Food Bioscience 41 (2021) 100887

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Antioxidant activity and phenolic profiles of ciders from the Basque Country

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ARTICLE INFO

Keywords:
Cider
Polyphenolic compounds
Antioxidant activity
Browning

ABSTRACT

The contribution of the individual polyphenols of Basque cider to the antioxidant activity and browning has been determined. Nineteen monovarietal musts were obtained using different varieties of cider apples throughout three seasons. Fermentation of these musts was monitored throughout 4 or 5 samplings, until their transformation into cider. Partial least squares regression was done to relate individual polyphenols with the parameters studied. The polyphenols which contributed most to the antioxidant activity were chlorogenic acid, 4-*p*-coumaroylquinic acid, (−)-epicatechin, phloretin 2′-*O*-xyloglucoside and isoquercitrine. The main contributors to browning were 4-*p*-coumaroylquinic acid and phloretin 2′-*O*-xyloglucoside. The antioxidant activity of Basque ciders was compared with that of other beverages, concluding that it was similar to that of red wines and greater than that of orange juices.

1. Introduction

Antioxidant compounds like phenols and flavonoids can prevent cancers and heart diseases to a certain extent by reducing or inhibiting the adverse effects of free radicals. These radicals are reactive and can attack biologically-relevant molecules, such as DNA and RNA, leading to cell damage. Consequently, there has been an increasing interest in determining the antioxidant potential of natural products such as phenols (Cerit et al., 2017; Parsons, 2017).

Cider is a beverage rich in polyphenols, which are important from the sensory quality point of view (Lea & Drilleau, 2003; Rodríguez et al., 2006). Even though they are natural antioxidants, no study has been done on the antioxidant activity of polyphenols of the ciders of the Basque Country (northern Spain), except one preliminary study (Zuriarrain et al., 2015). The different polyphenols present in ciders have different chemical properties, and the present study is focused on determining the contribution of the individual polyphenols to the antioxidant activity and browning. Polyphenols affect these parameters, which may have an effect on the taste, the visual aspect, and the preservation of ciders.

The antioxidant activity can be measured using many methods (Sochor et al., 2010), and the common trend is to use several of them together. The following have been chosen: FRAP assay (ferric reducing antioxidant power), ABTS assay (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) and FC assay (Folin-Ciocalteu). The FC assay is generally used as a measure of total polyphenol concentration in natural products, but its mechanism is an oxidation-reduction reaction, so it can be considered as another method for measuring the antioxidant activity.

These three assays have been chosen because they work using different mechanisms. FRAP and FC use an electron transfer mechanism, while the ABTS method uses both hydrogen atom transfer and single electron transfer mechanisms, depending on the antioxidant present (Shahidi & Zhong, 2015). They are also simple and fast, which is important when many measurements have to be made on many samples.

Browning is caused mainly by the soluble brown pigments resulting from the oxidation of polyphenols. This process takes place mainly when apples are crushed and pressed to obtain must. In the presence of oxygen, polyphenol oxidase (Enzyme Commission 1.14.18.1) catalyses the oxidation of polyphenols to *ortho*-quinones, which rapidly polymerize, either with themselves or with amino acids or proteins, to form brown pigments. (Deutch, 2018; Gacche et al., 2009; Park et al., 2018). This parameter is measured because some browning is accepted in Basque cider, but excessive browning reduces the visual quality of the product, and also leads to consumer rejection.

In this study, many monovarietal musts have been obtained using different varieties of apples from the Basque Country. Monovarietal musts were chosen to maximize differences in polyphenolic profiles. By

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https://doi.org/10.1016/j.fbio.2021.100887
Received 7 February 2020; Received in revised form 18 December 2020; Accepted 17 January 2021
Available online 17 February 2021

spontaneous fermentation of these musts, carried out by indigenous yeasts and lactic acid bacteria (LAB), the usual procedure in the Basque Country (del Campo et al., 2008), 19 monovarietal ciders have been obtained throughout three seasons (15 different, 4 repeated). The changes of the musts have been followed for 6–8 months by measuring the concentration of individual polyphenols and the parameters throughout 4 or 5 samplings. A multivariate analysis has been done to relate individual polyphenols with the parameters.

2. Materials and methods

2.1. Chemicals and reagents

A L-malic acid enzymatic assay kit was supplied by BioSystems S.A. (Barcelona, Spain). A Total Antioxidant Status assay kit was obtained from Rel Assay Diagnostics (Gaziantep, Turkey). Acetic acid, Folin-Ciocalteu reagent, hydrochloric acid, sodium acetate, sodium carbonate and sodium hydroxide were purchased from PanReac Química (Castellar del Vallès, Barcelona, Spain). Methanol for high-performance liquid chromatography (HPLC) was provided by Romil Ltd. (Cambridge, UK). Avicularin was obtained from LGC Standards (Barcelona, Spain). (+)-Catechin, isoquercitrin, hyperin and quercitrin were supplied by Apin Chemicals (Abingdon, UK). Procyanidin B1, procyanidin B2, procyanidin B5, 4-*p*-coumaroylquinic acid and phloretin 2′-*O*-xyloglucoside were provided by Polyphenols Biotech (Villenave d'Ornon, France). Acetonitrile, acrolein, caffeic acid, catechol, chlorogenic acid, (−)-epicatechin, 4-ethylcatechol, 4-ethylguaiacol, 4-ethyphenol, gallic acid, hydrocaffeic acid, iron(III) chloride hexahydrate, L-malic acid, *p*-coumaric acid, phloridzin, *p*-hydroxybenzoic acid, protocatechuic acid, tannic acid, 2,4,6-tripyridyl-*s*-triazine, (trans)-ferulic acid, Trolox and tyrosol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Potassium metabisulfite was supplied by Laffort (Bordeaux, France). Ascorbic acid was obtained from Apasa (Astigarraga, Gipuzkoa, Spain).

