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Original Article

Phenolic and antioxidant composition of cider

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ABSTRACT

Forty-four Asturian ciders were analysed for total phenolic content, phenolic profiles, and antioxidant capacity by the FRAP and the DPPH radical assays. The Folin index of ciders ranged between 446 and 1180 mg gallic acid/L. The phenolic profile of Asturian cider is mainly constituted by phenolic acids, flavan-3-ols, volatile phenols and dihydrochalcones. The methods to determine the antioxidant activity of ciders were optimised in terms of suitable reaction times, within-day and between-day repeatability. Thus, time courses of ciders in the DPPH and the FRAP assays were performed. Mean values for antioxidant activity of cider, expressed in ascorbic acid equivalents were 2.9 mM (as determined by the DPPH assay). When the FRAP assay was used, the antioxidant capacity of cider increased with the reaction time from 3.8 mM (4 min) to 5.4 mM (40 min). Multivariate approaches based on phenolic composition can be useful to predict the antioxidant capacity of cider. Folin index and flavanols and hydrocaffeic acid contents were the best predictors for antioxidant activity.

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1. Introduction

The well-recognised benefits of the Mediterranean diet, the French paradox or the popular proverb "an apple a day keeps the doctor away" are only a few of the many examples suggesting an association between diets rich in vegetables and fruits and reduced risk of cardiovascular diseases and certain types of cancer (Williamson and Manach, 2005). Many of these disorders are caused by free radicals, so the increasing interest in elucidating the mechanisms involved in the antioxidant activity of different compounds present in foods and beverages is justified.

Polyphenols work as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid oxidation. The ability of polyphenols to act as antioxidants depends on their structure, flavonoids being among the most potent antioxidants. Polyphenols are widely distributed in plants and they have been shown as the main contributors to the antioxidant activity of most of foods and beverages (Manach et al., 2004; Higdon and Frei, 2003); hence, many studies have been devoted to assess the accessibility, metabolism and sources of diet polyphenols (Manach et al., 2004; Pulido et al., 2003; Saura-Calixto et al., 2007).

The estimation of the antioxidant capacity of foods and beverages has been accomplished by various methods based on different reaction mechanisms (Roginsky and Lissi, 2005). Among them, those involving stable radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), or the ferric reducing antioxidant power (FRAP) assay are some of the most used to assess the antioxidant capacity in a variety of foods and beverages (De Beer et al., 2003; Lee et al., 2003; Pellegrini et al., 2000; Moreira et al., 2005). Most of the studies intend to explain the influence of the phenolic profile on the antioxidant capacity of different products. Although the conclusions inferred from those studies revealed significant relationships between antioxidant activity and different families of polyphenols, especially flavanols, flavonols and phenolic acids, it is difficult to estimate the antioxidant activity from phenolic contents. Synergistic relations among phenols (Tsao et al., 2005) as well as the influence of oligomeric procyanidins, which are not usually quantified are frequently proposed to explain differences between estimated and observed values for antioxidant capacities (Van der Sluis et al., 2002; Vanzani et al., 2005).

Cider is a popular drink in Europe, with consumption rates close to 10 million hectolitres a year (Association of the Cider and Fruit Wine Industry of the European Union, http://www.aicv.org). The polyphenolic profile of cider is influenced by several factors, on one hand, the characteristics of the apple mixture used as raw material, which is related to variety, climate and maturity (Mangas et al., 1999; Marks et al., 2007; Alonso-Salces et al., 2005), and, on the other hand, by the making process (Lea and Timberlake, 1978). Major polyphenols of Asturian cider are phenolic acids, dihydrochalcones and flavanols (Rodríguez Madrera et al., 2006), which are potentially important to the antioxidant activity of cider.

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The aim of this paper was to evaluate the antioxidant activity of Asturian cider in relation to its polyphenolic content by means of multivariate mathematical models. The antioxidant activity of ciders was determined by the widely used FRAP and DPPH radical assays. The results were expressed as vitamin C equivalents.

2. Materials and methods

2.1. Samples

Forty-four Asturian natural ciders available in the market and belonging to 43 cider makers were analysed. Prior to HPLC analysis, the cider samples were degassed in an ultrasonic bath during 10 min to remove all carbon dioxide and filtered through a 0.45 μm cellulose acetate membrane filter (Teknokroma, Barcelona, Spain).

