POLYPHENOL VARIABILITY IN THE FRUITS AND JUICES OF A CIDER APPLE PROGENY

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ABSTRACT

BACKGROUND: Polyphenols have a favorable antioxidant potential on human health, suggesting that their high content in apple is responsible for the beneficial effects of apple consumption. They are also linked to the quality of apple juices and ciders since they are predominantly responsible for astringency, bitterness, color and aroma. Major phenolic compounds were quantified by liquid chromatography in fruits and juices from a cider apple progeny harvested for three years. The total content of procyanidins and their average degree of polymerization (DPn) were also determined in fruits by phloroglucinolysis. Variability and extraction yield of these compounds were determined.

RESULTS: The variability observed in the progeny was representative of the variability observed in many cider apple varieties. Hydroxycinnamic acids were the most extractable group, with an average extraction yield of 67%, whereas flavonols and anthocyanins were the least.

CONCLUSION: This study is the first one to introduce variability and extraction yields of the main phenolic compounds in both fruits and juices of a cider apple progeny. This dataset will be used for an upcoming QTL mapping study, an original approach that has never been undertaken for cider apple.

KEYWORDS: *Malus* x *domestica*; cider apple; phenolic compound; extractability; phloroglucinolysis.

INTRODUCTION

Apple is one of the most highly produced fruits in the world, with more than 69.5 million tons produced in 2010 over an area of 4.7 million hectares (FAO, http://faostat3.fao.org/). Cider is made using specific varieties of apple that are different from the ones used for dessert apple production. A large number of cider apple varieties are grown throughout the world, with a wide diversity in terms of organoleptic criteria (including astringency, bitterness, aroma, color and acidity), crop management and disease resistance.[1] This variability among varieties in different countries is very pronounced, particularly with regard to their polyphenol composition.[2-4] For these reasons, they can serve as a relevant genetic resource for breeders.

Six main phenolic groups are present in apple: flavanols, which are subdivided into monomers (i.e., catechins) and oligomers (i.e., procyanidins, also referred to as condensed tannins), hydroxycinnamic acids, dihydrochalcones, flavonols and anthocyanins. Hydroxycinnamic acids are mainly represented by 5-caffeoylquinic acid (often referred to as chlorogenic acid), 4-caffeoylquinic acid and 4-*p*-coumaroylquinic acid. Flavanol monomers are mainly represented by (+)-catechin and (-)-epicatechin, the latter being the most abundant in apple. Procyanidins are the group with the highest global concentration in apple and the largest number of compounds.[5] Each compound is differentiated by the nature of the constitutive flavanol units, the polymerization degree and the position of the interflavanic linkages. Therefore, this polydispersity (or the heterogeneity of the distribution of molecular masses) in apple makes it difficult to individually quantify procyanidin molecules with polymerization degrees above 3-4. However, the use of acidolysis in the presence of nucleophiles (i.e., thiolysis or phloroglucinolysis), coupled with HPLC analysis, makes it possible to characterize and quantify the global procyanidin fraction in crude apple samples.[6] Dihydrochalcones are mainly represented by phloridzin (phloretin 2'-*O*-glucoside) and

phloretin xyloglucoside in apple. Flavonols are essentially represented by quercetin glycosides, mainly avicularin (quercetin-3-*O*-arabinoside), hyperin (quercetin-3-*O*-galactoside), isoquercitrine (quercetin-3-*O*-glucoside), quercitrin (quercetin-3-*O*-rhamnoside), reynoutrin (quercetin-3-*O*-xyloside) and rutin (quercetin-3-*O*-rutinoside). They are mainly present in the skin of the fruit, along with anthocyanins, essentially represented by ideain (cyanidin-3-*O*-galactoside).[4]

Phenolic compounds are directly linked to the major organoleptic criteria of apples and their products (apple juice, cider, etc.). Procyanidins are directly responsible for the astringent sensation resulting from their complexation with salivary proteins. They are also involved in the bitter taste of cider as the result of specific interactions with bitterness receptors in the mouth. The intensity of these sensory properties is directly linked to the procyanidin structures and, in particular, their degree of polymerization.[7] Chlorogenic acid is the preferential substrate of the polyphenol oxidase. In the presence of oxygen, it is enzymatically converted into *O*-quinone, which further reacts with catechins, procyanidins and dihydrochalcones, resulting in the formation of oxidation products including yellow-orange molecules responsible for the color of apple juice and cider.[8-10] Moreover, during the fermentation step of cider making, the ester hydrolysis of hydroxycinnamic acids could be the precursor of some aromatic compounds.[11]

In addition, it has been reported that phenolic compounds are involved in the health benefits of fruit and vegetable-rich diets.[12] Apples are widely consumed throughout the world and are rich in strong antioxidant polyphenols, including quercetin, (+)-catechin, phloridzin and 5-caffeoylquinic acid. Epidemiologic studies have shown that apple consumption is linked to the reduced risk of some cancers, cardiovascular disease, diabetes and asthma.[13, 14]

A wide variability of phenolic compound concentrations is observed among apple varieties, and cider apple are usually more concentrated in polyphenols than dessert apple varieties.[2, 15]

A considerable difference between the phenolic content of fruits and juices has already been reported. The work of Guyot et al.[6] on hydroxycinnamic acids, flavanols and dihydrochalcones has shown that the transferability of phenolic compounds during juice processing can be very different according to the variety. For example, it ranges from 57% for the 'Avrolles' variety, to 77% for the 'Kermerrien' variety.[6] However, the juice of polyphenol-rich varieties has a higher phenolic compound content. This observation is also true for all phenolic groups. Differences among fruits and juices were mainly explained by the extraction yield of phenolic compounds during juice processing. Monomers and polymers of flavanols had the smallest transferability rate as a result of their high retention in the mash, explained by their affinity for cell wall components.[16] The centrifugation of the juice further reduces the concentration of procyanidins and the mean polymerization degree by removing tannins associated with fruit solid parts suspended in the raw juice.[6] Moreover, the release of polyphenoloxidase (PPO) during grinding in addition to juice oxygenation causes the degradation of some phenolic compounds.[8] The addition of sodium fluoride during juice processing makes it possible to inactivate the PPO and to limit the degradation of compounds by oxidation.[17]

