Research Article

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Polyphenolic profile in cider and antioxidant power

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Abstract

BACKGROUND: The aim of this work was to find the effect of polyphenolic compounds in Basque ciders on the following parameters: antioxidant activity, browning, protein-precipitating capacity, turbidity and reduction potential. These five parameters are highly important, as they affect the taste, the visual aspect and the preservation of cider, and are mainly related to polyphenolic compounds.

RESULTS: Procyanidin B1 and procyanidin B2 showed a significant positive effect on antioxidant activity. *p*-Coumaric acid, (−)-epicatechin and hyperin had a significant positive effect on protein-precipitating capacity. Tyrosol had a significant negative effect on reduction potential.

CONCLUSION: Procyanidin B1 and procyanidin B2 are the most powerful antioxidants in Basque cider, while *p*-coumaric acid, (−)-epicatechin and hyperin are those with greatest capacity to precipitate proteins. Ciders with higher tyrosol concentration will have less reduction potential and higher antioxidant reservoir.
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Keywords: cider; polyphenolic compounds; antioxidant activity; antioxidant reservoir

INTRODUCTION

Cider apple varieties contain relatively large amounts of polyphenolic compounds, which can be divided into four classes: hydroxycinnamic acid derivatives, flavan-3-ols, either monomeric (catechins) or oligomeric (procyanidins), flavonols and dihydrochalcones.[1]

Polyphenolic compounds, particularly procyanidins, are responsible for haze and sediment formation because of their interaction with proteins.[2,3] They are also involved in browning processes because of the effect of polyphenol oxidases.[4] From the organoleptic point of view, polyphenols are related to bitterness and astringency, whose balance defines the overall mouth feel of the beverage.[5]

The aim of this work was to find correlations between the individual polyphenolic compounds in Basque ciders and the following parameters: antioxidant activity, browning, protein-precipitating capacity, turbidity and reduction potential. These five parameters are related mainly to polyphenols and are vital as they affect the taste, the visual aspect and the preservation of the cider.

Among the different methods available to measure the antioxidant activity,[6] we have chosen the ferric-reducing antioxidant power (FRAP) assay because it is simple and fast. It is based on the reduction of the Fe^{3+} complex of 2,4,6-tripyridyl-*s*-triazine, $Fe(TPTZ)^{3+}$, to the intensely blue-coloured Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and are usually expressed as micromolar equivalents of an antioxidant standard such as Trolox or ascorbic acid. The reaction is non-specific, so the change in absorbance is directly related to the total reducing power of the antioxidants present in the reaction mixture.

Enzymatic browning is one of the most limiting factors when fruits are cut or crushed, such as happens to apples in cider production. The vegetal cells are broken, the enzymes are liberated from tissues and they come into contact with their main substrates, the polyphenolic compounds. The major enzyme involved in the browning reaction is polyphenol oxidase (mixture of EC 1.14.18.1 and EC 1.10.3.1). In the presence of oxygen, this enzyme catalyses the oxidation of phenol to *ortho*-quinones, which rapidly polymerize to form brown pigments.[7] Browning is measured as the absorbance at 420 nm, the absorption maximum of these pigments.[8]

Many polyphenolic compounds have the ability to precipitate proteins. In fact, the interaction between polyphenolics and proteins is the most frequent cause of haze in beverages.[3] The method we have chosen to quantify the protein-precipitating capacity measures the amount of polyphenolic compounds precipitated by a standard protein, bovine serum albumin. The precipitate is dissolved at high pH in the presence of a detergent, and the coloured iron–phenolate complex is determined by spectrophotometry. The method is robust and works well with virtually all

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Table 1. Calibration data for $N = 5$ and general equation: peak area = $a \times C$ (mg L^{-1}) + b

Polyphenol	Linear range (mg L^{-1})	a	b	r	LOD (mg L^{-1})
Hydroxycinnamic acids					
Caffeic acid	2.0–10.0	346.0	−34.7	0.9998	0.2
Chlorogenic acid	23.8–237.5	158.3	−316.8	0.9998	5.4
p-Coumaric acid	0.5–6.1	432.0	2.7	0.9999	0.1
Hydrocaffeic acid	9.9–99.2	56.5	−389.5	0.9864	5.8
(*trans*)-Ferulic acid	1.3–5.0	242.8	24.3	0.9815	1.3
Flavan-3-ols					
(+)-Catechin	4.7–18.7	39.0	−13.5	0.9998	0.4
(−)-Epicatechin	18.3–183.2	46.7	−63.7	0.9996	6.5
Dihydrochalcones					
Phloridzin	2.0–20.1	120.5	11.7	0.9993	1.0
Flavonols					
Avicularin	2.7–20.7	129.1	−69.2	0.9948	2.7
Hyperin	0.2–2.0	114.5	−2.5	0.9998	0.05
Isoquercitrin	2.0–20.0	121.8	−2.3	0.9985	1.4
Quercitrin	0.2–2.0	98.7	−3.9	1.0000	0.02
Benzoic acids					
Gallic acid	2.2–10.5	164.2	−59.2	0.9990	0.5
p-Hydroxybenzoic acid	0.5–6.0	92.9	−1.9	1.0000	0.04
Protocatechuic acid	1.0–8.3	92.8	−0.8	0.9986	0.5
Volatile polyphenols					
Catechol	1.6–7.8	67.2	−4.1	0.9868	1.6
Tyrosol	4.9–49.4	36.4	−8.0	0.9999	1.1

LOD, limit of detection.

Table 2. Calibration data for $N = 1$

Polyphenol	Standard (mg L^{-1})	Response factor	LOD (mg L^{-1})
Hydroxycinnamic acids			
4-*p*-Coumaroylquinic acid	104.2	71.7	2.3
Flavan-3-ols			
Procyanidin B1	45.2	4.7	4.0
Procyanidin B2	113.4	7.8	6.4
Procyanidin B5	52.6	13.6	5.8
Dihydrochalcones			
Phloretin 2′-*O*-xyloglucoside	98.6	22.7	3.3

LOD, limit of detection.

plant extracts.[9] Besides, the turbidity caused by polyphenolics or by other factors in the must (newly pressed apple juice) or the cider can be measured with a conventional turbidimeter/nephelometer.

The reduction potential gives information on the ability of a redox couple to be oxidized or reduced. The methodologies developed for the measurement of the antioxidant activity, including FRAP, are kinetic methods that provide information about the most reactive compounds. On the other hand, the reduction potential is a thermodynamic quantity that accounts for all antioxidants present in the must or the cider, including the slowest but most efficient ones, as they represent the antioxidant reservoir of the beverage.[10]

In this work, we have obtained six different musts by using five different varieties of apples of the Basque Country (northern Spain). Five are monovarietal and the sixth is obtained by mixing the varieties used in the other five musts in equal weight proportions. Monovarietal musts have been used mainly in order to obtain polyphenolic profiles as different as possible. By spontaneous fermentation of these musts, six different ciders have been obtained. The evolution of the ciders has been followed during 5 months, by measuring the concentration of polyphenolics and the five parameters mentioned throughout five samplings, and a multivariate linear regression study has been performed.

EXPERIMENTAL

All chemicals used were of analytical reagent grade. Solutions were prepared with doubly distilled water (hereinafter 'water').

Obtaining musts and fermentation

Six musts were obtained from six 250 kg lots of cider apples harvested in October 2010 in an experimental orchard of Hondarribia (Basque Country, Spain). Each lot was crushed separately and pressed in a vertical press, and the must obtained was introduced into a 250 L stainless steel tank where the fermentations took place. The temperature was kept at 15 °C. Alcoholic and malolactic fermentations took place spontaneously by indigenous microflora. The tanks were kept uncovered for 38 days until the tumultuous phase of fermentation had finished. Immediately afterwards, they were hermetically sealed and connected to a CO_2 cylinder, with an overpressure of 0.2 atm, in order to protect the ciders from oxidation. After 114 days of fermentation, the ciders were racked to remove the sediments deposited at the bottom of the tanks.