Solutions were prepared with double-distilled water (from this point on "water").

2.2. Obtaining the musts and fermentation process

The word *must* refers to the newly obtained or the fermenting apple juice and the word *cider* refers to the final product.

Apple musts were obtained from 300 kg lots of cider apples harvested in September–October 2012, 2013 and 2014, as usual in the Basque Country. Most of them were grown in an experimental orchard of Hondarribia (43°21′45″N, 1°47′29″W, Basque Country, Spain), and some varieties were grown in private orchards located in the same geographic area. Each lot was washed and crushed separately, and pressed in a Bucher Vaslin pneumatic press (Chalonnes-sur-Loire, France), which applied several pressing cycles of 2 bars. No filtration was done. The must was put into a 150 L stainless steel tank where the fermentation took place. The temperature was kept between 14 and 18 °C. Alcoholic and malolactic fermentations took place spontaneously by the indigenous microflora. The development of alcoholic fermentation was followed using the decrease in density (see section 2.4) and the malolactic fermentation using the decrease in L-malic acid content (see section 2.5). The tanks were kept uncovered until the completion of the tumultuous phase of the alcoholic fermentation (density ≈ 1000 g L^{-1}). Immediately afterwards, they were hermetically sealed and connected to a CO_2 cylinder with an overpressure of 0.2 atm, to protect the ciders from oxidation. After the completion of the malolactic fermentation (L-malic acid concentration <0.5 g L^{-1}), the ciders were racked (moved to a clean tank) to remove the sediments deposited at the bottom of the tanks. Some months later (2–5) and following the usual practice in Basque Country, 200 mg L^{-1} ascorbic acid were added to each tank, to avoid browning of the ciders, and 20 mg L^{-1} potassium metabisulfite, as a preservative. Three wk later the ciders were bottled.

All musts were monovarietal, obtained from the indigenous varieties *Aritza, Errezila, Frantzes, Gezamina, Goikoetxea, Manttoni, Merabi, Moko, Mozoloa, Narbarte-Gorria, Txalaka, Udare-Marroi, Urdin, Urtebi-Haundi* and *Urtebi-Txiki*. These 15 varieties are commonly used in the Basque Country to prepare cider, with the exception of *Narbarte-Gorria*, a strongly acid variety, rich in polyphenols, which was found in a private orchard. The varieties cover a wide range of acidity and polyphenolic content, which introduces a desirable variability. The varieties studied in the 2012 season were *Gezamina, Goikoetxea, Moko, Txalaka, Urtebi-Haundi* and *Urtebi-Txiki*; in the 2013 season, *Frantzes, Gezamina, Merabi, Mozoloa, Narbarte-Gorria, Urdin and Urtebi-Txiki*; in the 2014 season, *Aritza, Errezila, Frantzes, Manttoni, Mozoloa* and *Udare-Marroi*. With respect to *Narbarte-Gorria*, there were a few apples of this variety available and only 40 L of must could be obtained, which was put into a 50 L stainless steel tank. In all, 19 musts were studied, 15 different, 4 repeated.

In the cases where alcoholic fermentation became stuck (*Moko*, 2012), 30 g hL^{-1} commercial yeasts (*Saccharomyces cerevisiae*, Darnstar Ferment AG, Zug, Switzerland) were added to restart it. In the case of the malolactic fermentation (*Moko*, 2012; *Frantzes, Merabi, Narbarte-Gorria* and *Urtebi-Txiki*, 2013), it was restarted by adding 5 g hL^{-1} commercial LAB (*Oenococcus oeni*, Chr. Hansen Holding A/S, Hørsholm, Denmark) and the tank temperature increased to 20 °C, until the completion of the fermentation.

2.3. Sampling

Must or cider samples of about 250 mL were taken from the tanks for 6–8 months each season from September–October to March–June at: 1) just after pressing the apples; 2) half of alcoholic fermentation (density ≈ 1020 g L^{-1}); 3) end of alcoholic fermentation (density ≈ 1000 g L^{-1}); 4) end of malolactic fermentation (L-malic acid concentration <0.5 g L^{-1}), immediately before racking; and 5) before bottling (immediately before adding ascorbic acid and potassium metabisulfite).

The first sampling always corresponded to 0 days of fermentation, but the others had great variability owing to the differences in fermentation rate between the varieties and between the seasons. In the 2014 season, the second sampling was not done because it was found in the previous two seasons, that it was difficult to capture the time of half of the alcoholic fermentation. It was also not done with *Narbarte-Gorria* in the 2013 season because there was little must of this variety. Besides, samplings 4 and 5 were not done with *Mozoloa* in the 2014 season because a strong Brett character was observed in sampling three, as explained later.