2.2. Reagents and solvents

Folin-Ciocalteu reagent was supplied by Merck (Darmstadt, Germany). Ascorbic acid, 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ), and 2,2′-diphenyl-β-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Polyphenol standards were supplied as follows: gallic acid, (+)-catechin, (−)-epicatechin, phloridzin, tyrosol, catechol, hydroxycinnamic acids (*p*-coumaric acid, caffeic acid, ferulic acid and chlorogenic acid), 3-phenylpropionic acids (hydrocoumaric acid, hydrocaffeic acid and hydroferulic acid) by Sigma (St. Louis, MO, USA), and procyanidin B2 and quercetin glycosides (hyperin, avicularin and quercitrin) by Extrasyntèse (Genay, France). Phloretin-2′-xyloglucoside and trimer C1 were kindly furnished by Dr. Lea (Reading, UK). Water was purified with a Milli-Q system from Millipore (Bedford, MA, USA). Reagents and solvents were purchased from Panreac (Barcelona, Spain) and were of analytical or HPLC grade.

2.3. Analytical procedures

HPLC analysis of polyphenols was performed according to the method validated by our research group (Suárez et al., 2005), by means of a Waters system, equipped with a 717 automatic injector, a column oven, two pumps (model 510), a diode array detector (model 996) and Millennium software v.2.1 data module. Separation of polyphenols was carried out on a reversed-phase Nucleosil 120 C_{18} (250 mm × 4.6 mm I.D., 3 μm) column from Teknokroma (Barcelona, Spain). The column was thermostated at 25 °C and a flow rate of 0.8 mL/min was used. The elution solvents were aqueous 2% acetic acid (solvent A) and 100% methanol (solvent B). The samples were eluted according to the following gradient: a linear step from 0 to 45% of solvent B in 55 min and a final isocratic step of 20 min. Injection volume was 50 μL.

Identification of compounds was achieved by comparing their spectra and retention times with those of standards when available. Quantification was done by the external standard method at 313 nm for hydroxycinnamic acids, at 355 nm for flavonols and 280 nm for the rest of the phenolic compounds. For those components available in small quantities or without commercial standards, quantification was achieved from compounds belonging to the same family. Thus, trimer C1 and procyanidin B2 were quantified as (−)-epicatechin, phloretin-2′-xyloglucoside as phloridzin, flavonol glycosides as quercitrin, *p*-coumaric acid derivatives (λ = 313.0 nm) as *p*-coumaric acid and hydroxycinnamic acid derivatives (λ_{max} = 326.3; sh. at 297.7 nm) as chlorogenic acid.

Total phenol analyses were done by spectrophotometry by the Folin-Ciocalteu, according to that described by the European Union Official Methods of Analysis (European Union Official Methods of Analysis, 1998), by means of a Perkin-Elmer Lambda 35 equipped with an autosampler and a 1-cm optical quartz flow cell. The reaction is developed in 100-mL volumetric flasks, where the different reactants were added in this order: 1 mL of sample, 50 mL of water, 5 mL of Folin-Ciocalteu reagent, 20 mL of 20% sodium carbonate, and water to reach the final volume. The absorbance was measured at 750 nm, after 30 min at room temperature. Results are expressed as mg/L of gallic acid.

2.4. Antioxidant activity

2.4.1. DPPH assay

The scavenging activity was done by spectrophotometry in 1-cm disposable plastic cells. To begin with, 100 μL of sample (previously diluted 1:5 with a 6% ethanol solution) or standards were added to 3.9 mL of DPPH solution (40 mg/L) in methanol, and left to stand in the dark. Absorbances at 515 nm were measured for the DPPH$^{\bullet}$ reactant at the beginning ($A_{control}$) and for the sample at different intervals of time ($A_{t(min)}$). A final time of 90 min was selected, when the reaction reached the steady state.

The percentage of remaining DPPH$^{\bullet}$ was calculated as follows:

$$\%\mathrm{DPPH}^{\bullet}_{rem} = \frac{A_t}{A_{\mathrm{control}}} \times 100$$

The absorbance at the steady state of ciders was related with vitamin C equivalent antioxidant capacity (VCEAC) using standard curves of ascorbic acid.