Five musts were monovarietal, obtained from the indigenous varieties Goikoetxea, Manttoni, Moko, Patzuloa and Txalaka, widely used to elaborate cider in the Basque Country. These varieties are

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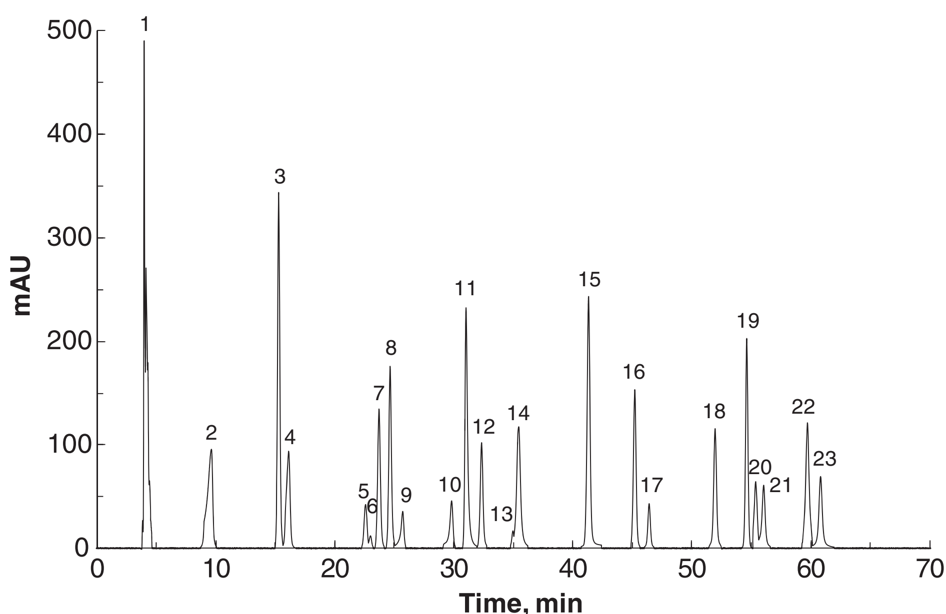

Figure 1. Chromatogram obtained at 280 nm with pure standards: 1, ascorbic acid; 2, gallic acid; 3, catechol; 4, protocatechuic acid; 5, tyrosol; 6, procyanidin B1; 7, *p*-hydroxybenzoic acid; 8, hydrocaffeic acid; 9, (+)-catechin; 10, procyanidin B2; 11, chlorogenic acid; 12, caffeic acid; 13, (−)-epicatechin; 14, 4-*p*-coumaroylquinic acid; 15, *p*-coumaric acid; 16, (*trans*)-ferulic acid; 17, procyanidin B5; 18, phloretin 2′-*O*-xyloglucoside; 19, phloridzin; 20, hyperin; 21, isoquercitrin; 22, avicularin; 23, quercitrin.

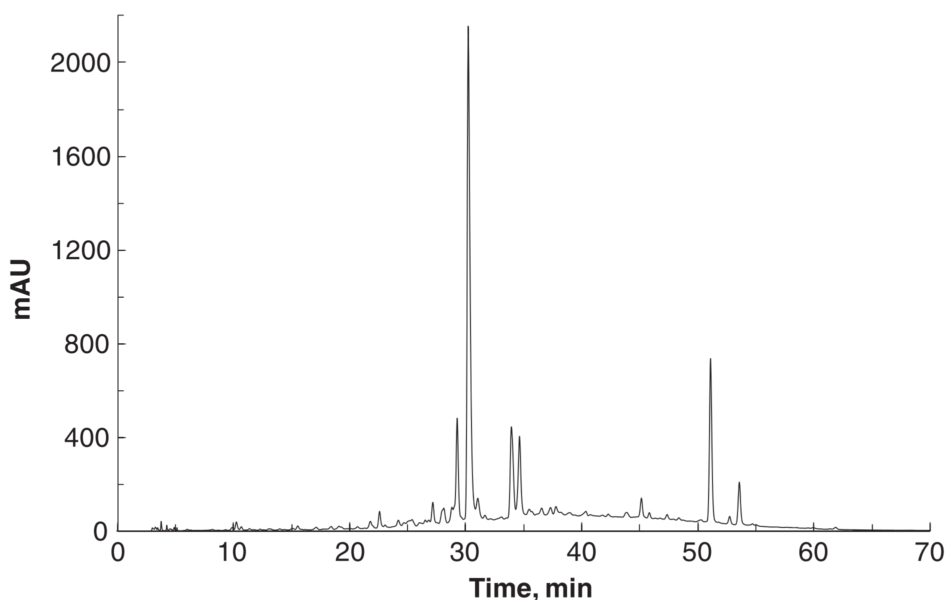

Figure 2. Chromatogram obtained at 280 nm with a cider sample of Moko variety.

quite different regarding polyphenolic content and acidity, which introduces variability in the study.

The sixth must, called Mixture, was obtained by crushing together 50 kg of each of the five varieties (total 250 kg). Cider in the Basque Country is always obtained from a mixture of varieties, so we considered it convenient to also include a must like this one.

Sampling

Must or cider samples of about 250 mL were taken from the six tanks during 5 months, from October 2010 to March 2011, at 0, 30, 60, 90 and 140 day intervals (there were minor differences in the number of days for the different tanks).

Each sample was homogenized by shaking and then degasified (except initial musts). Immediately afterwards, reduction potential and turbidity were measured. A 100 mL portion was centrifuged at $10\,000 \times g$ for 20 min and used to measure Folin–Ciocalteu index, FRAP, absorbance at 280 nm (total polyphenol index), absorbance at 320 nm (hydroxycinnamic acid derivatives), absorbance at 420 nm (browning) and protein-precipitating capacity. A fraction of the centrifuged sample was filtered through a 0.45 µm filter and distributed into two vials for later determination of polyphenolic compounds. The vials were stored in a freezer at −20 °C until analysis. The sampling procedure described was always performed in 1 day to avoid oxidation of polyphenols and continuation of fermentation.

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Table 3. Phenolic compound contents (mg L^{-1}) and parameters in Goikoetxea must[a]