Each sample was mixed using manual shaking and degasified (except initial musts) with a stirrer and a vacuum pump. Immediately afterwards, density and L-malic acid content were measured. A 100 mL portion was divided in two 50 mL centrifuge tubes and centrifuged at 9000×*g* (8200 rpm) for 20 min at room temperature (20–25 °C) in a Thermo Scientific™ Sorvall ST8 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was used for the FRAP, ABTS and FC assays and also to measure the absorbance at 420 nm (browning). A fraction of the centrifuged sample was filtered through a 0.45 μm nylon filter Chromafil Xtra PA-45/25 (Macherey-Nagel, Düren, Germany) and distributed in two vials for later determination of polyphenolic compounds. The vials were stored in the freezer at −20 °C for a maximum of 8 months, until their analysis was done. The sampling procedure described was carried out in one day to avoid oxidation of polyphenols and continuation of fermentation.

The musts or ciders are referred to by the name of the variety followed by the year of the season, e.g., "*Gezamina*-2012". When necessary, the sampling number will also be indicated, e.g., "*Gezamina*-2-2012".

2.4. Density and browning index

Density was measured using two precision hydrometers with scale 1.00–1.05 and 0.99–1.00 g cm^{-3} (Ludwig Schneider Messtechnik

GmbH, Wertheim, Germany). The browning index or absorbance at 420 nm (Ree et al., 2016) was measured in 10 mm quartz cuvettes against water using an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Cuvettes of smaller optical paths (5 or 2 mm) were used when the samples were darker, and the values obtained were corrected to a 10 mm optical path.

2.5. L-malic acid

This acid, as well as FRAP, ABTS and FC assays, were determined using the Automatic Analyser Y15 from BioSystems S.A. The analyser was provided with a halogen lamp, 340, 405, 420, 480, 520, 560, 600, 670 and 750 nm filters with a bandwidth of 10 nm, a reusable methacrylate rotatory tray with 120 cuvettes of 6 mm optical path for absorbance measurements, and a thermal heating system at 37 °C.

L-Malic acid was determined with a commercial enzymatic assay from BioSystems S.A. L-Malic acid in the sample reacts with NAD^+ in the presence of the enzyme L-malate dehydrogenase (L-MDH) to produce oxoaloacetate and NADH. The amount of NADH produced, measured by its absorbance at 340 nm, is stoichiometric to the amount of L-malic acid in the sample. The equilibrium of this reaction lies on the side of L-malate, so the enzyme glutamate-oxoaloacetate transaminase (GOT) and L-glutamate were added to displace the equilibrium by removal of oxaloacetate, which was converted to L-aspartate.

$$\text{L-malate} + NAD^+ \xrightarrow{\text{L-MDH}} \text{Oxaloacetate} + NADH$$
$$\text{Oxaloacetate} + \text{L-glutamate} \xrightarrow{\text{GOT}} \text{L-aspartate} + \text{2-oxoglutarate}$$

Procedure: 3 μL sample were mixed with 240 μL L-glutamate 45 mmol L^{-1} + L-MDH 37.5 U mL^{-1} + GOT >2.5 U mL^{-1} reagent (1 U = 1 μmol min^{-1} substrate converted). After 1 min, 60 μL NAD^+ >35 mmol L^{-1} were added. After 10 min, the absorbance was measured at 340 nm. A 2 g L^{-1} L-malic acid standard was used for calibration.

2.6. FRAP assay

The FRAP assay was done according to the Benzie and Strain (1996) procedure but using Trolox as a standard instead of ascorbic acid and with some changes to adapt the method to the Automatic Analyser Y15. The following solutions were prepared: a) acetate buffer 300 mM pH 3.6; b) 2,4,6-tripyridyl-*s*-triazine 10 mM in 40 mM HCl; c) $FeCl_3 \cdot 6H_2O$ 20 mM; d) working FRAP reagent prepared at the time of use (mixture of the previous solutions in the volume ratio of 10:1:1, respectively).

The assay was done as follows. Sample or standard (3 μL) was mixed with 300 μL working FRAP reagent, and absorbance at 600 nm was measured after 4 min. Trolox standards (0–5 mmol L^{-1}) were used to construct the calibration curve (absorbance versus Trolox mmol L^{-1}).

2.7. ABTS assay

This assay was measured according to the procedure described by Erel (2004) and carried out with the Total Antioxidant Status assay kit. The commercial kit provides a 0.4 mmol L^{-1} acetic/acetate buffer solution of pH 5.8 and a 30 mmol L^{-1} solution of ABTS radical cation.

The assay was done as follows (instructions of the manufacturer): Sample or standard (4 μL) was mixed with 200 μL acetate buffer and 25 μL ABTS radical cation reagent, and absorbance at 670 nm was measured after 5 min. Trolox standards (0–5 mmol L^{-1}) were used to construct the calibration curve (absorbance versus Trolox mmol L^{-1}).

2.8. FC assay

It was determined according to the Official Methods of Analysis of the International Organisation of Vine and Wine, OIV (2013), although some changes were made to adapt the method to the Automatic Analyser Y15.

