.12	0.75 ± 0.01	0.96 ± 0.00	0.66 ± 0.20	0.57 ± 0.09	0.79bc	1.12a	0.98ab	0.68c
Ethyl tetradecanoate[a]	0.44 ± 0.08	0.48 ± 0.23	0.39 ± 0.06	0.40 ± 0.06	0.25 ± 0.06	0.29 ± 0.06	0.25 ± 0.05	0.26 ± 0.02	0.35	0.38	0.32	0.33
Ethyl hexadecanoate[a]	1.16 ± 0.06	2.85 ± 0.51	2.31 ± 0.03	2.70 ± 0.85	2.05 ± 0.59	2.79 ± 0.15	1.67 ± 0.32	1.69 ± 0.36	1.60b	2.82a	1.99b	2.19ab
Ethyl linoleate	1.28 ± 0.34	3.54 ± 0.91	2.92 ± 0.09	3.93 ± 1.32	2.58 ± 0.56	3.48 ± 0.30	1.37 ± 0.59	1.98 ± 0.33	1.93b	3.51a	2.15b	2.95ab
Ethyl benzoate	0.90 ± 0.19	1.01 ± 0.03	0.93 ± 0.02	1.04 ± 0.11	0.55 ± 0.03	0.95 ± 0.06	0.41 ± 0.00	0.47 ± 0.04	0.73b	0.98a	0.67b	0.75b

Table 3 (Continued)

	Harvest 2010				Harvest 2011				Mean*			
	Levuline	Levuline + enzyme	S.c. 3′	H.u. 283	Levuline	Levuline + enzyme	S.c. 3′	H.u. 283	Levuline	Levuline + enzyme	S.c. 3′	H.u. 283
Ethyl lactate	1.68 ± 0.02	1.71 ± 0.02	2.46 ± 0.11	5.59 ± 0.47	1.70 ± 0.32	1.74 ± 0.05	1.09 ± 0.05	1.31 ± 0.15	1.69b	1.73b	1.77b	3.45a
Methyl octanoate	0.12 ± 0.04	0.31 ± 0.03	0.13 ± 0.00	n.d.	n.d.	0.18 ± 0.01	0.10 ± 0.00	n.d.	0.06c	0.24a	0.11b	n.d.
Methyl decanoate	0.07 ± 0.02	0.25 ± 0.04	0.06 ± 0.00	n.d.	n.d.	0.11 ± 0.01	0.03 ± 0.00	n.d.	0.03b	0.18a	0.05b	n.d.
3-Methyl-1-butyl octanoate	0.10 ± 0.03	0.12 ± 0.00	0.39 ± 0.05	0.03 ± 0.01	0.06 ± 0.00	0.08 ± 0.00	0.25 ± 0.06	0.12 ± 0.05	0.08b	0.10b	0.32a	0.07b
Sum of fatty acids ethyl esters	11.82	15.04	20.15	5.44	9.85	11.59	14.51	3.74	10.83	13.31	17.32	4.58
Sesquiterpenes												
Farnesol isomer	1.09 ± 0.05	1.84 ± 0.22	1.52 ± 0.14	1.54 ± 0.13	1.32 ± 0.12	1.78 ± 0.03	1.15 ± 0.12	1.46 ± 0.09	1.20c	1.81a	1.34bc	1.50b
Farnesene isomer	0.46 ± 0.12	0.45 ± 0.02	0.61 ± 0.07	0.41 ± 0.02	0.23 ± 0.03	0.29 ± 0.01	0.28 ± 0.13	0.39 ± 0.14	0.35	0.37	0.44	0.40
Nerolidol isomer	0.78 ± 0.11	1.40 ± 0.19	0.85 ± 0.15	0.78 ± 0.10	0.89 ± 0.06	1.43 ± 0.05	0.81 ± 0.15	0.91 ± 0.08	0.84b	1.42a	0.83b	0.85b

[a] Included in sum of fatty acids ethyl esters.
* Different letters mean significant differences at $p < 0.05$.

enzymatic activities due to inadequate purification. On the other hand, both indigenous yeasts showed β-glucosidase activity, while β-xylosidase activity was only detected in the assays with the Levuline strain.