Numerous studies have focused on the genetic bases of phenolic compounds.[18-21] Anthocyanins have been particularly well studied in fruits since they are major contributors to fruit quality.[22-24] A cluster of three MYB genes involved in the anthocyanin content was identified following a QTL study on grape.[22] In apple, two studies have recently been published on QTL detection of phenolic compounds measured in dessert apple progenies.[25, 26] Chagne et al.[25] reported the quantification of 16 and 23 phenolic compounds in two different harvesting years using an ultra-high performance liquid chromatograph (UHPLC) coupled to a UV-PDA detector. Khan et al.[26] reported the quantification of 81 phenolic compounds belonging to the two groups of phenylpropanoids and polyphenols in the skin and the flesh,

using a high performance liquid chromatograph (HPLC) coupled to a mass spectrometer associated with MSClust software. To our knowledge, these are the only studies that have been done on the variability of phenolic content existing within an apple progeny.

Since cider apples contain many more phenolic compounds than dessert apples, a progeny derived from a cross between a cider and a dessert apple was studied to perform a genetic study on phenolic content. An initial study was first carried out to develop a liquid chromatography method suitable for the quantification of major phenolic compounds in juices (Verdu et al., submitted).

MATERIAL AND METHODS

Phenolic standards and chemicals

LC/MS-grade MeOH was purchased from Carlo Erba reagents (Val de Reuil, France). Formic acid and acetic acid of LC/MS grade were obtained from Fisher Scientific (Illkirch, France). Ultrapure water was obtained from a MilliQ water purification system (Millipore S.A., Molsheim, France). Standards of procyanidins B1 and B2, 4-*p*-coumaroylquinic acid, 4-caffeoylquinic acid and phloretin xyloglucoside were obtained from Polyphenol Biotech (Bordeaux, France). (+)-catechin, (-)-epicatechin, 5-caffeoylquinic acid, phloridzin and rutin were purchased from Sigma-Aldrich (Lyon, France). Hyperin, isoquercitrine and quercitrin were obtained from Extrasynthese (Genay, France), and avicularin was obtained from LGC Standards SARL (Molsheim, France). (-)-Epicatechin-phloroglucinol adduct was purified in the laboratory. Reynoutrin was identified according to its m/z ratio and its retention time.[27]

Plant material

The material was a cider apple progeny consisting of 385 individuals derived from a cross between the hybrids X5210 and X8402. The former (X5210) is derived from the cider variety,

'Kermerrien', whereas the latter (X8402) is a dessert apple hybrid whose grandparents include the two varieties, 'Florina' and 'Prima'.

The cross between X5210 and X8402 was made in 2000. Plantlets were selected in a greenhouse for scab and powdery mildew resistance. Trees were planted in 2003 at the INRA Horticulture Experimental Unit in Angers, France, with their roots.

This study was carried out both on fruit extracts and apple juices. Fruit extracts were prepared from 92 apples harvested in 2008 and 137 harvested in 2009 (referred to as F08 and F09, respectively, in this paper). Apple juices were prepared from 209 and 123 hybrids from the progeny harvested in 2009 and 2010, respectively (J09 and J10, respectively). The fruits were collected at the mature stage "when 50% of the fruits have fallen off the tree", which is the harvest stage used in commercial cider orchards.

Sample preparation

Fruit sampling and sample preparation

For each individual, 30 fruits were randomly collected from one tree and divided into three batches of ten fruits. For each batch, fruits were mechanically cut according to a systematic procedure that made it possible to randomly select four small pieces per fruit that were immediately frozen in liquid nitrogen.[28] Samples were then freeze-dried and reduced to a fine and homogeneous powder with an electrical crusher (Retsch, model YGG, Bioblock Scientific). The powders were then kept under vacuum in a desiccator until analysis.

Apple juice preparation

The apple juice preparation consisted of mixing 330 g of cored apples using a juice extractor (Philips HR1865), adding sodium fluoride (200 mg L^{-1} of apple juice) to limit oxidation of phenolic compounds, and centrifuging at 15000 g during 15 min (Sigma 4K15) to recover the

clear apple juice. Following this preparation, one volume of methanol was added and the mixture was filtered on PTFE filters (0.2 μm, Uptidisc, Interchim, France) before chromatographic analysis.

Quantification of phenolic compounds

Analysis method for fruit extracts

Methanol extraction of freeze-dried powders

Simple polyphenols, including monomeric catechins, low molecular weight procyanidins, hydroxycinnamic acids, flavonols, dihydrochalcones and anthocyanins, were extracted from the powders using acidified methanol. Precisely weighted aliquots of powders (in the 50-100 mg range) were extracted using 1.2 ml of pure methanol containing 1% v/v acetic acid for 15 min in an ultrasonic bath (Brasson 2200, USA). The mixture was then filtered on PTFE filters (0.45 μm, Uptidisc, Interchim, France).

Acidolysis of procyanidin oligomers and polymers in the presence of phloroglucinol (phloroglucinolysis)

In previously published studies,[5, 6, 29] acidolysis of procyanidins was applied on apple powders using an excess of benzylmercaptan as a nucleophilic agent (thiolysis reaction). Phloroglucinol rather than benzylmercaptan was used in this study because of its advantages (odorless and non-toxic).[30, 31] Moreover, comparative assays using thiolysis or phloroglucinolysis revealed no significant differences in their efficiency to quantify and characterize the procyanidin fraction in crude apple samples.[32] Interestingly, the phloroglucinolysis reaction leads to the depolymerization of procyanidin structures making the distinction between terminal and extension units of procyanidins.Then, quantitative HPLC analysis of the phloroglucinolysis media allows the determination of the total

procyanidin concentration, the nature and the proportion of the constitutive units of procyanidins in the crude samples. This makes it possible to calculate their average degree of polymerization (DPn).[29] .