Compound	Fermentation days				
	0	30	63	98	140
Hydroxycinnamic acids					
Caffeic acid	0.73 ± 0.08	1.03 ± 0.12	ND	ND	0.24 ± 0.03
Chlorogenic acid	120.0 ± 0.6	124.5 ± 0.6	23.4 ± 0.1	9.21 ± 0.05	ND
p-Coumaric acid	1.47 ± 0.03	1.53 ± 0.03	0.31 ± 0.01	ND	ND
4-*p*-Coumaroylquinic acid	61.3 ± 0.2	92.2 ± 0.3	85.8 ± 0.2	80.8 ± 0.2	74.7 ± 0.2
Hydrocaffeic acid	7.37 ± 0.01	29.29 ± 0.06	79.1 ± 0.2	84.2 ± 0.2	84.2 ± 0.2
(*trans*)-Ferulic acid	ND	2.3 ± 0.2	2.6 ± 0.2	2.1 ± 0.1	2.2 ± 0.1
Flavan-3-ols					
(+)-Catechin	1.9 ± 0.4	6.5 ± 1.2	ND	ND	1.1 ± 0.2
(−)-Epicatechin	ND	13.12 ± 0.09	10.95 ± 0.07	ND	ND
Procyanidin B1	ND	20.9 ± 1.1	30.9 ± 1.6	36.4 ± 1.9	33.4 ± 1.7
Procyanidin B2	8.42 ± 0.01	4.99 ± 0.01	ND	ND	43.84 ± 0.06
Procyanidin B5	ND	ND	ND	ND	ND
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	52.2 ± 0.3	229.3 ± 1.3	36.9 ± 0.2	13.81 ± 0.08	15.09 ± 0.09
Phloridzin	ND	13.4 ± 3.5	29.8 ± 7.7	26.4 ± 6.8	23.4 ± 6.0
Flavonols					
Avicularin	ND	ND	ND	ND	ND
Hyperin	ND	0.15 ± 0.01	0.23 ± 0.01	0.19 ± 0.01	0.15 ± 0.01
Isoquercitrin	ND	ND	ND	ND	ND
Quercitrin	ND	0.58 ± 0.04	0.90 ± 0.07	0.78 ± 0.06	0.69 ± 0.05
Benzoic acids					
Gallic acid	ND	ND	ND	ND	ND
p-Hydroxybenzoic acid	0.13 ± 0.05	1.1 ± 0.4	1.5 ± 0.6	1.7 ± 0.6	1.7 ± 0.6
Protocatechuic acid	ND	0.9 ± 0.2	1.8 ± 0.4	1.8 ± 0.3	ND
Volatile polyphenols					
Catechol	ND	3.2 ± 0.1	12.8 ± 0.2	ND	12.4 ± 0.2
Tyrosol	1.20 ± 0.01	22.6 ± 0.1	22.6 ± 0.1	23.5 ± 0.1	23.0 ± 0.1
Overall polyphenols					
FCI (g tannic acid L^{-1})	1.37 ± 0.03	0.96 ± 0.01	0.97 ± 0.02	0.93 ± 0.02	0.80 ± 0.01
TPI ($A_{280\,nm}$)	31.0 ± 0.1	24.8 ± 0.1	21.6 ± 0.1	19.4 ± 0.1	18.4 ± 0.1
Hydroxycinnamics ($A_{320\,nm}$)	24.0 ± 0.1	17.7 ± 0.1	11.9 ± 0.1	9.4 ± 0.1	8.6 ± 0.1
Parameters					
FRAP (mmol Trolox L^{-1})	2.8 ± 0.1	2.9 ± 0.1	3.5 ± 0.2	4.2 ± 0.1	4.1 ± 0.1
PPC (g tannic acid L^{-1})	0.551 ± 0.008	0.431 ± 0.005	0.425 ± 0.010	0.394 ± 0.009	0.397 ± 0.002
Turbidity (NTU)	610 ± 2	304 ± 1	58 ± 1	19.8 ± 0.2	16.6 ± 0.1
Browning ($A_{420\,nm}$)	3.38 ± 0.02	1.32 ± 0.01	1.02 ± 0.01	0.684 ± 0.001	0.221 ± 0.001
E (mV)	324 ± 2	235 ± 2	167 ± 1	166 ± 1	175 ± 1

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

Turbidity and reduction potential (*E*)

Turbidity was measured with a Hanna HI 83749-02 turbidimeter (Hanna Instruments SL, Eibar, Spain) and is given in nephelometric turbidity units (NTU). Reduction potential was measured with a Mettler Inlab® Redox Pro redox combination electrode (Mettler–Toledo, S.A.E., Barcelona, Spain) against an AgCl/Ag reference and is given in mV.

Folin–Ciocalteu index (FCI)

This index gives an idea of the total polyphenolic content of the must or the cider. All polyphenolic compounds are oxidized by means of the so-called Folin–Ciocalteu reagent. This reagent is available commercially and is formed from a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}). The blue coloration produced has a maximum absorption in the region of 750 nm and is proportional to the total quantity of polyphenolic compounds originally present. The result is expressed as an index, absorbance at 750 nm. It was determined as follows according to the OIV method.[11]

Introduce into a 100 mL volumetric flask, strictly in the indicated order, 1 mL of the sample, 50 mL of distilled water, 5 mL of Folin–Ciocalteu reagent and 20 mL of sodium carbonate (200 g L^{-1} solution), then make up to 100 mL with distilled water. Mix to dissolve. Leave for 30 min for the reaction to stabilize. Determine

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Table 4. Phenolic compound contents (mg L^{-1}) and parameters in Manttoni must[a]

Compound	Fermentation days 0	30	62	97	139
Hydroxycinnamic acids					
Caffeic acid	nd	0.25 ± 0.03	0.36 ± 0.04	nd	nd
Chlorogenic acid	17.04 ± 0.06	34.5 ± 0.1	12.26 ± 0.04	nd	nd
p-Coumaric acid	nd	0.6 ± 0.3	nd	nd	nd
4-*p*-Coumaroylquinic acid	nd	72.46 ± 0.05	65.73 ± 0.04	56.88 ± 0.04	57.42 ± 0.04
Hydrocaffeic acid	7.37 ± 0.04	19.4 ± 0.1	7.58 ± 0.04	35.0 ± 0.3	35.6 ± 0.3
(trans)-Ferulic acid	nd	nd	nd	nd	nd
Flavan-3-ols					
(+)-Catechin	1.3 ± 0.2	2.4 ± 0.2	nd	0.7 ± 0.1	2.3 ± 0.2
(−)-Epicatechin	nd	nd	nd	nd	nd
Procyanidin B1	nd	nd	18.4 ± 0.6	9.5 ± 0.3	6.6 ± 0.2
Procyanidin B2	19.0 ± 2.8	nd	16.9 ± 2.5	10.2 ± 1.5	nd
Procyanidin B5	nd	nd	nd	nd	nd
Dihydrochalcons					
Phloretin 2′-*O*-xyloglucoside	nd	20.78 ± 0.07	nd	nd	nd
Phloridzin	nd	2.5 ± 0.5	5.0 ± 0.9	1.4 ± 0.3	1.2 ± 0.2
Flavonols					
Avicularin	nd	nd	nd	nd	nd
Hyperin	nd	nd	nd	nd	nd
Isoquercitrin	nd	nd	nd	nd	nd
Quercitrin	nd	nd	0.3 ± 0.1	nd	nd
Benzoic acids					
Gallic acid	nd	0.6 ± 0.1	0.5 ± 0.1	nd	1.6 ± 0.2
p-Hydroxybenzoic acid	0.64 ± 0.03	0.56 ± 0.03	1.19 ± 0.06	1.03 ± 0.05	0.83 ± 0.04
Protocatechuic acid	nd	0.9 ± 0.2	0.6 ± 0.1	nd	nd
Volatile polyphenols					
Catechol	nd	4.35 ± 0.04	10.2 ± 0.1	9.68 ± 0.09	9.78 ± 0.09
Tyrosol	nd	22.0 ± 0.8	23.9 ± 0.9	21.1 ± 0.8	21.7 ± 0.8
Overall polyphenols					
FCI (tannic acid g L^{-1})	0.87 ± 0.02	0.72 ± 0.01	0.64 ± 0.02	0.54 ± 0.01	0.40 ± 0.01
TPI (A_{280nm})	19.8 ± 0.1	11.6 ± 0.1	11.4 ± 0.1	9.8 ± 0.1	9.0 ± 0.1
Hydroxycinnamics (A_{320nm})	13.7 ± 0.1	7.3 ± 0.1	6.5 ± 0.1	4.9 ± 0.1	4.2 ± 0.1
Parameters					
FRAP (trolox mmol L^{-1})	1.56 ± 0.07	1.65 ± 0.06	1.61 ± 0.03	1.73 ± 0.04	1.88 ± 0.06
PPC (tannic acid g L^{-1})	0.657 ± 0.053	0.296 ± 0.008	0.292 ± 0.003	0.277 ± 0.001	0.269 ± 0.009
Turbidity (NTU)	717 ± 1	60.1 ± 0.1	29.2 ± 0.1	21.95 ± 0.07	8.44 ± 0.05
Browning ($A_{420\ nm}$)	3.012 ± 0.012	0.410 ± 0.001	0.491 ± 0.002	0.211 ± 0.001	0.000 ± 0.001
E (mV)	341 ± 3	204 ± 2	183 ± 2	183 ± 2	180 ± 2

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

the absorbance at 750 nm through a path length of 10 mm with respect to a blank prepared with water in place of the sample. Making use of the Folin–Ciocalteu index (FCI), we calculated the total polyphenolic compounds in g tannic acid L^{-1} by reference to a calibration curve prepared using tannic acid standards ($(2-16) \times 10^{-3}$ g L^{-1}).