A working FC reagent was prepared by diluting 7.5 mL commercial reagent to 100 mL with water. The FC index was measured as follows: Sample (4 μL) was mixed with 400 μL working FC reagent and 200 μL 12% w/v Na_2CO_3 solution. After 10 min, absorbance at 750 nm (called the FC index) was measured. From this absorbance, the result was also calculated in tannic acid g L^{-1} units by reference to a calibration curve, using tannic acid standards (0–5 g L^{-1}).

2.9. Polyphenolic compounds

Twenty-five individual polyphenolic compounds were determined: caffeic acid, chlorogenic acid, *p*-coumaric acid, 4-*p*-coumaroylquinic acid, hydrocaffeic acid, (trans)-ferulic acid (hydroxycinnamic acids), (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2, procyanidin B5 (flavan-3-ols), phloretin 2′-*O*-xyloglucoside, phloridzin (dihydrochalcons), avicularin, hyperin, isoquercitrin, quercitrin (flavonols), gallic acid, *p*-hydroxybenzoic acid, protocatechuic acid (benzoic acids), catechol, tyrosol (volatile polyphenols), 4-ethylcatechol, 4-ethylguaiacol and 4-ethyphenol (Brett character polyphenols).

The determination was done on the filtered samples using HPLC. They were thawed at room temperature and used directly, without any extraction, to avoid time-consuming treatments and oxidation of polyphenols. This is known as the “dilute and shoot” approach (Sapozhnikova, 2014). The HPLC method by Suárez et al. (2005) was used, as described by Zuriarrain et al. (2015). Briefly, an Agilent 1100 Series liquid chromatograph (Agilent Technologies) with a diode-array detector was used, with a Nucleosil® 120-3 C_{18} column, 250 × 4.6 mm, 3 μm (Macherey-Nagel). The flow rate was 0.8 mL min^{-1}, the column temperature was 25 °C, and the injected volume was 50 μL. The elution solvents were aqueous 20 mL L^{-1} acetic acid (solvent A) and methanol (solvent B). The samples were eluted according to the following gradient: a linear increase from 0 to 45% solvent B in 55 min, followed by a 20 min isocratic step, and finally, a return to the initial conditions (0% solvent B), allowing 5 min for stabilisation. Column effluents were monitored at three wavelengths: 280 nm for flavan-3-ols, dihidrochalcons, benzoic acids, volatile polyphenols and Brett character polyphenols; 313 nm for hydroxycinnamic acids; and 355 nm for flavonols. Phenolic compounds were quantified (mg L^{-1}) using the external standard method from peak areas, assuming all peaks responded equally and all were in their Beer-Lambert law’s region. Peak areas were calculated using the software of the instrument. They were identified by means of their retention time and their 190–900 nm spectra, which were previously obtained by injecting the pure compounds.

Brett character polyphenols, i.e., 4-ethylcatechol, 4-ethylguaiacol and 4-ethyphenol were not determined in the previous study. They eluted at 42.9, 62.3 and 58.7 min, respectively, and did not overlap with any other polyphenol.

2.10. Acrolein

This compound was determined using gas chromatography, together with other volatile compounds, e.g., ethanol and methanol, not included in this article. An Agilent HP 6890N gas chromatograph was used (Agilent Technologies), with a Restek-Stabilwax capillary column, 60 m × 0.53 mm, 1.00 μm (Restek Corp., Bellefonte, PA, USA), and a flame ionisation detector. Helium at 7 mL min^{-1} was the carrier gas. The temperature program was 1 min at 40 °C, an increase to 65 °C at 5 °C min^{-1}, 1 min at 65 °C, another increase to 125 °C at 15 °C min^{-1}, and 1 min at 125 °C. To clean the column, the temperature was increased to 200 °C and kept there for 4 min. The injector and detector temperatures were 200 and 250 °C, respectively. A 3 μL volume of each previously filtered must or cider sample was directly injected in a 1:15 split mode. Acrolein retention time was 4.7 min, and it was quantified from the peak area using acetonitrile as an internal standard. A calibration graph was obtained with acrolein standards in the range 5–50 mg L^{-1}.

2.11. Statistical analysis

Partial Least Squares Regression (PLS) was used to explore the effect of the individual phenol content on the parameters ABTS, FRAP, FC and browning in a multivariate way. The PLS regression ($p \leq 0.05$) was done with Unscrambler® X, version 10.3 (CAMO Software AS, Oslo, Norway).

3. Results and discussion

3.1. Classification of cider apple varieties

The English classification of cider apple varieties divides them into 4 technological groups: bittersharp (>4.5 g L^{-1} L-malic acid, >2 g L^{-1} tannic acid), sharp (>4.5 g L^{-1} L-malic acid, <2 g L^{-1} tannic acid), bittersweet (<4.5 g L^{-1} L-malic acid, >2 g L^{-1} tannic acid) and sweet (<4.5 g L^{-1} L-malic acid, <2 g L^{-1} tannic acid) (Jolicoeur, 2013). Most apple varieties used for cidermaking in the Basque Country are bittersharp or sharp. *Aritza, Merabi, Moko, Narbarte-Gorria* and *Urdin* varieties are bittersharp; *Errezila, Frantzes, Goikoetxea, Txalaka, Udare-Marroi, Urtebi-Haundi* and *Urtebi-Txiki* are sharp; *Gezamina* and *Mozoloa* are bittersweet; and *Manttoni* is sweet.

The English classification was used because there is no systematic classification of cider apple cultivars in the Basque Country.