The phloroglucinolysis method was adapted from Kennedy and Jones.[30] Freeze-dried apple powders (30 mg) were treated with a solution of 0.3 M HCl in MeOH containing 75 g L^{-1} phloroglucinol and 10 g L^{-1} ascorbic acid at 50°C for 50 min, and then combined with 1.2 mL of aqueous sodium acetate to stop the reaction. The mixture was then filtered on PTFE filters (0.45 µm, Uptidisc, Interchim, France).

RP-HPLC of methanol extracts and phloroglucinolysis reaction media

HPLC analysis was performed using a Waters 2690 separation module equipped with an autosampler, a cooling system set to 4°C, and a Waters 996 photodiode array detector. The column was a 250mm×4mm ID with a bead diameter of 5µm, and an end-capped Purospher RP 18 column (Merck) maintained at 30°C. The mobile phase contained (A) 2.5% acetic acid and (B) acetonitrile, which was previously degassed and then continuously sparged with high-purity helium during analysis. The solvent system was a gradient of aqueous acetic acid, 2.5% v/v (solvent A), and acetonitrile (solvent B). The following gradient was applied at a constant flow rate of 1 $ml.min^{-1}$: initial, 3% B; 0-5 min, 9% B linear; 5-15 min, 16% B linear; 15-45 min, 50% B linear, followed by washing and reconditioning of the column. The volume of injection was 10 µL.

The acquisition, integration and processing of the signal was controlled using Millennium software 2010, version 2.1. Simultaneous monitoring was performed at 280 nm for flavan-3-ols and dihydrochalcones, 320 nm for hydroxycinnamic acids, 350 nm for flavonols, and 520 nm for anthocyanins. Spectra were recorded between 200 and 600 nm. Phenolic compounds were identified on the basis of their retention times and their characteristic spectra in

comparison to available standards. Except for the series of flavonols that were all quantified at 350 nm as hyperoside equivalent, the other phenolic compounds and phloroglucinolysis products were quantified using their own calibration curve: 5-caffeoylquinic acid and 4-*p*-coumaroylquinic acid were quantified at 320 nm; (-)-epicatechin, (+)-catechin, procyanidins B1 and B2, phloretin xyloglucoside, phloridzin and epicatechin-PLG adduct were quantified at 280 nm ; and ideain, the only molecule from the anthocyanin class considered in this study, was quantified at 520 nm according to its own calibration curve.

Analytic method for apple juices

UHPLC analyses were performed using a Thermo Accela High Speed LC system (Thermo Scientific, Gometz le Châtel, France) equipped with a refrigerated autosampler. Samples were injected into a Zorbax Eclips Plus C18 column (50mmx2.1mm, 1.8 µm; Agilent) using a 10-µL loop in partial loop mode. The column was heated at 30°C and was equipped with an in-line filter (0.2 µm) (Thermo Scientific). The solvent system was a gradient of aqueous formic acid, 0.1% v/v (solvent A), and methanol (solvent B). The following gradient was applied at a constant flow rate of 250 $\mu L.min^{-1}$: initial, 0% B; 0-1 min, 10% B linear; 1-3 min, 18% B linear; 3-11 min, 18.5% B linear; 11-13 min, 21.5 % B linear; 13-17 min, 25.5% B linear; 17-21 min, 29% B linear; 21-23 min, 32% B linear; 23-35 min, 50% B linear, followed by washing and reconditioning of the column. The volume of injection was 1µL. The MS experiments were performed with a Thermo TSQ Quantum Access MAX equipped with an electrospray interface (ESI) operating in the negative ionization mode. Each standard was infused into the electrospray ion source at 5 $\mu g\ mL^{-1}$ in MeOH using a syringe pump at a flow rate of 250 $\mu L\ min^{-1}$ to determine the collision energy, the tube lens offset and the SRM transitions chosen to be the most sensitive with the lowest collision energy for each compound. The Selective Reaction Monitoring (SRM) mode was used to quantify phenolic

compounds. The ESI conditions were as follow: spray voltage, 3500 V; vaporizer temperature, 350°C; sheath gas pressure, 48 arbitrary units (au); ion sweep gas, 1 au; auxiliary gas pressure, 13 au; capillary temperature, 200°C; skimmer offset, 0 au. The collision gas used was argon at a pressure of 1.5 mTorr. The data were processed using Xcalibur software (2.1).

Phenolic compounds were identified on the basis of their retention times and their characteristic fragmentation pattern in comparison with available standards. Quantifications were performed in SRM mode, using the calibration curves of standards.

In addition to procyanidins B1 and B2, ten other major procyanidins were individually quantified by UHPLC-UV at 280 nm with a Thermo Accela PDA detector in juices prepared in 2010 to estimate the total content of flavanols.

Statistical analyses

Statistical analyses were performed with R software version 2.13.1.[33] and GraphPadPrism 5.01 (GraphPad Software, San Diego, CA, USA).

Two separate analyses of variance (ANOVA) were performed for fruits and juices, to evaluate the genetic effect, the harvest year effect and the interaction genetic x year.

Principal component analyses (PCA) were performed for fruits and juices to estimate the correlation between different traits. Each year was analyzed separately because there were not enough individuals in common between the two harvest years.

Using the method of Guyot et al.,[6] the extraction yields were calculated for each compound, and each individual was analyzed for both fruit and juice phenolic content in 2009 as follows: Yield (%) = (([C_j]*R)/[C_f]*d)*100 where [C_j] and [C_f] were the concentration of the considered polyphenol in the juice (mg L^{-1}) and in the fruit (mg kg^{-1}), respectively. R was the

extraction yield of the juice (kg of juice per kg of fresh fruits) and d was the density of the juice (kg L^{-1}).

RESULTS AND DISCUSSION

Phenolic compounds in fruits

Four flavanols ((+)-catechin, (-)-epicatechin, procyanidins B1 and B2), six flavonols (avicularin, hyperoside, isoquercitrin, quercitrin, reynoutrin and rutin), two hydroxycinnamic acids (5-caffeoylquinic acid and 4-*p*-coumaroylquinic acid), two dihydrochalcones (phloridzin and phloretin xyloglucoside) and the anthocyanin ideain, were measured by HPLC-UV for two harvest years. The phloroglucinolyse reaction was used to estimate the total content of procyanidins and the average degree of polymerization of flavanols.