Ferric-reducing antioxidant power

This parameter was measured according to the procedure of Benzie and Strain[12] but using Trolox as a standard instead of ascorbic acid. To perform the assay, the following solutions are prepared.

- Acetate buffer 300 mmol L^{-1}, pH 3.6: 3.1 g of sodium acetate trihydrate, 16 mL of glacial acetic acid and distilled water until 1 L.
- 2,4,6-Tripyridyl-*s*-triazine 10 mmol L^{-1} in 40 mmol L^{-1} HCl.
- $FeCl_3 \cdot 6H_2O$ 20 mmol L^{-1}.
- Working FRAP reagent: mixture of the above three reagents in the ratio 10:1:1 prepared at the time of use.

The assay is achieved as follows. The sample (100 μL) is mixed with 3 mL of working FRAP reagent thermostated at 37 °C and the absorption at 593 nm (*A*) is measured after 4 min. Trolox standards

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Table 5. Phenolic compound contents (mg L^{-1}) and parameters in Mixture must[a]

Compound	Fermentation days 0	30	58	92	139
Hydroxycinnamic acids					
Caffeic acid	1.0 ± 0.3	18.6 ± 5.9	6.1 ± 1.9	1.2 ± 0.4	0.9 ± 0.3
Chlorogenic acid	106.0 ± 2.0	208.4 ± 3.9	178.3 ± 3.4	107.2 ± 2.0	75.7 ± 1.4
p-Coumaric acid	1.0 ± 0.2	1.9 ± 0.3	1.6 ± 0.3	1.0 ± 0.2	0.8 ± 0.1
4-*p*-Coumaroylquinic acid	81.1 ± 1.3	205.6 ± 3.2	207.9 ± 3.2	199.9 ± 3.1	194.8 ± 3.0
Hydrocaffeic acid	11.48 ± 0.05	ND	14.25 ± 0.07	80.7 ± 0.4	92.7 ± 0.4
(*trans*)-Ferulic acid	ND	1.4 ± 0.3	1.3 ± 03	ND	ND
Flavan-3-ols					
(+)-Catechin	3.4 ± 0.3	13.1 ± 1.1	10.1 ± 0.8	11.4 ± 0.9	8.2 ± 0.7
(−)-Epicatechin	32.7 ± 3.3	62.7 ± 6.4	69.4 ± 7.0	57.1 ± 5.8	31.5 ± 3.2
Procyanidin B1	5.8 ± 0.3	148.9 ± 7.1	149.4 ± 7.1	112.4 ± 5.3	87.6 ± 4.2
Procyanidin B2	281 ± 18	293 ± 19	313 ± 20	278 ± 18	176 ± 11
Procyanidin B5	ND	8.3 ± 0.8	9.0 ± 0.8	9.5 ± 0.9	ND
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	20.8 ± 0.4	188.4 ± 3.9	201.1 ± 4.2	200.5 ± 4.1	162.2 ± 3.4
Phloridzin	ND	15.1 ± 1.6	18.2 ± 1.9	19.0 ± 2.0	12.1 ± 1.3
Flavonols					
Avicularin	ND	ND	ND	ND	ND
Hyperin	ND	0.31 ± 0.02	0.31 ± 0.02	0.27 ± 0.01	0.15 ± 0.01
Isoquercitrin	ND	ND	ND	ND	ND
Quercitrin	ND	1.07 ± 0.01	1.03 ± 0.01	0.84 ± 0.01	0.409 ± 0.004
Benzoic acids					
Gallic acid	ND	0.82 ± 0.03	1.07 ± 0.04	1.02 ± 0.04	1.00 ± 0.04
p-Hydroxybenzoic acid	0.08 ± 0.01	ND	ND	ND	1.4 ± 0.2
Protocatechuic acid	ND	3.7 ± 0.2	2.4 ± 0.2	1.20 ± 0.08	1.27 ± 0.08
Volatile polyphenols					
Catechol	ND	ND	ND	2.8 ± 0.2	7.8 ± 0.6
Tyrosol	ND	24.4 ± 0.7	26.0 ± 0.8	26.0 ± 0.8	26.7 ± 0.8
Overall polyphenols					
FCI (g tannic acid L^{-1})	2.06 ± 0.04	1.83 ± 0.04	1.70 ± 0.02	1.63 ± 0.03	1.35 ± 0.03
TPI ($A_{280\,nm}$)	40.3 ± 0.1	36.2 ± 0.1	34.2 ± 0.1	32.2 ± 0.1	32.2 ± 0.1
Hydroxycinnamics ($A_{320\,nm}$)	29.5 ± 0.1	26.8 ± 0.1	22.8 ± 0.1	18.2 ± 0.1	17.0 ± 0.1
Parameters					
FRAP (mmol Trolox L^{-1})	5.0 ± 0.2	5.4 ± 0.3	6.0 ± 0.4	5.8 ± 0.3	5.6 ± 0.3
PPC (g tannic acid L^{-1})	0.776 ± 0.017	0.662 ± 0.014	0.658 ± 0.012	0.627 ± 0.007	0.662 ± 0.007
Turbidity (NTU)	732 ± 4	486.5 ± 0.7	41.15 ± 0.07	17.85 ± 0.07	5.7 ± 0.1
Browning ($A_{420\,nm}$)	2.53 ± 0.03	1.14 ± 0.01	0.871 ± 0.004	0.615 ± 0.001	0.999 ± 0.001
E (mV)	328 ± 3	284 ± 2	228 ± 2	210 ± 2	200 ± 2

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

(0.2–1 mmol L^{-1}), as well as a blank, are processed in the same way to construct the calibration curve (*A versus* mmol Trolox L^{-1}). The results are given in mmol Trolox L^{-1}.

Absorbances at 280, 320 and 420 nm

These were measured in 10 mm cuvettes against water by means of an Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The absorbance at 280 nm is proportional to the total polyphenolic content and is called the total polyphenol index (TPI). The absorbance at 320 nm is proportional to the total hydroxycinnamic acid content.[13] Dilutions, usually 1:50, were made with water when necessary and the absorbance was corrected by the dilution factor. In the case of absorbance at 420 nm (browning), where dilutions are not allowed, cuvettes of smaller optical paths were used and the value obtained was corrected to a 10 mm optical path.[11]

Protein-precipitating capacity (PPC)

To measure this parameter, the following reagents are prepared.[9]

- Buffer A: 0.20 mol L^{-1} acetic acid, 0.17 mol L^{-1} NaCl, pH adjusted to 4.9 with NaOH (11.4 mL of glacial acetic acid, 9.86 g of NaCl dissolved in about 800 mL of water, adjust to pH 4.9 with a solution of NaOH, make up to a final volume of 1 L with water).
- BSA: 1 mg mL^{-1} bovine serum albumin (BSA) in buffer A.