2.4.2. FRAP assay

The working FRAP reagent was prepared freshly every day by mixing 2.5 mL of TPTZ (10 mM in 40 mM hydrochloric acid), 2.5 mL of ferric chloride (20 mM) and 25 mL of sodium acetate buffer (300 mM, pH = 3.6). The FRAP assay was carried out at 37 °C, in 1-cm disposable plastic cells. 900 μL of the FRAP reagent were mixed with 90 μL of water and 30 μL of cider (diluted 1:25 with methanol solution). After 4 and 40 min, the absorbances at 595 nm were measured.

The ascorbic acid equivalents (mM) were obtained using standard curves of ascorbic acid.

2.5. Statistical analyses

Pearson correlation analyses were performed by means of the SPSS v.11.0 for Windows statistical package. PLS regression analyses were performed with the PARVUS v 3.0 statistical package (Forina et al., 1988), taking the antioxidant activity of ciders, expressed as mmol L^{-1} of vitamin C as dependent variables, and Folin index and the individual phenols determined by HPLC (23 compounds) contents as predictor variables.

3. Results and discussion

3.1. Antioxidant activity of ciders

Three ciders, covering a range of Folin index of 0.4–1.2 g gallic acid/L were used to optimise the methods for antiradical activity determination. This was done by measuring in triplicate the absorbance at 515 nm at different interval times, as shown in Fig. 1 for the DPPH assay (A), and at 595 nm for FRAP measurements (B).

3.2. DPPH assay

The DPPH assay has been widely used for measuring the antiradical activity of foods and beverages. Reaction time is dependent on the kind of sample to be analysed, and therefore, this parameter was optimised. The steady stage for scavenging activity

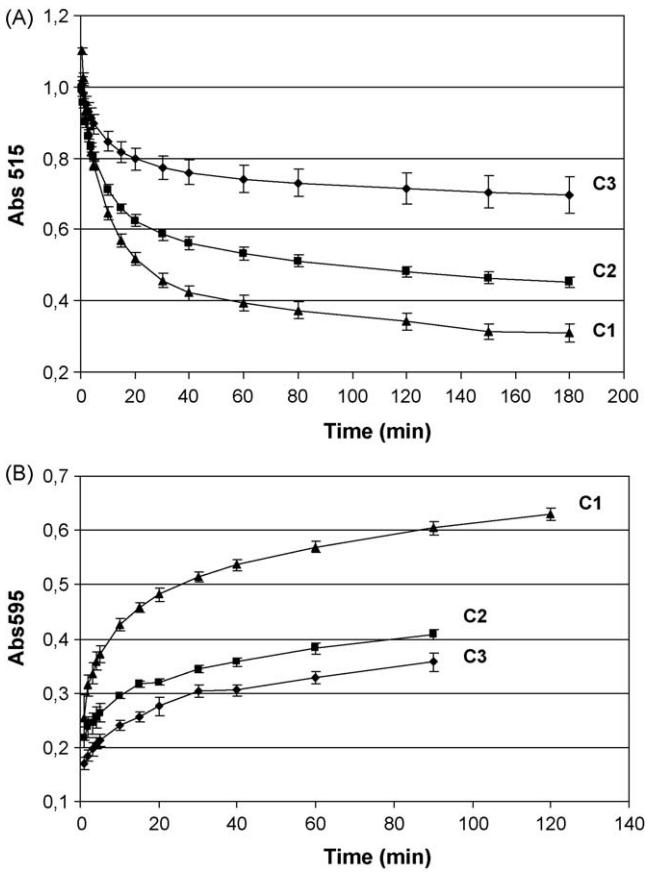

Fig. 1. Time courses of cider in the DPPH assay (A) and the FRAP assay (B). C1, cider 1 (Folin index 1180 mg gallic acid/L); C2, cider 2 (Folin index 783 mg gallic acid/L); C3, cider 3 (Folin index 465 mg gallic/L).

was reached at 80 min, so a final reaction time of 90 min was selected. The radical scavenging capacity of the above mentioned ciders was determined five times within one day for within-day repeatability, while between-day repeatability was examined in triplicate for two consecutive days. The relative standard deviations in terms of ascorbic acid equivalents were between 1.9 and 5.7% for within-day repeatability, and between 7.0 and 10.6% for between-day repeatability.