The total concentration of polyphenols in fruit samples was comprised between 1058 and 6418 mg kg^{-1} of fresh weight (FW), with an average concentration of 2707 mg kg^{-1} (Table 1). These results are in agreement with total polyphenol concentrations determined in different cider and dessert apple varieties (Table 2). The lowest concentration obtained in our progeny was close to the ones determined for the 'Golden Delicious' (a dessert apple variety) and 'Judor' varieties (1040 mg kg^{-1} of FW), and the highest was close to that of the cider variety 'Jeanne Renard' (6990 mg kg^{-1} of FW).[2] This wider range observed in our progeny could be explained by the parents: X5210, a descendant of the cider apple variety, 'Kermerrien' (4500 mg kg^{-1} of FW in Sanoner et al.[2]), and X8402 (1400 mg kg^{-1} of FW in our experiment), a descendant of the dessert apple variety, 'Florina' (2240 mg kg^{-1} of FW in Wojdylo et al.[34]). Phloroglucinolysis coupled to HPLC analysis revealed that the most concentrated group was the flavanols, which represented 65% of average total polyphenols in the fruit (Table 1). The total content of flavanols ranged from 592 to 4769 mg kg^{-1} of FW, with an average

concentration of 1761 mg kg^{-1}. These results were consistent with other results obtained on cider varieties, with a total flavanol content ranging from 621 to 6195 mg kg^{-1} of FW.[2] The most concentrated compounds within flavanols were (-)-epicatechin and procyanidin B2, with both concentrations close to 210 mg kg^{-1} FW (Fig. 1). They ranged from 33 to 574 mg kg^{-1} of FW for (-)-epicatechin, and from 49 to 460 mg kg^{-1} of FW for procyanidin B2 (without one outlier at 730 mg kg^{-1}). These concentrations were consistent with previous studies (Table 2). The polymerization degree (DPn) of the flavanol class in the progeny was 3.0, ranging from 2.1 to 5.6. This result was consistent with major cider varieties, mainly comprised between 3.7 and 7.5. However, some varieties such as 'Guillevic' and 'Avrolles' could have a DPn that was higher than 40.[2, 3]

The second most concentrated group was hydroxycinnamic acids, with 26.4% of average total polyphenols (Table 1). The concentration of hydroxycinnamic acids in the progeny ranged from 86 to 2000 mg kg^{-1} of FW, with an average concentration of 715 mg kg^{-1}. 5-caffeoylquinic acid was the most concentrated hydroxycinnamic acid and the most concentrated phenolic compound in fruits, with an average concentration close to 610 mg kg^{-1} FW and ranging from 79 to 1865 mg kg^{-1} (Figure 1). These results were consistent with previous studies done on cider apples and high compared to dessert apples (Table 2). In some varieties such as 'Ellis Bitter' or 'Harry Masters Jersey', (-)-epicatechin is the major fruit phenolic compound.[4]

Dihydrochalcones represent 4.8% of average total polyphenols in the fruit (Table 1). Phloridzin and phloretin xyloglucoside had proximate average concentrations in the progeny, close to 60 mg kg^{-1} of FW and ranging from 17 to 190 mg kg^{-1} for phloridzin (without one outlier at 250 mg kg^{-1}), and from 6 to 180 mg kg^{-1} for phloretin xyloglucoside (Fig. 1). These results were consistent with previous studies on cider apples and higher than those on dessert apples (Table 2).

Flavonols represent 3.5% of total phenolic compounds, ranging from 20 to 274 mg kg^{-1} of FW, with an average concentration of 94 mg kg^{-1} (Table 1). These concentrations were low compared to previous studies performed on English cider apples where flavonol concentrations ranged from 149 to 1215 mg kg^{-1} of FW in peel.[4] However, the range between the most and the least concentrated individuals was higher in our progeny (14-fold) than in English varieties (8-fold). Quercitrin was the most concentrated flavonol, with 49% of total flavonol concentration ranging from 6 to 161 mg kg^{-1} of FW (Fig. 1). In previous works, hyperin was commonly more concentrated than quercitrin.[4, 34] On average, it represented 34% and 33%, whereas quercitrin represented only 13% and 23% of the total flavonols in English cider and dessert apple varieties, respectively. However, in some varieties such as the ancestor, 'Florina', quercitrin was the most concentrated flavonol, with a concentration of 45 mg kg^{-1} of FW, whereas the concentration of hyperin was 29 mg kg^{-1}.[34] Rutin was the least concentrated flavonol (3.8%) and the least concentrated phenolic compound, with concentrations ranging from 1 to 10 mg kg^{-1} of FW (without one outlier at 39 mg kg^{-1}; Fig. 1). Anthocyanins quantified in fruits represented 0.2% of total phenolic compounds (Table 1). The maximum concentration of ideain obtained in the progeny was 58 mg kg^{-1} of FW, which was low compared to previous studies on cider apples but higher than concentrations in dessert apples (Table 2).

ANOVA was carried out with 14 common individuals harvested in 2008 and 2009. The genetic effect, harvest year effect and the interaction genetic x year were significant for all compounds (Supplementary data, Table S1). For most compounds, the genetic factor is the most important. However, for the flavonols avicularin, hyperin, isoquercitrin and reynoutrin, the year effect was more important than the genetic effect. These results were obtained with only 14 individuals and they may not be very representative. Nevertheless, the instability and

low repeatability of flavonols was previously reported in *Malus x domestica* germplasm grown in New Zealand.[35] These variations were mainly explained by the sensitivity of flavonols to light and temperature.