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Table 6. Phenolic compound contents (mg L^{-1}) and parameters in Moko must[a]

Compound	Fermentation days 0	30	58	92	142
Hydroxycinnamic acids					
Caffeic acid	2.1 ± 0.2	3.4 ± 0.4	5.9 ± 0.6	7.1 ± 0.8	6.6 ± 0.2
Chlorogenic acid	396.1 ± 4.5	429.5 ± 4.9	398.5 ± 4.6	420.5 ± 4.8	400.9 ± 0.2
p-Coumaric acid	1.9 ± 0.4	2.3 ± 0.4	2.7 ± 0.5	2.7 ± 0.5	2.7 ± 0.4
4-*p*-Coumaroylquinic acid	96.3 ± 1.6	106.1 ± 1.8	134.3 ± 2.3	134.2 ± 2.3	133.7 ± 0.3
Hydrocaffeic acid	13.9 ± 0.1	12.8 ± 0.1	15.8 ± 0.1	12.9 ± 0.1	13.2 ± 0.1
(*trans*)-Ferulic acid	1.58 ± 0.08	ND	ND	ND	ND
Flavan-3-ols					
(+)-Catechin	18.9 ± 2.7	18.2 ± 2.6	25.9 ± 3.7	24.6 ± 3.5	10.6 ± 1.1
(−)-Epicatechin	114.5 ± 1.8	92.2 ± 1.5	145.0 ± 2.3	166.2 ± 2.6	152.9 ± 0.2
Procyanidin B1	87.8 ±1.8	90.4 ± 1.9	93.5 ± 2.0	104.0 ± 2.2	83.3 ± 2.9
Procyanidin B2	674 ± 12	856 ± 15	855 ± 15	843 ± 15	771 ± 10
Procyanidin B5	23.5 ± 0.8	18.2 ± 0.6	23.9 ± 0.5	26.2 ± 0.8	23.2 ± 0.7
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	377 ± 15	460 ± 18	455 ± 18	452 ± 18	435 ± 6
Phloridzin	11.9 ± 0.7	11.2 ± 0.6	18.3 ± 1.1	28.5 ± 1.7	27.4 ± 0.2
Flavonols					
Avicularin	ND	ND	ND	ND	ND
Hyperin	0.60 ± 0.05	0.41 ± 0.03	0.54 ± 0.04	0.59 ± 0.05	0.58 ± 0.04
Isoquercitrin	0.33 ± 0.01	0.46 ± 0.02	0.56 ± 0.02	0.80 ± 0.03	0.30 ± 0.02
Quercitrin	1.25 ± 0.02	1.02 ± 0.02	1.47 ± 0.02	1.74 ± 0.03	1.64 ± 0.02
Benzoic acids					
Gallic acid	0.58 ± 0.02	ND	0.54 ± 0.02	0.56 ± 0.02	0.57 ± 0.01
p-Hydroxybenzoic acid	1.38 ± 0.07	ND	1.17 ± 0.06	1.71 ± 0.09	1.68 ± 0.04
Protocatechuic acid	ND	1.60 ± 0.01	2.73 ± 0.02	1.97 ± 0.02	2.01 ± 0.02
Volatile polyphenols					
Catechol	1.8 ± 0.2	1.7 ± 0.1	2.0 ± 0.2	1.7 ± 0.1	ND
Tyrosol	ND	12.2 ± 0.1	14.7 ± 0.3	14.6 ± 0.3	14.8 ± 0.2
Overall polyphenols					
FCI (g tannic acid L^{-1})	3.37 ± 0.04	3.04 ± 0.02	3.12 ± 0.03	2.86 ± 0.03	2.64 ± 0.02
TPI ($A_{280\,nm}$)	58.4 ± 0.1	59.1 ± 0.1	59.2 ± 0.1	59.2 ± 0.1	58.2 ± 0.1
Hydroxycinnamics ($A_{320\,nm}$)	36.0 ± 0.1	37.3 ± 0.1	37.1 ± 0.1	36.9 ± 0.1	36.2 ± 0.1
Parameters					
FRAP (mmol Trolox L^{-1})	9.8 ± 0.2	9.3 ± 0.2	10.0 ± 0.3	9.5 ± 0.3	9.3 ± 0.2
PPC (g tannic acid L^{-1})	1.055 ± 0.012	1.070 ± 0.009	1.121 ± 0.023	1.063 ± 0.004	1.079 ± 0.025
Turbidity (NTU)	432 ± 3	471 ± 3	138 ± 1	64.4 ± 0.2	15.1 ± 0.1
Browning ($A_{420\,nm}$)	1.56 ± 0.01	1.31 ± 0.01	0.953 ± 0.004	0.821 ± 0.001	0.959 ± 0.003
E (mV)	324 ± 2	275 ± 2	233 ± 2	214 ± 2	191 ± 1

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

- SDS/TEA: 50 mL L^{-1} triethanolamine (TEA), 10 g L^{-1} sodium dodecyl sulfate (SDS) (50 mL of TEA, 10 g of SDS, make up to 1 L with water).
- $FeCl_3$: 0.01 mol L^{-1} $FeCl_3$ in 0.01 mol L^{-1} HCl. To make 0.01 mol L^{-1} HCl, dilute 0.83 mL of concentrated HCl to 1 L with water. Dissolve 1.62 g of $FeCl_3$ in 1 L of the acid solution and allow it to sit for several hours. Gravity filter through paper.

The assay is performed as follows. Dispense 2 mL of BSA into a 15 mL centrifuge tube and add 1 mL of sample solution. The solution cannot contain any acetone, since even traces of acetone inhibit the precipitation reaction. Mix immediately and allow to sit for 24 h at 4 °C. Centrifuge at 3000 × *g* for 15 min and pour off the supernatant. Redissolve the pellet in 4 mL of SDS/TEA, add 1 mL of $FeCl_3$ and vortex immediately. After about 15 min, read the absorbance at 510 nm (*A*). Tannic acid standards (0.2–1.4 g L^{-1} in water), as well as a blank ($FeCl_3$ in SDS/TEA), are processed in the same way to construct the calibration curve (*A versus* g tannic acid L^{-1}), except that they need to sit for only 15 min at room temperature after mixing with BSA. The results are given in g tannic acid L^{-1}.

Polyphenolic compounds

The determination of individual polyphenolic compounds was performed by high-performance liquid chromatography (HPLC) using the filtered samples. The method of Suárez *et al.*[14] was employed. An Agilent 1100 Series liquid chromatograph (Agilent

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Table 7. Phenolic compound contents (mg L^{-1}) and parameters in Patzuloa must[a]