3.2. Antioxidant activity of ciders

In Table 1, the antioxidant activity of the monovarietal ciders obtained in this study is shown, together with averages of other alcoholic and non-alcoholic beverages. The values of ciders corresponded to the mean of the last two samples, as ciders were usually finished or almost finished with the penultimate sample. With *Goikoetxea*-2012 and *Urdin*-2013 ciders, the value of the 4th sample is given because, for the reasons explained later, the antioxidant activity values of the 5th samples were rejected. ABTS values are shown to make comparisons, since they are most often used with ciders. FRAP values are more limited and FC values are normally used to report total polyphenol concentration (Pior et al., 2005; Shahidi & Zhong, 2015).

The antioxidant activity range of the monovarietal ciders, 5.7–23.8 Trolox mmol L^{-1}, was greater than that of white or rosé wines and was similar to that of red wines. The antioxidant activity of wines is often quoted as a beneficial aspect. Moreover, the antioxidant activity of the ciders studied was greater than that of other alcoholic beverages like beer, Cognac, Armagnac and rum. With respect to non-alcoholic beverages, the antioxidant activity of the ciders is similar to that of coffee and is greater than that of tea, orange juice, olive oil, sunflower oil and cola. The comparison with orange juice is particularly interesting because this drink is always quoted as a model of a healthy and antioxidant beverage.

Table 1
Antioxidant power of monovarietal ciders and other beverages.

Ciders[a]	ABTS	Other	ABTS
	Trolox mmol L^{-1}	beverages	Trolox mmol L^{-1}
Gezamina-2012	17.6	Red wine[b]	2.3–25.2
Goikoetxea-2012	9.3	Rosé wine[b]	1.5–3.2
Moko-2012	17.6	White wine[b]	0.1–1.9
Txalaka-2012	6.8	Beer[c]	0.6–2.0
Urtebi-Haundi-2012	6.2	Cognac[d]	0.7–4.9
Urtebi-Txiki-2012	8.4	Armagnac[d]	0.6–1.2
Merabi-2013	23.8	Rum[e]	<0.1
Narbarte-Gorria-2013	16.9		
Urdin-2013	8.8	Cola[f]	<0.1
Urtebi-Txiki-2013	9.7	Coffee (100 g L^{-1})[g]	7.8–12.3
Aritza-2014	14.8	Tea (15 g L^{-1})[h]	0.8–6.3
Frantzes-2014	7.9	Orange juice[i]	0.7–7.1
Manttoni-2014	5.7	Olive oil[j]	0.4–1.8
Udare-Marroi-2014	7.7	Sunflower oil[j]	0.1–1.2

[a] Mean of the last two samples, except in *Goikoetxea*-2012 and *Urdin*-2013, where the value of the 4th sample is given.
[b] mainly Spanish and French wines (Landrault et al., 2001; Pellegrini et al., 2003; Pulido et al., 2003; Saura-Calixto & Goñi, 2006; Villaño et al., 2004).
[c] beers from all over the world (Zhao et al., 2010).
[d] (Schwarz et al., 2009).
[e] (Pellegrini et al., 2003).
[f] (Pellegrini et al., 2003; Pulido et al., 2003; Saura-Calixto et al., 2006).
[g] filter coffees from all over the world (Parras et al., 2007).
[h] infusion, 5 min in boiling water (Almajano et al., 2008).
[i] fresh juices of Italian oranges (Rapisarda et al., 1999).
[j] Italian oils (Pellegrini et al., 2003; Pulido et al., 2003; Saura-Calixto & Goñi, 2006).

3.3. Defects of ciders

Acrolein is a compound that occasionally arises in cider, completely spoiling its quality by generating bitter tastes. It is produced from glycerol by some types of *Lactobacillus* bacteria (Garai-Ibabe et al., 2008). It was found in *Frantzes*-4-2013, *Frantzes*-5-2013, *Errezila*-4-2014 and *Errezila*-5-2014 musts, and was considered to be a "defect". The concentrations of acrolein found were between 7.6 and 18.3 mg L^{-1}.

The Brett character or phenolic off-flavour is another alteration that may occur in cider, especially in ciders rich in polyphenols, caused by some types of *Brettanomyces* yeasts and *Lactobacillus* bacteria. They degrade the hydroxycinnamic acids to give compounds with unpleasant flavours and aromas, mainly 4-ethylphenol, 4-ethylguaiacol and 4-ethylcatechol (Buron et al., 2011, 2012; Steensels et al., 2015). The Brett character was observed in *Gezamina*-4-2013, *Gezamina*-5-2013, *Mozoloa*-4-2013, *Mozoloa*-5-2013 and *Mozoloa*-3-2014 musts, which were also considered to be defects. The three spoilage compounds were found in the musts, but the main one was 4-ethylcatechol with concentrations of 46.2–138 mg L^{-1}. These compounds were not determined in the 2012 season because no phenolic off-flavours were observed.

3.4. Multivariate study

Tables S1–S19 (supplementary material) show the concentrations obtained throughout the samplings for the individual polyphenolic compounds, as well as the values for density, L-malic acid, acrolein, FRAP, ABTS, FC and browning. The content of individual phenolics in Basque apples and ciders was previously reported (Alonso-Salces et al., 2004a, 2004b, 2006), and therefore, these data are not discussed here as the emphasis was on the effect of the individual polyphenols on the parameters of interest.