PCA analyses were performed separately each year with all phenolic compounds because there were not enough common individuals between the two harvest years (Fig. 2). The first dimension was around 33% and 30%, and the second around 19% and 16% in 2008 and 2009 respectively. Although these dimensions explain only 50% of all variables, these PCA can highlight existing correlations between variables best representing in the plane. For both years, a good correlation was observed within flavanols, on the one hand, and within flavonols on the other. No correlation between these two groups was observed. Previous studies on the 'Granny Smith' apple variety had already shown that phenolic compounds were highly correlated within their chemical groups.[36] Similar observations were also reported in dessert apple progenies studied by Chagne et al.[25] and Khan et al.,[26] with strong correlations between compounds of the same phenolic group. In contrast to our results, hyperin and reynoutrin were correlated with procyanidins in the skin.[25] These results tend to show the regulation systems of the biosynthetic pathway of phenolic compounds which act more on an entire group of compound than one particular compound.

Phenolic compounds in apple juices

In cider industries, apples are rapped and pressed mechanically to get the juice. An enzymatic treatment can also be applied before pressing to hydrolyze the cell walls (Grimi et al., 2011). These devices are not suitable for the small amount of fruit that we had available, so we decided to prepare our juice samples with a centrifuge although we are conscious that this juice preparation procedure strongly differed from the current industrial processes.

Nevertheless, decanter centrifuges are still used sometimes to produce industrial apple juices. Twelve major phenolic compounds of apple juices were measured by UHPLC-MS/MS for two harvest years: four flavanols ((+)-catechin, (-)-epicatechin, procyanidins B1 and B2), four flavonols (avicularin, hyperin, quercitrin and rutin), three hydroxycinnamic acids (5-caffeoylquinic acid, 4-caffeoylquinic acid and 4-*p*-coumaroylquinic acid) and the dihydrochalcones, phloridzin. In 2010, the dihydrochalcone, phloretin xyloglucoside, and ten other major procyanidins were also quantified. Since the addition of these 11 compounds makes comparisons with J09 difficult, only the results for J10 are presented below.

The total concentration of polyphenols in the juices was comprised between 740 and 3742 mg L^{-1} of juice, with an average concentration of 1994 mg L^{-1} (Table 3). These results are in agreement with total polyphenol concentrations previously determined in juices prepared from cider apple varieties (Table 2). The Basque cider variety, 'Larrabetzu', was particularly concentrated, with a total phenolic content of 13600 mg L^{-1}. The second most concentrated variety in this study was 'Mendexa 10', with a total polyphenol concentration of 4300 mg L^{-1}.[3] However, total concentrations determined in dessert and German cider varieties were lower than ours (Table 2).

The largest group was the flavanols, with 50.7% of average total phenolic content in the juice (Table 3). The total concentration of flavanols ranged from 451 to 2168 mg L^{-1}, with an average concentration of 1011 mg L^{-1}. These results are consistent with a previous study where the total flavanol content of Basque cider apple juice determined with phloroglucinolysis analysis ranged from 347 to 3511 mg L^{-1}, with an average concentration of 968.5 mg L^{-1}.[3] Procyanidin B2 was the most concentrated flavanol with an average concentration of 289 mg L^{-1}, ranging from 120 to 650 mg L^{-1} (Fig. 3). The second one was (-)-epicatechin, with concentrations ranging from 61 to 433 mg L^{-1}, with an average

concentration of 206.9 mg L^{-1}. Procyanidin B2 represented an average of 28.6% and (-)-epicatechin an average of 20.4% of the total flavanol content. Their concentrations were consistent with those determined for Basque cider varieties (Table 2). Depending on the variety, procyanidin B2 or (-)-epicatechin was the major flavanol compound. (-)-epicatechin represented between 6.5% and 29.7% and procyanidin B2 between 7.9% and 20.6% of the total flavanol content.[3] The least concentrated compound was (+)-catechin, with concentrations comprised between 13 and 143 mg L^{-1} of juice (without one outlier at 178 mg L^{-1}). (Fig. 3). These results were consistent with previous studies on Basque cider varieties (Table 2).

The second major phenolic group was hydroxycinnamic acids, with 43.3% of total phenolic compounds (Table 3). The total content ranged from 137 to 1788 mg L^{-1}, with an average concentration of 863 mg L^{-1}. 5-caffeoylquinic acid is the most concentrated hydroxycinnamic acid and the most concentrated phenolic compound in juices, ranging from 77 to 1413 mg L^{-1}, with an average concentration of 700 mg L^{-1} (Fig. 3). The range of this compound in our progeny is particularly high (18-fold) compared to previous works on Spanish (4-fold), German (6-fold) and Basque (6-fold) cider apple varieties (Table 2).

Dihydrochalcones represented 3.6% of the total phenolic content in juices (Table 3). Total dihydrochalcones determined in our study ranged from 31 to 244 mg L^{-1}, with an average concentration of 73 mg L^{-1}. These results were consistent with previous studies on German cider varieties (Table 2). Phloretin xyloglucoside is more concentrated than phloridzin in our progeny, as previously reported in most cultivars (Fig. 3).[3, 37, 38]

Flavonols were the least concentrated compounds in our progeny, with 2.4% of the total content of phenolic compounds present in juices (Table 3). The concentration of this group ranged from 16 to 179 mg L^{-1}, with an average concentration of 47 mg L^{-1}. These results were very high compared to those previously obtained (Table 2). Quercitrin is the most

concentrated flavonol, with 55% of total flavonol concentration, ranging from 5 to 98 mg L^{-1} (without one outlier at 130 mg L^{-1}). Rutin is the least concentrated flavonol (1.4%) and the least concentrated phenolic compound, with concentrations ranging from 0.06 to 3.35 mg L^{-1} of juice. These high concentrations compared to previous studies could be due to the extraction method used for juice preparation since we used a juice extractor, whereas pressing was used in previous studies.[3, 38]

The same profiles were obtained for common compounds quantified in both J09 and J10. However, average concentrations obtained in J09 for catechins (207 mg L^{-1}), hydroxycinnamic acids (755 mg L^{-1}) and flavonols (31 mg L^{-1}) were lower than those obtained in J10. A wider range was obtained for these groups (catechins: from 22 to 584 mg L^{-1}; hydroxycinnamic acids: from 82 to 2053 mg L^{-1}; flavonols: from 8 to 118 mg L^{-1}), which could be explained by the larger number of individuals studied (209 individuals in J09 and 123 in J10).