Compound	Fermentation days 0	30	58	92	134
Hydroxycinnamic acids					
Caffeic acid	0.24 ± 0.06	2.3 ± 0.3	2.1 ± 0.4	1.2 ± 0.1	1.2 ± 0.1
Chlorogenic acid	180.2 ± 1.9	292.6 ± 3.2	197.1 ± 2.1	118.4 ± 1.3	72.3 ± 0.8
p-Coumaric acid	2.35 ± 0.09	2.88 ± 0.11	2.11 ± 0.08	1.37 ± 0.05	0.95 ± 0.04
4-*p*-Coumaroylquinic acid	218.6 ± 0.5	585.7 ± 1.4	545.5 ± 1.3	504.3 ± 1.2	481.7 ± 1.2
Hydrocaffeic acid	7.37 ± 0.04	55.9 ± 0.3	92.4 ± 0.4	149.4 ± 0.7	168.5 ± 0.8
(*trans*)-Ferulic acid	ND	3.22 ± 0.08	3.19 ± 0.08	2.80 ± 0.07	2.41 ± 0.06
Flavan-3-ols					
(+)-Catechin	2.5 ± 0.1	13.6 ± 0.7	18.1 ± 1.0	14.8 ± 0.8	12.2 ± 0.6
(−)-Epicatechin	30.1 ± 7.5	33.6 ± 8.4	36.6 ± 9.1	31.9 ± 8.0	26.5 ± 6.6
Procyanidin B1	ND	130.5 ± 7.8	151.9 ± 9.1	142.4 ± 8.5	137.9 ± 8.2
Procyanidin B2	100.1 ± 0.5	114.4 ± 0.6	109.7 ± 0.5	112.1 ± 0.6	112.3 ± 0.6
Procyanidin B5	ND	ND	ND	ND	ND
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	5.01 ± 0.01	169.1 ± 0.5	160.2 ± 0.5	143.4 ± 0.4	120.6 ± 0.4
Phloridzin	ND	4.07 ± 0.06	4.26 ± 0.06	3.11 ± 0.04	3.11 ± 0.04
Flavonols					
Avicularin	ND	ND	ND	ND	ND
Hyperin	ND	ND	ND	ND	ND
Isoquercitrin	ND	ND	ND	ND	ND
Quercitrin	ND	1.25 ± 0.02	1.61 ± 0.03	0.87 ± 0.01	0.68 ± 0.01
Benzoic acids					
Gallic acid	ND	1.31 ± 0.01	1.38 ± 0.01	1.33 ± 0.01	1.39 ± 0.01
p-Hydroxybenzoic acid	0.64 ± 0.06	3.5 ± 0.3	ND	ND	2.8 ± 0.3
Protocatechuic acid	ND	1.9 ± 0.1	2.1 ± 0.1	1 .81 ± 0.09	1.76 ± 0.09
Volatile polyphenols					
Catechol	ND	4.3 ± 0.3	6.0 ± 0.4	4.2 ± 0.2	5.4 ± 0.3
Tyrosol	ND	25.6 ± 0.1	25.2 ± 0.1	25.2 ± 0.1	25.4 ± 0.1
Overall polyphenols					
FCI (g tannic acid L^{-1})	2.33 ± 0.03	1.90 ± 0.01	1.90 ± 0.02	1.77 ± 0.01	1.64 ± 0.01
TPI ($A_{280\,nm}$)	61.8 ± 0.1	44.1 ± 0.1	41.3 ± 0.1	37.8 ± 0.1	35.4 ± 0.1
Hydroxycinnamics ($A_{320\,nm}$)	60.7 ± 0.1	41.6 ± 0.1	36.4 ± 0.1	30.1 ± 0.1	26.2 ± 0.1
Parameters					
FRAP (mmol Trolox L^{-1})	5.39 ± 0.04	5.28 ± 0.05	6.11 ± 0.07	6.17 ± 0.07	6.32 ± 0.06
PPC (g tannic acid L^{-1})	1.07 ± 0.01	0.65 ± 0.01	0.60 ± 0.02	0.56 ± 0.01	0.546 ± 0.007
Turbidity (NTU)	1023 ± 1	390 ± 6	155.5 ± 0.7	114.0 ± 0.5	38.8 ± 0.5
Browning ($A_{420\,nm}$)	6.11 ± 0.03	1.29 ± 0.01	0.963 ± 0.007	0.987 ± 0.007	0.550 ± 0.005
E (mV)	280 ± 2	255 ± 2	175 ± 1	163 ± 1	157 ± 1

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

Technologies) was used with a Nucleosil® 120-3 C_{18} column (250 mm ×4.6 mm, 3 µm; Macherey-Nagel, Düren, Germany). The flow rate was 0.8 mL min^{-1}, the column temperature 25 °C and the injected volume 50 μL. The elution solvents were aqueous 20 mL L^{-1} acetic acid (solvent A) and pure 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 stabilization. Column effluents were monitored at four wavelengths: 280 nm for benzoic acids, flavan-3-ols, dihydrochalcones, catechol and tyrosol, 313 nm for hydroxycinnamic acids and 355 nm for flavonols. Phenolic compounds were quantified by the external standard method from peak areas. They were identified by means of their retention times and their 190–900 nm spectra, which were previously recorded by injecting the pure compounds.

The polyphenolic compounds determined are listed in Tables 1 and 2, where calibration data are also given. Polyphenols of Table 2 were calibrated to a single point owing to their high cost. Polyphenols of Table 1 were dissolved in 900 mL L^{-1} ethanol containing 10 g L^{-1} ascorbic acid to avoid oxidation. By dilution of these stock solutions with water, standards of appropriate concentrations were obtained, in which ethanol was adjusted to 60 mL L^{-1} to avoid precipitation, and ascorbic acid to 10 g L^{-1} to avoid oxidation. Polyphenols of Table 2 were dissolved in 60 mL L^{-1} ethanol containing 10 g L^{-1} ascorbic acid.

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Table 8. Phenolic compound contents (mg L^{-1}) and parameters in Txalaka must[a]

	Fermentation days				
Compound	0	30	64	98	139
Hydroxycinnamic acids					
Caffeic acid	0.91 ± 0.02	8.5 ± 0.2	8.9 ± 0.2	2.6 ± 0.6	1.1 ± 0.2
Chlorogenic acid	54.6 ± 4.2	17.5 ± 1.4	45.2 ± 3.5	40.6 ± 3.1	36.3 ± 2.8
p-Coumaric acid	0.40 ± 0.04	0.32 ± 0.03	0.17 ± 0.02	0.37 ± 0.04	0.20 ± 0.02
4-*p*-Coumaroylquinic acid	89.9 ± 6.4	119.9 ± 8.5	128.1 ± 9.1	124.3 ± 8.9	117.9 ± 8.4
Hydrocaffeic acid	10.32 ± 0.02	ND	10.89 ± 0.02	18.77 ± 0.04	21.50 ± 0.04
(*trans*)-Ferulic acid	ND	ND	ND	ND	ND
Flavan-3-ols					
(+)-Catechin	4.3 ± 0.3	12.3 ± 0.9	4.6 ± 0.4	8.6 ± 0.7	5.1 ± 0.4
(−)-Epicatechin	23.5 ± 1.8	52.3 ± 4.0	63.9 ± 4.9	60.9 ± 4.6	49.6 ± 3.8
Procyanidin B1	27.5 ± 1.1	47.6 ± 1.8	62.5 ± 2.3	57.4 ± 2.2	57.2 ± 2.1
Procyanidin B2	130 ± 1	263 ± 3	266 ± 4	262 ± 3	230 ± 3
Procyanidin B5	ND	6.4 ± 0.6	7.4 ± 0.7	7.8 ± 0.7	6.0 ± 0.6
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	17.5 ± 1.0	32.1 ± 1.9	35.9 ± 2.1	37.7 ± 2.2	32.6 ± 1.9
Phloridzin	1.4 ± 0.1	5.3 ± 0.4	7.9 ± 0.5	9.9 ± 0.7	6.2 ± 0.4
Flavonols					
Avicularin	ND	ND	ND	ND	ND
Hyperin	ND	0.24 ± 0.01	0.29 ± 0.01	0.32 ± 0.02	0.17 ± 0.01
Isoquercitrin	ND	ND	ND	ND	ND
Quercitrin	ND	0.46 ± 0.03	0.63 ± 0.04	0.83 ± 0.05	0.49 ± 0.03
Benzoic acids					
Gallic acid	0.55 ± 0.07	0.68 ± 0.08	0.78 ± 0.09	0.82 ± 0.09	0.82 ± 0.10
p-Hydroxybenzoic acid	ND	ND	ND	ND	2.4 ± 0.1
Protocatechuic acid	ND	2.8 ± 0.2	2.2 ± 0.2	1.5 ± 0.1	1.6 ± 0.1
Volatile polyphenols					
Catechol	ND	ND	ND	ND	ND
Tyrosol	ND	13.59 ± 0.04	15.62 ± 0.05	16.35 ± 0.05	16.34 ± 0.05
Overall polyphenols					
FCI (g tannic acid L^{-1})	1.62 ± 0.01	1.29 ± 0.02	1.24 ± 0.01	1.13 ± 0.01	0.98 ± 0.01
TPI ($A_{280\,nm}$)	22.8 ± 0.1	22.3 ± 0.1	21.8 ± 0.1	21.2 ± 0.1	19.5 ± 0.1
Hydroxycinnamics ($A_{320\,nm}$)	12.1 ± 0.1	11.3 ± 0.1	11.2 ± 0.1	10.0 ± 0.1	8.4 ± 0.1
Parameters					
FRAP (mmol Trolox L^{-1})	4.10 ± 0.09	3.47 ± 0.07	3.98 ± 0.08	3.95 ± 0.06	3.60 ± 0.06
PPC (g tannic acid L^{-1})	0.71 ± 0.02	0.56 ± 0.02	0.53 ± 0.01	0.520 ± 0.006	0.525 ± 0.008
Turbidity (NTU)	244 ± 4	544 ± 2	99.6 ± 0.4	36.8 ± 0.3	15.2 ± 0.1
Browning ($A_{420\,nm}$)	0.870 ± 0.007	0.585 ± 0.005	0.490 ± 0.005	0.493 ± 0.002	0.000 ± 0.001
E (mV)	308 ± 2	231 ± 2	173 ± 2	177 ± 1	164 ± 1