3.2. *Apple pomace spirits*

The spirits did not show significant differences in alcoholic strength, with a mean value of 60.0 ± 0.53% (v/v) for all spirits, in keeping with the alcoholic strength of the fermented apple pomace and the use of the same equipment and procedure for distillation. Likewise, the contents in ethanol fall within the limits established in the European Regulation (EC 110/2008), between 37.5% (v/v) and 86% (v/v). Furthermore, no significant differences in pH or volatile acidity were detected, with respective mean values of 5.27 ± 0.07 and 4.08 ± 0.03 g acetic acid/hL of absolute alcohol (AA).

Significant differences were detected, however, in the content in methanol (Table 3), a substance originating from the demethylation of the pectins in the raw material whose ingestion in large quantities is poisonous to the central nervous system and can cause blindness, coma or even death. Apple pomace fermentations showed higher concentrations of methanol when the enzymatic preparation was employed, being above the permissible limits of 1500 g/hL AA established by the European Regulation (EC 110/2008). This fact can be explained by the pectin methylesterase activity detected in the fermentation with the commercial enzyme (Table 2). In any case, it should be noted that a careful distillation could reduce the methanol content, although decreasing the alcoholic yield.

Quantitatively, higher alcohols (1-propanol, 2-methyl-1-propanol, 1-butanol, 2-butanol, 1-hexanol, 2-propenol, 2-methyl-1-butanol, 3-methyl-1-butanol, 1-hexanol and 2-phenylethanol) constitute the main group of components in the distillates. As can be seen in Table 3, all the spirits had higher alcohols above 200 g/hL AA, the minimum content required by the European Regulation, with values ranging between 261 g/hL AA and 509 g/hL AA for *H.u.* 283 and *S.c.* 3′, respectively. These differences are in keeping with the lower levels reported for the genus *Hanseniaspora* in other papers (Moreira et al., 2008; Satora and Tuszynski, 2010). This fact could be important because although the higher alcohols are considered an important part of the aroma of alcoholic beverages, some authors note that higher alcohols could have a negative effect when present in excess (Romano et al., 2006). Quantitatively, the sum of 1-propanol, 2-methyl-propanol and amyl alcohols (2-methyl-1-butanol and 3-methyl-1-butanol) accounted for more than 98% of the higher alcohols in all spirits; 3-methyl-1-butanol and 2-methyl-propanol were higher in the distillates obtained with the strain *S.c.* 3′, and 1-propanol and 2-methyl-1-butanol were higher in the distillates produced with Levuline. On the other hand, a higher concentration of 1-hexanol, a varietal and prefermentative aroma linked to 1-butanol (Rodríguez Madrera and Mangas Alonso, 2005), was detected in the spirits from apple pomace subject to enzymatic treatment as a result of hydrolysis of glycosidic precursors (Schwab and Schreier, 1990). Furthermore, the absence of 2-propen-1-ol and 2-butanol, two volatiles associated with raw material spoilage and unpleasant flavours in the final product (Christoph and Bauer-Christoph, 2007; Da Porto, 1998; López-Vázquez et al., 2012) is worth noting. The only alcohol found in more abundance in the distillates obtained from *H.u.* 283 was 6-methyl-5-heptanol. This could be due to a higher capacity of this species to reduce

6-methyl-5-heptenone, which is less abundant in these spirits. On the other hand, 6-methyl-5-heptenone could be formed during apple pomace drying by oxidation of α-farnesene (Whitaker and Saftner, 2000).

Carbonylic compounds can be responsible for sharp and aggressive flavours. Acetaldehyde is often the most abundant aldehyde in distillates, having its origin both in the fermentation and in the distillation (Rodríguez Madrera et al., 2006). Levels of this volatile and its derivative, acetal, did not show significant differences (Table 3), the concentrations of both compounds being similar to reported values in different spirits (López-Vázquez et al., 2010, 2012; Soufleros et al., 2004). Similarly, significant differences were not detected in the contents of benzaldehyde, a major aldehyde in the analyzed distillates as a result of hydrolysis of the amygdalin present in the seeds forming part of the apple pomace. The only carbonylic compound for which significant differences were detected depending on the inoculated yeast was furfural (Table 3), an aldehyde imparting a caramel aroma. The highest concentrations were detected in the fermentation with Levuline plus the enzymatic preparation, followed by Levuline and, finally, the two cider yeasts. These differences could be explained on the basis of the hydrolysis (acid and/or enzymatic) of the major polymers present in apple pomace, such as pectin or hemicelluloses. The degradation of these polymers explains the presence of non-fermentable pentoses such as arabinose and xylose (furfural precursors), their subsequent thermal degradation during distillation yielding the furfural detected in the spirits (Rodríguez Madrera et al., 2003). As shown in Table 1, α-L-arabinofuranosidase activity was detected when the ENOVIN enzyme was used, while β-xylosidase activity was detected in both fermentations employing Levuline, which explains the higher levels of furfural in their corresponding distillates.