ANOVA was performed with 57 common progenies harvested in 2009 and 2010 for the 12 compounds quantified in both years. The genetic effect, harvest year effect and the interaction genetic x year were significant for all compounds except for (+)-catechin and avicularin, which did not have significant effects for harvest year and the genetic x year interaction, respectively (Supplementary data, TableS2). The genetic effect was always the biggest one, except for hyperin that had a dominant year effect.

PCA analyses were performed separately for both years using all of the phenolic compounds. The first dimension was around 37% and 29%, and the second around 15% and 16% in 2009

and 20109 respectively. The results obtained were close to those obtained in fruits. A good correlation was observed for flavanols, on the one hand, and for flavonols on the other, and no correlation between these two groups was observed. The two acids, 4-caffeoylquinic acid and 4-*p*-coumaroylquinic acid, were also correlated in 2010 (Fig. 4).

Extractability of polyphenols from the fruit to the juice

Globally, similar profiles were obtained in fruits and juices with flavanols as the major group, followed by hydroxycinnamic acids, dihydrochalcones and flavonols. However, in fruits, flavanols and hydroxycinnamic acids represented 65 and 26.4% of the average total phenolic content, respectively, whereas in juices, they represented 50.7 and 43.3%, respectively. These differences were directly linked to the extraction yield of compounds during fruit processing. The degradation of compounds by oxidation was minimized by the addition of sodium fluoride. The detailed polyphenol profiles of the fruits were thus compared to the corresponding juices (same year of harvest) according to the method used by Guyot et al.[6] The mean extraction yield was then calculated and is illustrated in Fig. 5. Hydroxycinnamic acid was the least affected group following the juice preparation, with an average extraction yield of 67%. Flavanol monomers were the second group that had the best extraction yield, around 48%. Since the total procyanidin content was not evaluated in juices in 2009, it was not possible to determine the extraction yield of this group. However, the average concentration of procyanidins determined in juices prepared in 2010 compared to the total procyanidin content obtained in fruits showed an average extraction yield of around 30%. These results are consistent with those obtained by Guyot et al.[6] The high association between procyanidins and the solid parts of the fruit, particularly cell wall materials, explains this low extraction yield.[16] However, individually, procyanidin B2 had a significantly higher extraction yield compared to monomers (CAT and ECAT on Fig. 5). This result could be in part

explained by the better solubility in water of procyanidin B2 compared to (-)-epicatechin, which had an octanol-water partition coefficient of 0.172 and 1.299, respectively.[39] Moreover, the tanning effect of procyanidins is directly related to the degree of polymerization.[40] Since procyanidin B2 is a dimer, its association with the proteins of the matrix was less than other more polymerized tannins. The extraction yield was particularly low for flavonols, around 18% (Fig. 5). In fruits, more flavonol compounds could be quantified compared to the juices. Isoquercitrin was not detected and reynoutrin was not sufficiently concentrated to be quantified in juices with our method. This low extraction yield could be explained by the localization of these compounds in the fruit skin. Nevertheless, in our progeny, the extraction yield determined for flavonols was better than that previously reported in cider apple varieties, ranging around 10%.[41] The difference could be due to the juice extraction method used since they used a commercial scale pressing method, whereas our fruits were cored and crushed with a juice extractor. In 2009, since the dihydrochalcones, phloretin xyloglucoside, was not quantified, the average extraction yield was only estimated for phloridzin, around 17% (Fig. 5). If we consider the average concentration of total dihydrochalcones determined in J10 compared to that determined in fruits, the extraction yield was better, nearly 33%. Nevertheless, these results are very low compared to previous results that showed an extraction yield of 80%.[6] However, to determine the fruit content, Guyot et al.[6] had only considered the flesh of apples, whereas the skin and the seeds, very concentrated in dihydrochalcones, were included in our study.[3, 42, 43] The extraction yield previously measured by Guyot et al.[6] had therefore probably been overestimated. Finally, ideain was exclusively found in fruits, which is the confirmation of its localization in the fruit skin.

Variability of polyphenol contents between two apple progenies

The variability observed in the progeny was representative of that observed for different cider apple varieties. In our progeny, 4-*p*-coumaroylquinic acid had the largest range in fruits (125-fold), and hyperin had the lowest (13-fold). The variability of DPn was estimated in a progeny for the first time. It was almost 3-fold depending on the individuals. In juices, rutin had the largest range since it was 56 times more concentrated in the most concentrated individual compared to the least concentrated. Procyanidin B2 had the lowest one, less than 6-fold. The variability observed in juices was less than that observed in fruits. This could be explained by the loss of some phenolic compounds during the juice preparation. However, rutin was the only compound for which the variability was greater in the juices than in the fruits.

The comparison with the New Zealand study on dessert apple progeny showed that the variability observed in our progeny was similar for 4-*p*-coumaroylquinic acid and reynoutrin, but was higher for isoquercitrin and quercitrin in our progeny. However, the variability observed in our progeny was low for flavanols, DHC and anthocyanins. Indeed, the difference between the most and the least concentrated individual in the dessert progeny was 172-fold for (+)-catechin and 203-fold for ideain in the skin, and 61-fold for phloridzin or phloretin xyloglucoside in the flesh.[25] In our cider progeny, they were 35-, 18-, 30- and 15-fold, respectively (Table 1). These comparisons were based on minimum and maximum values found in progenies. These results have to be examined with great care since extreme values were not critically representative of the entire progeny. In addition, the Chagne et al.[25] study was performed for skin and flesh separately, which could have accentuated the variability observed compared to the whole fruit.