As said elsewhere (Rodríguez Madrera et al., 2006), phenolic acids (hydroxy- and dihydroxycinnamic acids) constituted the main group in Asturian cider, followed by flavanols (catechin, epicatechin and procyanidins), volatile phenols and dihydrochalcones, whereas flavonols were minor components. Pearson test gave highly significant correlations ($p < 0.001$) between the VCEAC (mM vitamin C) and Folin index, the sum of HPLC phenols, flavanols, phenolic acids, and flavonols. Non-significant correlations were found for the sum of dihydrochalcones and volatile phenols (Table 1). The good relationship between Folin index and the antioxidant capacity of cider was expected, as it has been reported elsewhere for apples (Tsao et al., 2005) or wines (De Beer et al., 2003; Landrault et al., 2001). The content of trimer C1 showed the highest correlation coefficient with the antioxidant activity, followed by that of procyanidin B2 and (−)-epicatechin (Table 1). Flavan-3-ols and procyanidin oligomers have been described as powerful antioxidants (Saint-Cricq de Gaulejac et al., 1999a), whose ability is related to their degree of polymerisation and their substituents (Lotito et al., 2000). Likewise, the role played by phenolic acids on the antiradical capacity of cider was in agreement with previous research works (Moreira et al., 2005; Tsao et al., 2005).

3.3. FRAP assay

The original method reported by Benzie and Strain (1996) used a fixed time of 4 min to develop the reaction with the FRAP system. Pulido et al. (2000) studied the effect of the reaction time for several hours on the activity of different phenolics, and observed increasing activities after 4 min. On the basis of these results, three ciders were monitored at different time intervals. Comparing with the DPPH assay, this reaction did not reach a steady stage but exhibited a continuous increase with time, as seen in Fig. 1B. A final time of 40 min was selected for further analyses, yet keeping the 4-min interval of the original method for comparison. The ferric reducing activity of cider measured at 40 min was 1.4-fold higher than that obtained at 4 min (Table 1). The ciders used to optimise reaction time were analysed as explained before to determine precision of FRAP measurements. Mean relative standard deviations in terms of ascorbic acid equivalents were 4.7% and 3.3% for within-day repeatability at 4 and 40 min respectively, and 9.3 and 8.5% for between-day repeatability at 4 and 40 min.

The FRAP and DPPH assays are included among the electron transfer methods, thus, similar trends were observed for the antioxidant activities measured by both methods. The correlation between these methods was highly significant, and higher when the interval of 40 min was used (Table 1). As observed with the DPPH, Folin index showed the highest correlation coefficients, followed by that of flavanols (trimer C1 > B2 > epicatechin) and hydrocaffeic acid contents (Table 1). In general, higher correlations were found between the ferric reducing activity determined at 40 min and the phenolic contents of ciders, except for hydrocaffeic acid, coumaric acid derivative-1, phloridzin, avicularin and the unknown flavonol (Table 1).

3.4. Phenolic profile and antioxidant capacity of cider

The antioxidant ability of those compounds showing significant correlations with the antioxidant capacity of cider was measured, on the same mM basis using standard solutions (0.5 mM) (Table 2). As shown in Table 2, for the DPPH assay the order for antioxidant activity was procyanidin B2 > epicatechin > hydrocaffeic acid > catechin > quercitrin > caffeic ∼ chlorogenic acids, which agrees with previous reports (Chinnici et al., 2004; Tsao et al., 2005; Saint-Cricq de Gaulejac et al., 1999a). In the case of the FRAP assay, the order was procyanidin B2 > hydrocaffeic acid > chlorogen-chlorogenic ∼ caffeic acids > quercitrin > epicatechin ∼ catechin. In general, all the compounds tested increased their antioxidant capacity at 40 min, the hydrocaffeic acid being the less sensitive to reaction time. No activities were observed for *p*-coumaric, hydrocoumaric acids and phloridzin.