FCI, Folin–Ciocalteu index; TPI, total polyphenol index; FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; ND, not detected.
[a] Average of two measurements ± standard deviation.

RESULTS AND DISCUSSION

The following calibration equations were obtained from n experimental points for FCI, FRAP and PPC to calculate the concentration of the standard used (C) from the absorbance (A) at the given wavelength.

$$\text{FCI}: A_{750} = 88.8393C + 0.0448, C = \text{g tannic acid L}^{-1}, n = 8, R = 0.9999$$

$$\text{FRAP}: A_{593} = 1.3564C + 0.1160, C = \text{mmol Trolox L}^{-1}, n = 6, R = 0.9998$$

$$\text{PPC}: A_{510} = -0.4982C^2 + 1.8721C - 0.3034, C = \text{g tannic acid L}^{-1}, n = 7, R = 0.9997$$

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Table 9. Correlations between parameters and polyphenolics: Pearson coefficients[a]

Polyphenolic compound	FRAP	PPC	Turbidity	Browning	*E*
Hydroxycinnamic acids					
Caffeic acid	0.347	0.313	0.086	−0.128	0.105
Chlorogenic acid	**0.889**	**0.858**	0.178	0.180	0.316
p-Coumaric acid	**0.774**	**0.771**	0.281	0.343	0.300
4-*p*-Coumaroylquinic acid	0.294	0.077	−0.058	−0.013	−0.241
Hydrocaffeic acid	0.065	−0.280	−0.417	−0.251	**−0.546**
(*trans*)-Ferulic acid	0.091	−0.201	−0.133	−0.120	−0.250
Flavan-3-ols					
(+)-Catechin	**0.840**	**0.658**	−0.052	−0.148	0.042
(−)-Epicatechin	**0.866**	**0.806**	−0.068	−0.074	0.081
Procyanidin B1	**0.594**	0.283	−0.258	−0.279	−0.210
Procyanidin B2	**0.876**	**0.835**	0.008	−0.054	0.184
Procyanidin B5	**0.809**	**0.748**	−0.084	−0.116	0.124
Dihydrochalcones					
Phloretin 2′-*O*-xyloglucoside	**0.880**	**0.741**	−0.070	−0.067	0.133
Phloridzin	0.448	0.227	−0.437	−0.298	−0.314
Flavonols					
Hyperin	**0.739**	**0.629**	−0.177	−0.194	0.011
Quercitrin	**0.774**	**0.494**	−0.300	−0.270	−0.230
Benzoic acids					
Gallic acid	0.151	−0.105	−0.409	−0.422	−0.385
p-Hydroxybenzoic acid	0.114	−0.017	−0.264	−0.172	−0.282
Protocatechuic acid	0.387	0.171	−0.242	−0.344	−0.327
Volatile polyphenols					
Catechol	−0.292	**−0.511**	−0.454	−0.353	**−0.491**
Tyrosol	−0.096	**−0.498**	**−0.710**	**−0.640**	**−0.768**
Overall polyphenols					
FCI	**0.937**	**0.942**	0.306	0.299	0.398
TPI	**0.902**	**0.936**	0.365	0.440	0.352
Hydroxycinnamics	**0.719**	**0.810**	**0.514**	**0.617**	0.378

FRAP, ferric-reducing antioxidant power; PPC, protein-precipitating capacity; *E*, reduction potential against Ag/AgCl reference; FCI, Folin–Ciocalteu index; TPI, total polyphenol index.
[a] The significant correlations are indicated in **bold** type.

In the original description of the PPC assay, Hagerman and Butler[9] reported a straight line. However, we obtained a much better fit with a second-order polynomial, hence we used it in all calculations. Besides, in the original method the absorbance at 510 nm is plotted against mg tannic acid, while we considered it more convenient to express tannic acid content in concentration units, so we used g L^{-1} (mg mL^{-1}).

The polyphenolic compounds determined were the most habitual in Basque musts and ciders:[15,16]

- Hydroxycinammic acids: caffeic acid, chlorogenic acid, *p*-coumaric acid, 4-*p*-coumaroylquinic acid, hydrocaffeic acid and (*trans*)-ferulic acid.
- Flavan-3-ols: (+)-catechin, (−)-epicatechin, procyanidin B1, procyanidin B2 and procyanidin B5.
- Dihydrochalcones: phloretin 2′-*O*-xyloglucoside and phloridzin.
- Flavonols: avicularin, hyperin (or hyperoside), isoquercitrin and quercitrin.

Besides, we determined the benzoic acids gallic acid, *p*-hydroxybenzoic acid and protocatechuic acid as well as the volatile phenols catechol and tyrosol, which are determined by many authors.[17,18]

Figure 1 shows the chromatogram obtained with pure standards. The overlapping of some peaks (e.g. peaks 13 and 14) was avoided by measuring at three different wavelengths, as explained above. Figure 2 shows a representative chromatogram obtained with one of our cider samples.

Tables 3–8 show the concentrations obtained throughout the five samplings for the individual polyphenolic compounds, as well as the values for the parameters FRAP, PPC, turbidity, browning and reduction potential. From these data, correlations between the five parameters and the polyphenols were calculated as Pearson coefficients at the 0.01 level. They are listed in Table 9.

As expected, FRAP and PPC parameters are significantly correlated with FCI and TPI. These two indices are related to total polyphenolic content, and polyphenolic compounds have antioxidant and precipitating properties. Besides, FRAP and PPC are also significantly correlated with hydroxycinnamics, and so are turbidity and browning.

Regarding individual polyphenolics, multivariate analysis was performed on data in order to explore the effect of the individual phenol contents in a multivariate way. Separate multivariate linear regression (MLR) models were built for parameters FRAP, PPC, turbidity, browning and reduction potential as dependent *Y* variable and individual phenol contents as predictor *X*

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Table 10. MLR parameters for regression models[a] using significant phenol contents to estimate cider parameters and % estimation (mean ± standard deviation) obtained after applying MLR equations

Parameter	Multiple correlation	MLR equation		% estimation
		X variables[b]	*B* coefficients	
FRAP	0.944		b0: 2.3568	106 ± 27
		x1: **Caffeic acid**	b1: −0.1435	
		x2: **Procyanidin B1**	b2: 0.0193	
		x3: **Procyanidin B2**	b3: 0.0076	
PPC	0.964		b0: 0.5262	101 ± 12
		x1: *p*-Coumaric acid	b1: 0.1047	
		x2: (−)-Epicatechin	b2: 0.0018	
		x3: **Procyanidin B2**	b3: 0.0007	
		x4: **Procyanidin B5**	b4: −0.0247	
		x5: Hyperin	b5: 0.0388	
		x6: Tyrosol	b6: −0.0105	
Turbidity	0.787		b0: 625.93	210 ± 480
		x1: **Phloridzin**	b1: −16.6404	
		x2: Quercitrin	b2: 167.5879	
		x3: Gallic acid	b3: −211.6035	
		x4: **Tyrosol**	b4: −12.6223	
Browning	0.743		b0: 2.0655	127 ± 105
		x1: ***p*-Coumaric acid**	b1: 0.5001	
		x2: Gallic acid	b2: −0.4566	
		x3: Protocatechuic acid	b3: −0.2539	
		x4: Tyrosol	b4: −0.0517	
Potential	0.802		b0: 286.1248	102 ± 16
		x1: *p*-Coumaric acid	b1: 13.5755	
		x2: **Tyrosol**	b2: −4.5877	