Separate PLS regression models were built for the parameters ABTS, FRAP, FC and browning as dependent Y variables, and the 22 individual phenol contents as predictor X variables, to explore the effect of the individual phenols on these parameters in a multivariate way (diagram in Fig. 1) (the three Brett character polyphenols were not included, as they were not determined in all seasons). The samples considered were the 86 samplings. However, the 9 samples corresponding to the defect musts were rejected because it was considered appropriate to work only with healthy musts. Therefore, 77 × 22 matrices composed of 77 samples and 22 variables were included in the multivariate analysis. These samples were divided into two sets, the calibration set, including 65% of the samples, and the validation set, which included the rest. The split was random, ensuring that at least two samples of each variety were included in the calibration set. The algorithm used to calculate the models was NIPALS. All the variables were normalized using autoscaling, and the validation method was confirmed using full cross validation leave-one-out. Martens' uncertainty test (Martens & Martens, 2000) was also done to obtain the significance of the individual polyphenols in the calculated models. This test, based on cross validation, jack-knifing and stability plots, has been previously used for variable

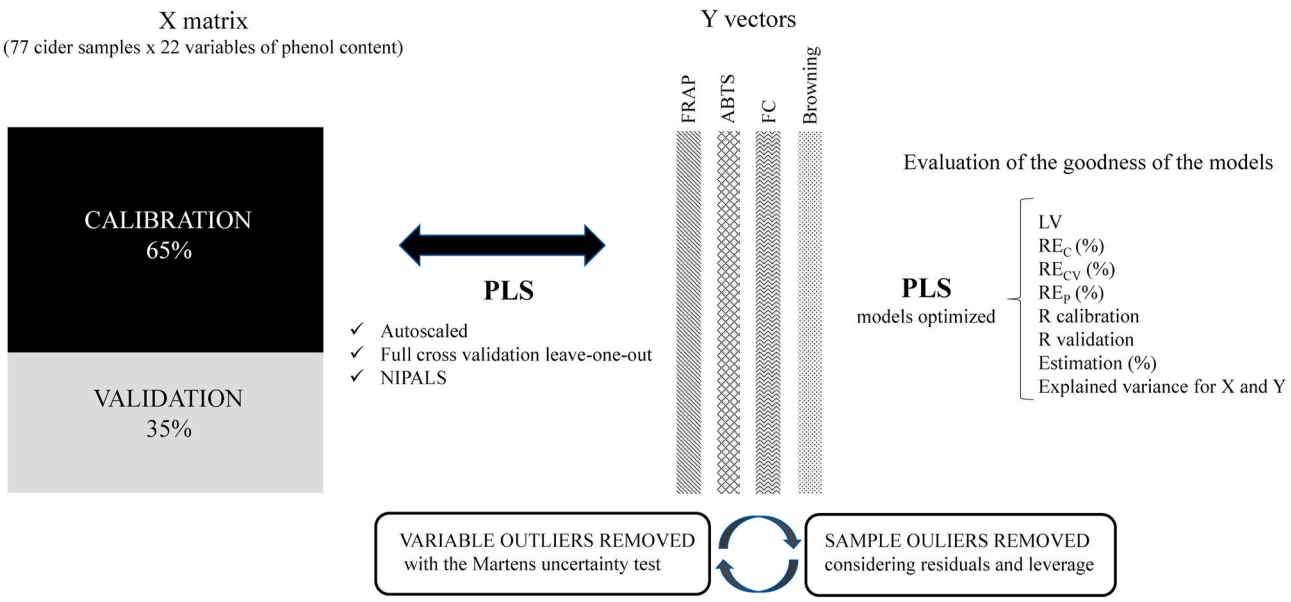

Fig. 1. Diagram showing the PLS models creation process.

selection with similar data (Baxter et al., 2005; Osorio et al., 2013) as well as spectroscopic data (Rambo et al., 2015; Woodcock et al., 2009).

To obtain PLS models that explain the maximum variance of the selected Y variables or parameters with the minimum amount of X variables or individual polyphenols, a PLS model was first obtained for each parameter which included all polyphenols. Then, the polyphenols were removed, one by one, beginning with the less important one according to Martens' uncertainty test until the significance of the remaining polyphenols was >95% ($p \leq 0.05$) according to the same test. In this process, the polyphenol removed each time was always the one with the lowest weighted regression coefficient (absolute value). In addition, the leverage and the residual of each sample, as well as the X–Y relation outliers plots, were also taken into account to detect possible outliers and eliminate them. Samples with both high leverage and high residual were poorly described by the PLS model, and they should be removed (Pedro & Ferreira, 2005; Šegan et al., 2016). On the other hand, the PLS X–Y relation outliers plot is also a powerful tool to detect non-linearities in the data that can be caused by outliers. Outlier removal can be done during the calibration procedure because a sample may not be considered an outlier according to X-variables but become an outlier to the Y-variables only when the X–Y relationship is considered (Cozzolino et al., 2011; The Unscrambler, 2006). By doing this, outliers that have an effect on the regression model are also removed (Cozzolino, 2014, p. 24).