CONCLUSION

The genetic basis of the progeny under study resulting from a cross between a dessert apple and a hybrid of a cider apple, allowed us to obtain mean values and ranges for each compound that were very similar to previous studies on phenotypic variability in cider apples for both fruit extracts and juices. As expected, fruits were more concentrated than juices for all the compounds detected. The differences observed between fruits and juices for each polyphenol compound can easily be explained by their various extractability properties: hydroxycinnamic acids were the most extractable group, followed by flavanol monomers, procyanidins, dihydrochalcones, flavonols and anthocyanins. However, phenolic profiles were similar between fruits and juices with 5-caffeoylquinic acid and rutin as the most and least concentrated compounds, respectively. ANOVA results have shown a high genetic effect for all compounds, which suggests a considerable part of genetic variability in the expression of these traits. However, for flavanols, a greater effect of the harvest year was observed in both fruits and juices, consistent with their high sensitivity to light and temperature. The high genetic effects will allow us to use this dataset for future QTL mapping analysis.

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Table 1. Mean concentration (mg kg^{-1} of fresh weight) of phenolic compounds present in the whole fruit (2008 and 2009 harvest year)[a]

	Total catechins	**Total PCA**	**Total flavanols**	**DPn**	**Total HA**	**Total DHC**	**Total flavonols**	**Ideain***	**Total polyphenols**
Average	253	1508	1761	30	715	131	94	13	2707
Median	237	1394	1644	29	654	127	88	11	2488
Minimum	33	376	592	21	86	35	20	3	1058
Maximum	656	4112	4769	56	2000	332	274	58	6418

[a]: PCA: procyanidins; DPn: average degree of polymerization of flavanols; HA: hydroxycinnamic acids; DHC: dihydrochalcones.

*: The average, median, minimum and maximum concentrations of ideain were determined for the 82 individuals for which ideain were detected. 134 individuals had no anthocyanins.

Table 2. Range of total polyphenol content, total procyanidins determined with acidolysis reaction, polymerization degree of flavanols and some other phenolic compounds in fruit (expressed in mg kg^{-1} of fresh weight) and juice (expressed in mg L^{-1}) of different apple varieties[a]

Number and type of apple varieties		Total polyphenols	Total PCA	ECAT	CAT	B2	DP flavanols	5CQA	References
19 English cider apples	peel (mg kg^{-1} of FW)	546-6306		116-2095	10-265	107-1362		30-1163	4
	flesh (mg kg^{-1} of FW)	485-4920		ND-2225	6-408	ND-1368		69-1766	
2 juices, 12 French and 1 English cider apples	(mg kg^{-1} of FW)	1040-6990	515-4731	tr-1410	tr-154		42-503	154-1195	2
67 dessert apples	(mg kg^{-1} of FW)	1046-5448	469-4679	13-551	2-145	137-400	6-57	3-592	34b
31 Basque cider apples	(mg L^{-1})	660-13600	347-3511	39-822	3.3-40.7	47-550	2.7-4.6	172-1099	3
7 German cider apples	(mg L^{-1})	261.2-970		29.8-189.1	3-60	29.2-138.4		80.6-487.6	38
46 Spanish cider apples	1994 season (mg L^{-1})	570-2060		4.1-234.6		ND-222.9		25.1-377.1	37
	1995 season (mg L^{-1})	750-2420		ND-206.5		ND-246.9		21.2-350.5	
4 dessert apples	(mg L^{-1})	154.4-178		15.1-51.4	2.5-7	29.6-42.5		32.7-54.1	38

Number and type of apple varieties		DHC	PLZ	XPL	Total flavonols	HY	QR	RU	ID	References
19 English cider apples	peel (mg kg^{-1} of FW)		25-1061	ND-201		40-520	18-236	2-6	ND-494	4
	flesh (mg kg^{-1} of FW)		16-159	ND-73		ND-1.2	ND-12		ND-12	
2 juices, 12 French and 1 English cider apples	(mg kg^{-1} of FW)		16-102	10-98						2
67 dessert apples	(mg kg^{-1} of FW)		1-61	3-41		2-107	4-138	ND-19	ND-20	34b
31 Basque cider apples	(mg L^{-1})		11-92	15-137		0.34-4	0.48-6.5			3
7 German cider apples	(mg L^{-1})	33.5-171	13.2-93.6	20.3-135.9	tr-26.7	tr-8.1	tr-4.6	ND-0.8		38
46 Spanish cider apples	1994 season (mg L^{-1})		3.7-36.6	4-159.1						37
	1995 season (mg L^{-1})		2.5-32.4	3.3-107.2						
4 dessert apples	(mg L^{-1})	9.8-35.2	4.1-9.3	2.7-25.9	tr-3.6	tr-2.2	tr-1.9			38

[a]: PCA: procyanidins; ECAT: (-)-epicatechin; CAT: (+)-catechin; B2: procyanidin B2; DP: polymerization degree; 5CQA: 5-caffeoylquinic acid; DHC: dihydrochalcones; PLZ: phloridzin; XPL: phloretin xyloglucoside; HY: hyperin; QR: quercitrin; RU: rutin; ID: ideain; ND: not detected; tr: trace.
[b]: Values presented by Wojdylo et al. (2008) were modified to be expressed in g of FW. We chose an arbitrary correction factor of 5.

Table 3. Concentration (mg L^{-1}) of phenolic compounds present in the juice (2010 harvest year)[a]

	Total catechins	Total PCA	Total flavanols	Total HCA	Total flavonols	Total DHC	Total polyphenols
Average	249	762	1011	863	47	73	1994
Median	231	716	988	883	43	65	1911
Minimum	61	339	451	137	16	31	740
Maximum	611	1604	2168	1788	177	244	3742