Esters are undoubtedly one of the most relevant groups in alcoholic beverages on account of their positive impact on aroma. Significant differences were detected in the content of these flavours for the different strains employed (Table 3). On the one hand, the fatty acid ethyl esters (hexanoic, octanoic, decanoic and dodecanoic acids) were more abundant in the spirits obtained from the *Saccharomyces* strains, especially the indigenous strain S.c. 3′. In contrast, the strain *H.u.* 283 provided spirits with a higher content in acetic acid esters (methyl, ethyl, 3-methyl-1-butyl and 2-phenylethyl acetates). The high values of ethyl acetate in distillates from the fermentation with *H.u.* 283, about 10 times higher than for the fermentations with Levuline, are worth noting. These results are in agreement with those reported by other authors (Moreira et al., 2008; Rojas et al., 2001) showing an increased production of acetic acid esters in different strains of *Hanseniaspora*, as opposed to *Saccharomyces* yeasts, the largest producers of fatty acid ethyl esters. On the other hand, the levels of ethyl lactate detected in all the spirits can be regarded as normal taking into account the values reported in apple and cider distillates (Versini et al., 2009; Rodríguez Madrera et al., 2010). Furthermore, the use of Levuline plus the enzyme resulted in a higher content in methyl esters of octanoic and decanoic acids, which could be justified by the higher levels of methanol in these experimental units.

Three sesquiterpenes were detected in the spirits (farnesene, farnesol and nerolidol), all of which were characterized by their aromatic impact. As shown in Table 3, the higher concentrations of nerolidol and farnesol isomers detected in the spirits obtained from Levuline together with the enzymatic preparation confirm the greater efficiency in hydrolyzing their precursors in apples.

Table 4 – Loadings of variables on PC 1 and PC2.

	PC 1	PC 2
Total acetaldehyde	−0.014	−0.312
Ethyl acetate	**−0.779**	0.545
Methanol	**0.859**	0.444
Higher alcohols	0.301	**−0.770**
Furfural	**0.868**	0.383
Ethyl esters of fatty acids	**0.647**	**−0.600**
Sum of acetates	−0.448	**0.752**
Methyl esters of fatty acids	**0.919**	0.170
Farnesol	0.480	**0.689**
Nerolidol	**0.776**	0.551
6-Methyl-5-heptanol	**−0.761**	0.330

The most relevant variables in each PC are highlighted in bold.

To facilitate the visualization of results, a principal component analysis (PCA) was carried out. First, some variables with the same technological or sensorial interest were grouped together. The sum of the alcohols: 1-propanol, 1-butanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 1-hexanol and 2-phenylethanol, was thus replaced by the variable *higher alcohols*; the sum (acetaldehyde + 0.37 × acetal) by the variable *total acetaldehyde*; the sum of the ethyl esters: hexanoate, octanoate, decanoate, dodecanoate, tetradecanoate and hexadecanoate, by the variable *ethyl esters of fatty acids*; the sum of methyl octanoate and methyl decanoate by the variable *methyl esters of fatty acids*; and the sum of methyl acetate, 3-methyl-1-butyl acetate and 2-phenylethyl acetate by the variable *sum of acetates*. Those variables which were highly correlated ($r > 0.7$; $p < 0.001$) but for which no significant differences were detected were then subsequently removed. The database was finally constituted by 11 new variables (Table 4) and 16 objects. A PCA was performed on this database, choosing three principal components (PC) with eigenvalues >1 which accounted for 87.8% of the variance.