Mean values for the observed antiradical activity of ciders expressed as vitamin C equivalent antioxidant capacities (VCEAC) were 2.9 mmol L^{-1} (Table 1). This figure is somewhat lower to those reported for extracts of flesh apple extracts (Chinnici et al., 2004; Kim et al., 2002) which should be explained on the basis of the Asturian cider-making process. In this process, the apples are exposed to the air for two or three days during pressing, thus, a strong oxidation is produced. Flavan-3-ols and oligomer procyanidins are quite sensitive to oxidation, leading to the formation of larger polymers (Lea and Timberlake, 1978; Nicoli et al., 2000). In advanced stages of oxidation, the increasing degree of polymerisation results in a decreasing scavenging activity of polyphenols due to steric hindrance (Saint-Cricq de Gaulejac et al., 1999b; Lotito et al., 2000). Likewise, many of the polyphenols are adsorbed onto the apple pulp, and therefore, they will not reach the must, explaining the subsequent decrease in its antioxidant activity, as compared with fresh apples (Van der Sluis et al., 2002). With regards to other fermented products, the antiradical capacity of

Table 1
Phenolic profile (mg/L) and antioxidant activity (vitamin C equivalents antioxidant capacity, mmol L^{-1}).

Phenolic composition of ciders (mg/L)					Pearson correlation coefficients		
	Mean	sd	Maximum	Minimum	DPPH	FRAP (4-min)	FRAP (40-min)
FI (mg gallic/L)	733	148	1180	447	0.898**	0.852**	0.854**
Protocatechuic	0.8	1.5	10.2	nd	ns	ns	ns
Hydrocaffeic	90.0	22.4	147.2	13.0	0.643**	0.715**	0.658**
iso-Chlorogenic	8.0	2.3	12.6	3.7	ns	ns	ns
Chlorogenic	1.1	5.3	32.2	nd	ns	ns	ns
Caffeic	0.1	0.2	1.0	nd	ns	ns	ns
Hydrocoumaric	10.2	7.0	52.3	nd	ns	ns	ns
Coumaric derivative 1	12.5	4.3	25.0	4.8	0.497**	0.483**	0.449**
Ferulic	0.6	0.2	1.1	0.1	0.304*	ns	ns
Ferulic derivative	0.2	0.2	1.0	nd	0.381*	0.298*	0.318*
Coumaric derivative 2	0.8	0.2	1.2	0.1	0.381*	0.490**	0.492**
Caffeic derivative	0.4	0.2	0.9	nd	ns	ns	ns
Sum of phenolic acids	124	28	184	37	0.617**	0.685**	0.637**
(+)-Catechin	0.3	0.8	3.0	nd	ns	ns	ns
(−)-Epicatechin	4.0	5.4	22.3	nd	0.493**	0.528**	0.606**
Trimer C1	23.0	14.1	66.1	nd	0.802**	0.774**	0.783**
Procyanidin B2	14.4	15.9	81.9	nd	0.677**	0.686**	0.713**
Sum of flavanols	45	38	169	nd	0.770**	0.765**	0.796**
Phloretin-2′-xyloglucoside	5.3	7.2	37.0	1.2	ns	ns	ns
Phloridzin	18.8	12.9	53.6	0.7	0.368*	0.409**	0.381*
Sum of dihydrochalcones	24	17	71	3	ns	0.372*	0.370*
Catechol	4.7	4.8	30.2	1.7	ns	ns	ns
Tyrosol	22.1	7.3	38.2	nd	−0.397**	−0.393**	−0.434**
Sum of volatile phenols	27	9	51	3	ns	ns	−0.298*
Hyperin	1.6	1.3	4.9	nd	0.557**	0.459**	0.458**
Unknown	0.5	0.4	1.5	nd	0.435**	0.492**	0.441**
Avicularin	0.5	0.4	1.5	nd	0.547**	0.510**	0.476**
Quercitrin	2.3	1.3	5.7	nd	0.374*	ns	ns
Sum of flavonols	5	3	14	nd	0.558**	0.471**	0.452**
VCEAC (mM, DPPH)	2.9	0.7	5.4	1.7		0.797**	0.821**
VCEAC (mM, FRAP-4)	3.8	1.0	7.2	1.9			0.960**
VCEAC (mM, FRAP-40)	5.4	1.3	10.0	2.8			

FI: Folin index; sd: standard deviation; nd: not detected; VCEAC: vitamin c equivalents antioxidant capacity; ns: not significant.
* Significant at the 5% level.
** Significant at the 1% level.

cider was in the range reported for white wines (De Beer et al., 2003; Landrault et al., 2001). On the same Folin index basis, cider results to be more (De Beer et al., 2003) or less efficient than white wines (Landrault et al., 2001). This could be attributed both to the respective phenolic compositions. Thus, the proportion of flavanols seems to be the clue to explain these facts. In the case of the South-African wines, the hydroxycinnamic acid derivative contents are 6-fold higher than that of flavanols (De Beer et al., 2003), whereas in the French wines the proportions of these phenolic families are similar (Landrault et al., 2001). In ciders, phenolic acid contents are 2-fold that of flavanols (Table 1).