The goodness of the calibration model for each parameter is given by calculating the statistic relative error of calibration (RE_C) and that of cross validation (RE_{CV}):

$$RE(\%) = 100\sqrt{\frac{\sum_{i=1}^{n}\left(\widehat{y}_i - y_i\right)^2}{\sum_{i=1}^{n} y_i^2}}$$

$\widehat{y}_i$ = predicted parameter in i sample

y_i = observed parameter in i sample
n = number of samples

The equation was applied to the calibration data in the case of RE_C and to the validation ones in the case of RE_{CV}.

Once a satisfactory model was obtained, a prediction of the parameter was done with the validation set to test the reliability of the calculated model. The predictions were always done using the optimal number of latent variables (LV) suggested by the statistical software for each model. The accuracy of the prediction of each parameter is given by calculating the statistic relative error of prediction (RE_P), with the same equation as before, applied to the prediction data. Model overfitting implies the inclusion of "too many" LV in the model, while underfitting implies the inclusion of "too few" (Gowen et al., 2011). In the same way, RE_P should be low and close to the RE_{CV} and RE_C values to avoid model overfitting (Roussel et al., 2014). In conclusion, overfitting is characterized by a good model (low RE_C and RE_{CV}) with bad predictions (high RE_P) by taking too many LV (Geladi & Grahn, 2018). Additionally, the value of each parameter was estimated for all samples cases ($\widehat{y}_i$) using the PLS model and compared with the observed value (y_i) according to the equation (Picinelli et al., 2009):

$$\%Estimation = \frac{\widehat{y}_i}{y_i} \times 100$$

Correlation coefficients (R) obtained with calibration and validation sets were also calculated. The results are shown in Table 2.

Zuriarrain et al. (2015) did a similar but simpler study during the 2010 season. However, in that study only 6 ciders were measured for a single season. Moreover, only the FRAP assay was used to measure the antioxidant power, whereas this time, FRAP, ABTS and FC assays have been used. Furthermore, in the previous study, the multivariate analysis was done using the Multivariate Linear Regression, whilst this time, the more powerful PLS Regression has been used. Consequently, the conclusions of this study are much more robust and reliable than those of the initial one.

3.5. Contribution of individual polyphenols to the antioxidant activity

During the calculation of the calibration models of FRAP, ABTS and FC assays some samples considered as outliers were removed: *Moko*-1-2012, *Goikoetxea*-5-2012 and *Urdin*-5-2013. In the calibration model for FC, *Narbarte-Gorria*-3-2013 was also eliminated. No sample was rejected in the validation set.

The PLS models provided low values for RE_C, RE_{CV} and RE_P and good estimations for the three parameters. Among the polyphenols with significant positive contribution ($p \leq 0.05$) as shown in Table 2, 5 are

Table 2
PLS results for regression models.

Parameter	Model results		PLS equation	
			X variables[a]	β
FRAP	LV[b]	2	β_0	0.6642
	RE_C (%)	12.53	4-*p*-Coumaroylquinic acid	0.3598
	RE_{CV} (%)	14.12	Chlorogenic acid	0.3594
	RE_P (%)	13.20	(−)-Epicatechin	0.1760
	R calibration	0.9650	Isoquercitrin	0.1452
	R validation	0.9572	Procyanidin B5	0.1397
	Estimation (%)[c]	110 ± 20	Phloretin 2′-*O*-xyloglucoside	0.1295
	Explained variance (%)	X: 65 ; Y: 94	Gallic acid	−0.1561
ABTS	LV	2	β_0	0.8216
	RE_C (%)	14.57	Chlorogenic acid	0.3226
	RE_{CV} (%)	16.25	4-*p*-Coumaroylquinic acid	0.2893
	RE_P (%)	16.48	(−)-Epicatechin	0.1565
	R calibration	0.9442	Phloretin 2′-*O*-xyloglucoside	0.1468
	R validation	0.9331	Procyanidin B1	0.1304
	Estimation (%)	100 ± 20	Procyanidin B5	0.1087
	Explained variance (%)	X: 79 ; Y: 90	Isoquercitrin	0.0878
FC	LV	2	β_0	0.9271
	RE_C (%)	14.12	Chlorogenic acid	0.4047
	RE_{CV} (%)	15.59	4-*p*-Coumaroylquinic acid	0.2986
	RE_P (%)	20.94	(−)-Epicatechin	0.2281
	R calibration	0.9559	Phloretin 2′-*O*-xyloglucoside	0.1719
	R validation	0.9482	Isoquercitrin	0.1507
	Estimation (%)	110 ± 30	Protocatechuic acid	−0.1216
	Explained variance (%)	X: 54 ; Y: 91	Tyrosol	−0.1243
Browning	LV	3	β_0	0.2064
	RE_C (%)	20.92	4-*p*-Coumaroylquinic acid	0.9575
	RE_{CV} (%)	25.14	Phloretin 2′-*O*-xyloglucoside	0.3410
	RE_P (%)	19.08	Quercitrin	−0.2720
	R calibration	0.9525	(+)-Catechin	−0.3693
	R validation	0.9347		
	Estimation (%)	110 ± 40		
	Explained variance (%)	X: 56 ; Y: 89		

[a] Significant at $p \leq 0.05$.
[b] LV = number of latent variables.
[c] mean ± standard deviation.

common to the three parameters. They are, in decreasing order, chlorogenic acid > 4-*p*-coumaroylquinic acid > (−)-epicatechin > phloretin 2′-*O*-xyloglucoside > isoquercitrine. Chlorogenic and 4-*p*-coumaroylquinic acids, as well as phloretin 2′-*O*-xyloglucoside and isoquercitrine reversed their order in FRAP. Consequently, these are the polyphenols that most contributed to the antioxidant power. No significant negative contributions common to the three parameters were obtained.