Fig. 2 shows the projection of distillates onto the first and second axes, allowing three groupings. On the left of the graph, related to negative values of PC 1 (ethyl acetate and 6-methyl-5-heptanol, Table 4), are situated spirits made from apple pomace fermented with *H.u.* 283, while distillates made with Levuline plus the enzymatic preparation are situated in the positive region of this axis (high values of *methyl esters of fatty acids*, furfural, methanol, nerolidol and *ethyl esters of fatty acids*, Table 4), with the distillates from the *S. cerevisiae* strains in between the two groups. In turn, the set constituted by the distillates made with Levuline and S.c. 3′ can be split into two along the axe of PC2, both on the negative region. The distillates elaborated with the S.c. 3′, characterized by the highest levels of ethyl esters of fatty acids and higher alcohols are displayed below those from the Levuline strain. Likewise, the distillates from *H. uvarum* 283 and Levuline plus enzyme are

Table 5 – Results from hedonic valuations of the spirits by a panel of consumers (mean values ± standard deviation).

	Harvest 2010 ($n = 40$)	Harvest 2011 ($n = 41$)
Levuline	5.6 ± 1.6	5.5 ± 1.6
Levuline + enzyme	5.4 ± 1.4	5.3 ± 1.5
S.c. 3′	5.4 ± 2.1	5.4 ± 1.7
H.u. 283	5.0 ± 2.1	5.1 ± 1.8

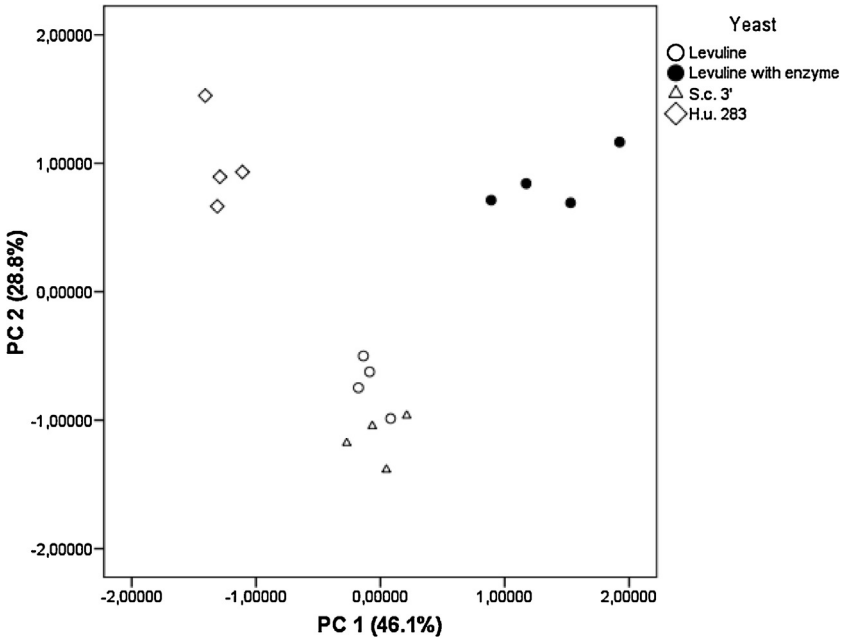

Fig. 2 – Projection of the spirits onto the plane formed by principal components 1 and 2.

located in the positive region of PC 2, associated with higher values of the sum of acetates and farnesol.

A sensory analysis was carried out to provide initial information on the acceptance of the apple pomace spirits obtained. Table 5 shows the results corresponding to hedonic scores of the spirits by a panel of consumers. As can be seen, the mean values were equal to or higher than 5 points (defined as '*correct*') in all the cases, with no significant differences between the spirits, presenting mean values ranging from 5.0 (*H.u. 283*, harvest 2010) to 5.6 (Levuline, harvest 2010). Furthermore, the scores for distillates made with each yeast were similar for both years. Thus, the method employed to process and preserve the apple pomace as well as the selected yeasts used to ferment it permit both the reproducibility of the final product and its acceptance by consumers.

4. Conclusions

In summary, the results of this study showed that drying apple pomace enables its preservation in good condition, thereby solving the problem of the seasonality of the raw material used to produce apple pomace distillates. Moreover, using different yeast species permits the production of spirits with important differences in aromatic composition, which is certainly interesting from a commercial point of view. Furthermore, none of the spirits showed detectable levels of 2-propen-1-ol or 2-butanol, associated with bacterial spoilage of the raw material and aromatic defects in the distillates. According to the results of this work, treatment with enzymes with pectin methylesterase activity led to excessive levels of distilled methanol and hence its use is not advisable. In contrast, the indigenous yeasts employed in this study yielded the lowest concentrations of methanol.

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