Table 2
Millimolar concentration of a vitamin C solution having the antioxidant activity equivalent to 0.5 mM standard solution of the substance assayed: mean ± standard deviation ($n = 3$).

Phenolic standards	DPPH (90 min)	FRAP (4 min)	FRAP (40 min)
Hydrocaffeic acid	0.7 ± 0.0	1.2 ± 0.1	1.3 ± 0.0
Chlorogenic acid	0.4 ± 0.0	0.7 ± 0.0	1.2 ± 0.0
Caffeic acid	0.4 ± 0.0	0.7 ± 0.0	1.2 ± 0.0
Coumaric acid	–	–	–
Hydrocoumaric acid	–	–	–
(+)-Catechin	0.7 ± 0.1	0.6 ± 0.0	1.0 ± 0.0
(−)-Epicatechin	1.0 ± 0.0	0.6 ± 0.0	1.1 ± 0.0
Procyanidin B2	1.4 ± 0.0	1.5 ± 0.0	2.3 ± 0.0
Phloridzin	–	–	–
Quercitrin	0.4 ± 0.0	0.6 ± 0.0	1.0 ± 0.0

Partial least squares regression analyses were performed taking the observed antioxidant activity of ciders determined by the DPPH or the FRAP assays (VCEAC, mmol L^{-1}) as dependent variables, and Folin index and the individual phenol contents (mg/L) as predictor variables. Once computed, the antioxidant activity of ciders was estimated for each mathematical model and compared with the observed values according to the expression:

$$\%\text{Estimation} = \frac{\text{VCEAC}_{\text{estimated}}}{\text{VCEAC}_{\text{observed}}} \times 100$$

The regression models with two latent variables for antioxidant activity provided high values for multiple correlation coefficients, explained and cross-validated explained variance as shown in Table 3. The relevant variables were, in the case of the DPPH

Table 3
Partial least square (PLS) parameters of multiple regression analysis with two latent variables to determine the antioxidant activity of cider as a function of its phenolic profile and Folin index.

	Explained variance (%)	R^2 (%)	Cross-validated explained variance (%)	Standard error of the estimate	(%) Accounted antioxidant activity: estimated × 100/observed ($n = 42$)			
					Mean	sd	Maximum	Minimum
DPPH	83.03	83.84	80.30	0.249	99.9	10.2	127.7	83.8
FRAP-4 min	84.08	82.54	84.84	0.300	100.4	8.5	114.3	77.3
FRAP-40 min	87.29	87.90	86.66	0.354	100.2	7.9	114.4	75.2

assay: procyanidin B2 > trimer C1 > Folin > hydrocaffeic acid > epicatechin > phloridzin > coumaric acid derivative 1. The same order was obtained for the two FRAP assays performed, excepting that hydrocaffeic acid and Folin index reversed their relative positions. Phloridzin and the coumaric acid derivative 1 contributed with negative coefficients to the mathematical models, and flavonols had a minor contribution, due to their low concentration in cider. This order agrees with the antioxidant activity reported for the individual standards (Table 2) and the mean concentration found in cider for each compound. All these mathematical models were robust and good at predicting the antioxidant activity of ciders (Table 3), although nearly 20% of the antioxidant activity is still unaccounted for.

4. Conclusions

Antioxidant capacity of cider can be accurately determined by means of the DPPH or the FRAP assays, both of them being simple and reproducible, as far as proper reaction times and reagent to analyte excess are optimised. With the DPPH assay, the analyses were performed at the steady stage, whereas for FRAP, significant differences were found between the results obtained at 4 or 40 min, thus the measures of reducing capacity of cider should be done at 40 min. Multivariate approaches based on phenolic composition were useful to predict the antioxidant activity of cider, Folin index and the contents of flavanols and hydrocaffeic acid being the most relevant variables.

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