The literature about the contribution of individual polyphenols of apples and apple derivatives to antioxidant activity reports a great variety of different results and different methods to obtain them, which makes it difficult to establish any comparison. The results are only partially consistent with previously published values. For example, Tsao et al. (2005) reported that the principal contributors to antioxidant activity in apple flesh (the part mainly found in the must), measured using the FRAP assay, were (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2 and, to a lesser extent, chlorogenic acid, 4-*p*-coumaroylquinic acids and phloretin 2′-*O*-xyloglucoside. However, they used Pearson correlation to decide which were the main contributors, and multivariate calibration is a more appropriate statistical technique to find relationships between these data than a set of simple paired X–Y regressions.

Chinnici et al. (2004) gave as the principal contributors in apple flesh (−)-epicatechin, chlorogenic acid and, to a lesser extent, procyanidin B2 and another unspecified procyanidins. However, to calculate this contribution, they simply multiplied the concentration found for each polyphenol by the value of the antioxidant activity of the standard pure polyphenols. This supposes that more concentrated polyphenols always give greater antioxidant activity, obviating the possible interactions of polyphenols between themselves or with other compounds. On the other hand, they used the DPPH assay (DPPH = 1,1-diphenyl-2-picrylhydrazyl radical) to measure the antioxidant activity, but this assay gives similar results as the ABTS assay (Leong & Shui, 2002).

Picinelli et al. (2009) measured the antioxidant activity in ciders from Asturias (northern Spain), where the production methods are similar to those used in the Basque Country. They reported as the main polyphenolic contributors to the antioxidant activity, in descending order, procyanidin B2 > hydrocaffeic acid > epicatechin. They used FRAP and DPPH assays to measure the antioxidant activity of ciders and PLS multivariate calibration to decide which were the main contributors. Despite using a similar procedure, the results were different. The difference may be due to the cider apples used in Asturias. Basque ciders are more acidic, more phenolic (more bitter), and more sparkling than Asturian ciders. The polyphenolic compositions were different and so were the interactions between polyphenols, which led to the different results observed.

3.6. Contribution of individual polyphenols to browning

More difficulties arose when the PLS model for browning was calculated. All the first samples had to be eliminated before calculation of the model. They corresponded to freshly obtained musts, which were highly oxygenated as a consequence of milling and pressing, and they had much higher browning values than the subsequent samples. It was not possible to obtain a PLS model including these high values. Moreover, while calculating the model, three more samples were rejected as they were outliers (high leverage and/or high residual): *Narbarte-Gorria*-3-2013, *Narbarte-Gorria*-4-2013 and *Urdin*-2-2013. Furthermore, *Narbarte-Gorria*-5-2013 and *Urdin*-3-2013 had to be eliminated from the validation set as they were distorting the prediction for being samples

not properly represented in the calibration matrix. Consequently, all the *Narbarte-Gorria* samples were eliminated.

Low values for RE_C, RE_{CV} and RE_P, and good estimation for browning were obtained with the PLS model, although they were not as satisfactory as in the preceding models. The optimum number of LV was 3, and no model overfitting was, therefore, observed. The main significant positive contributions to this parameter corresponded to 4-*p*-coumaroylquinic acid > phloretin 2′-*O*-xyloglucoside; hence, these are the polyphenols that most contribute to the browning of apple musts. Significant negative contributions were obtained with (+)-catechin and quercitrin.

A correlation between initial chlorogenic acid content and final browning has been reported (Robards et al., 1999). Therefore, attempts were made to relate the polyphenols of the first samples (eliminated in the previous calibration) with the browning value of the last ones, but no satisfactory models were obtained. This may be because the data matrix used was smaller, i.e., 14 samples × 15 variables.

Much work has been done to determine the path for enzymatic oxidation of polyphenols to give brown pigments in apples and apple products. However, no relationship has been found between browning and the individual polyphenols, and different conclusions have been reported (Alberti et al., 2014; Le Deun et al., 2015; Nicolas et al., 1994; Persic et al., 2017; Song et al., 2007). According to Février et al. (2017), browning in apple juices must be considered as a multifactorial process, and the main factors involved are polyphenol oxidase enzyme activity, pH and concentration of the different polyphenols. Consequently, browning in apples and ciders is a subject that needs further research.

4. Conclusions

The antioxidant activity of the monovarietal ciders of the Basque Country was similar to that of red wines and greater than that of orange juices. The polyphenols which most contributed to the antioxidant activity were chlorogenic acid, 4-*p*-coumaroylquinic acid, (−)-epicatechin, phloretin 2′-*O*-xyloglucoside and isoquercitrine. The main contributors to browning were 4-*p*-coumaroylquinic acid and phloretin 2′-*O*-xyloglucoside.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to the Basque Government, the University of the Basque Country EHU/UPV, the Gipuzkoa Natural Cider Association and the Fraisoro Agro-Environmental Laboratory for their financial and technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2021.100887.

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