[a] The regression models are significant at 0.01 level.
[b] Variables in **bold** type are significant at 0.01 level.

variables. Those phenols showing individual correlations higher than 0.300 (absolute value) with the parameter under evaluation were used to perform the MLR analysis. MLR models provided information about phenols with a significant effect on the studied parameter. Individual phenols showing a significant effect ($P = 0.05$) were selected to compute a final MLR model for each of the parameters. The regression models were significant at 0.01 level in all cases and the variables shown in Table 10 are the significant ones at 0.05 level. Finally, the value of the parameter was predicted ($\text{PARAMETER}_{\text{predicted}}$) for all samples using the MLR equation (Table 10) and compared with the observed value ($\text{PARAMETER}_{\text{observed}}$) according to the expression[19]

$$\%\ \text{estimation} = \left(\text{PARAMETER}_{\text{predicted}}/\text{PARAMETER}_{\text{observed}}\right) \times 100$$

MLR models provided high values for multiple correlation coefficients and good estimation percentages for FRAP and PPC. Lower multiple correlation values were obtained for turbidity, browning and potential. However, the last of these was quite well predicted, with an average estimation value of 102%. Estimated values corresponding to turbidity and browning show that, even though significant regression models are obtained when individual phenol contents are used as predictor variables, these variables do not provide enough information to predict these two parameters properly.

Procyanidin B1 and procyanidin B2 show a significant positive effect on FRAP, so they are the polyphenolic compounds which most contribute to the antioxidant power in Basque ciders. On the other hand, *p*-coumaric acid, (−)-epicatechin, procyanidin B2 and hyperin have a significant positive effect on PPC, being therefore the polyphenolic compounds which most contribute to the precipitating capacity. With respect to reduction potential *E*, only *p*-coumaric acid shows a positive effect on it.

It should be stressed that the main positive contribution to antioxidant power, precipitating capacity or reduction potential does not come necessarily from the most concentrated polyphenols. Although chlorogenic acid, 4-*p*-coumaroylquinic acid, procyanidin B2 and phloretin 2′-*O*-xyloglucoside are the most concentrated polyphenols in the studied ciders, only procyanidin B2 shows a significant effect on antioxidant power and precipitating capacity, and none of them does on reduction potential. On the other hand, less concentrated polyphenols such as *p*-coumaric acid and (−)-epicatechin offer remarkable contributions to some of these parameters. Even minor polyphenols such as hyperin contribute.

Although some significant effects were found on turbidity and browning, the regression models did not provide satisfactory predictions for these parameters. Some authors report a correlation between initial chlorogenic acid content and final browning,[20] but we have not seen this in our results.

With respect to negative contributions, caffeic acid has a significant negative effect on FRAP, and procyanidin B5 and tyrosol on PPC. However, the most remarkable negative contribution is that of tyrosol (a fermentation product) on reduction potential. This is interesting because it means that ciders with higher tyrosol

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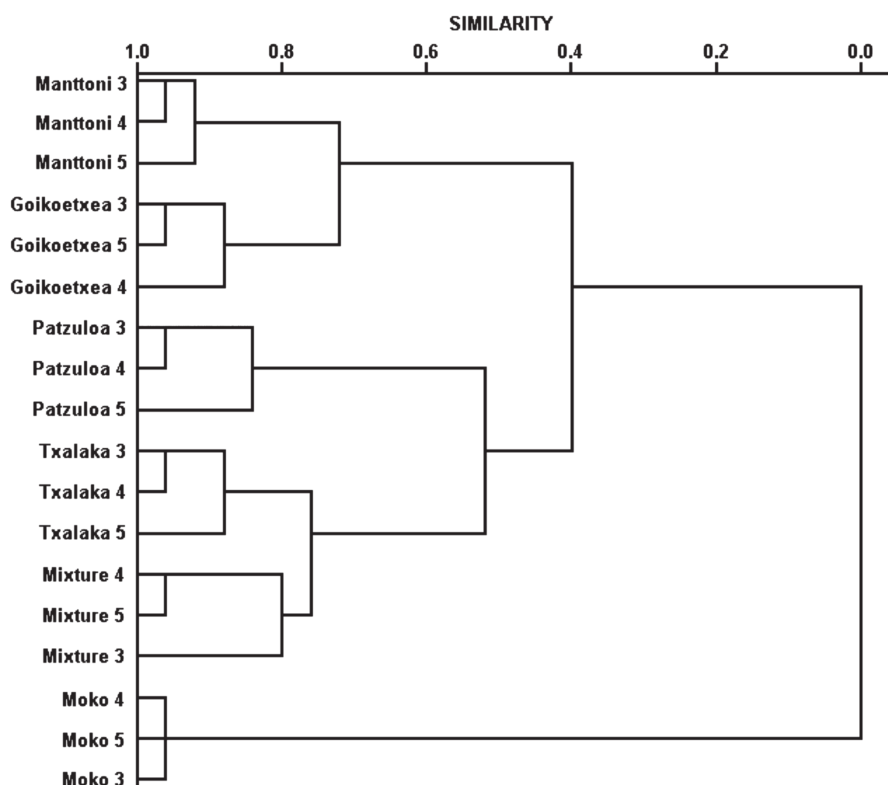

Figure 3. Cluster analysis: dendogram for last three samplings.

concentration will have less reduction potential, which indicates higher antioxidant reservoir.

Tyrosol is a metabolite from tyrosine formed by yeasts during alcoholic fermentation and is present in wine, olive oil and other plant-derived products. Increasing interest has been focused on this compound because of its antioxidant, anticarcinogenic, cardioprotective and antimicrobial properties.[21] It has been determined in many beverages such as Spanish Jerez red wines (20.38–44.46 mg L^{-1}),[21] Suavia 'Le Rive' white wine (17.06 mg L^{-1}),[22] Spanish white wines (10.15–24.77 mg L^{-1})[23] and Spanish Merlot red wine (31.36 mg L^{-1}).[24] It has been suggested that white wines can provide cardioprotection similar to red wines, although containing lower quantities of polyphenols, if they are rich in tyrosol.[25–27] This is consistent with the negative effect of tyrosol on reduction potential found in our work. As the tyrosol contents in our ciders (14.8–26.7 mg L^{-1}) and in white wines are similar, ciders are supposed to provide similar health benefits to white wines.

To conclude the study, we have used hierarchical cluster analysis in order to find patterns of clustering between the ciders obtained, based on the individual polyphenol profile. The data matrix included as objects the last three samplings (3, 4 and 5) of the six ciders studied (18 objects). These samplings correspond to ciders with completed alcoholic and malolactic fermentation, which means that they are finished or almost finished. The variables were the 22 individual polyphenols determined. Data were normalized by autoscaling. Square Euclidean distance was used as similarity criterion and furthest neighbour as clustering method. The results are presented in Fig. 3 as a dendogram. Six groups are obtained, corresponding to the six ciders elaborated. Consequently, ciders can be classified attending only to their polyphenol composition. The smallest difference is obtained between Txalaka and Mixture ciders. As Mixture cider was obtained by mixing the other five, it is not surprising that it is similar to some of them.

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