[a] : Abbreviations: see Table 1

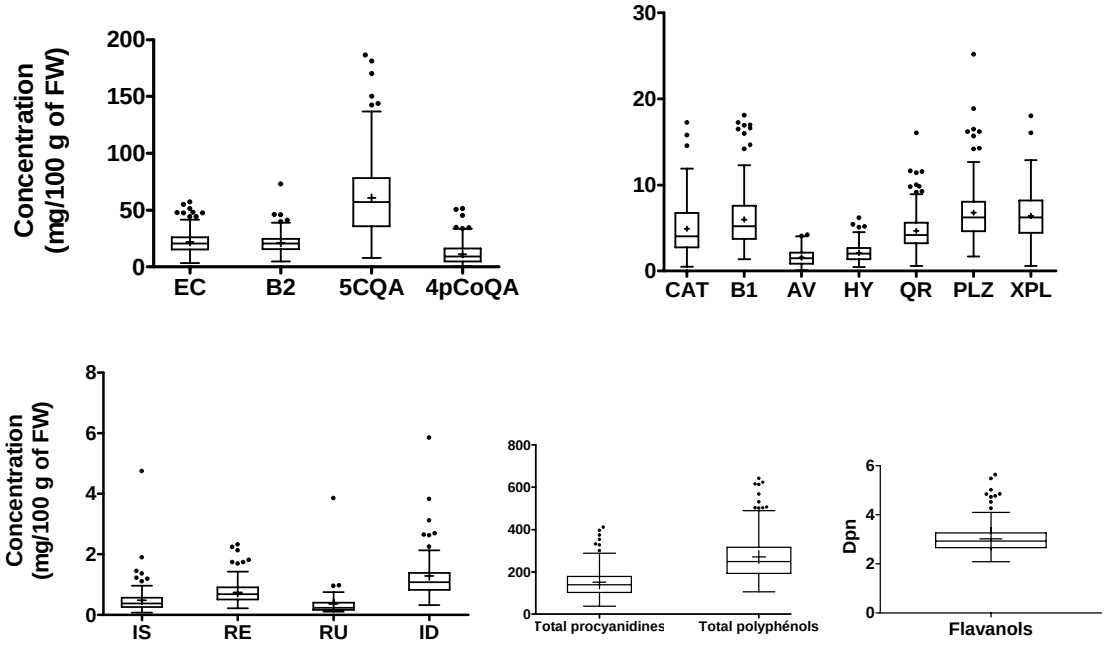

Figure 1. Turkey boxplot for the mean polyphenol concentration (mg/100g of fresh weight) in the whole fruit (2008 and 2009 harvest years). EC: (-)-epicatechin; CAT: (+)-catechin; B1: procyanidin B1; B2: procyanidin B2; 5CQA: 5-caffeoylquinic acid; 4pCoQA: 4-*p*-coumaroylquinic acid; AV: avicularin; HY: hyperin; IS: isoquercitrin; QR: quercitrin; RE: reynoutrin; RU: rutin; PLZ: phloridzin; XPL: phloretin xyloglucoside; ID: ideain; DPn: mean polymerization degree. Outliers and means are represented with circles and crosses, respectively.

Source file: GraphPadPrism 5.01

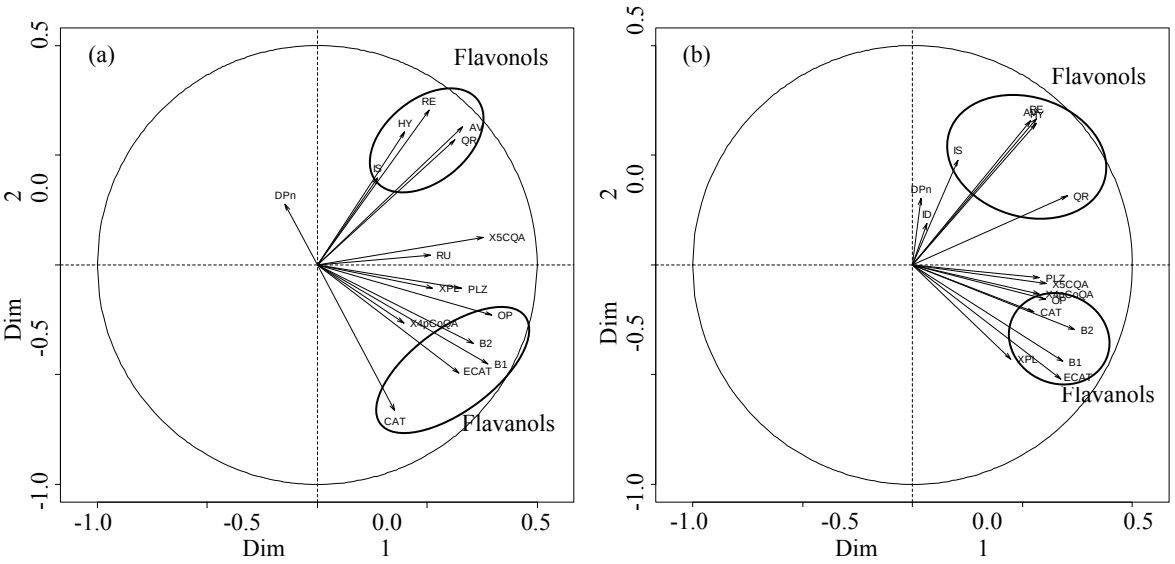

Figure 2. PCA of phenolic compounds quantified in fruits harvested in 2008 (a) and 2009 (b). Abbreviations: see Fig. 1; DPn: polymerization degree of flavanols; OP: total procyanidins without procyanidins B1 and B2; X4pCoQA: 4-*p*-coumaroylquinic acid; X5CQA: 5-caffeoylquinic acid.

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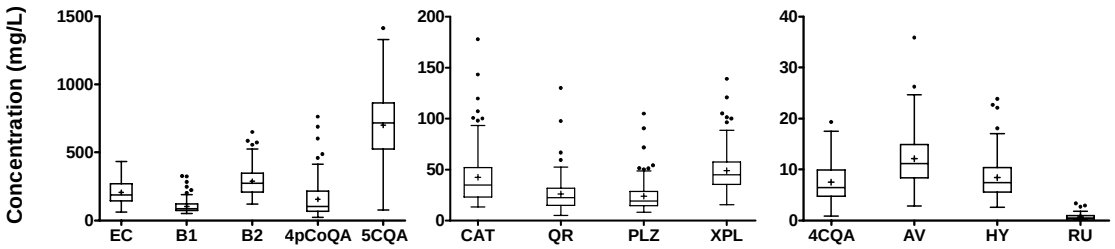

Figure 3. Turkey boxplot for the mean polyphenol concentration (mg/L) in juices prepared in 2010. Abbreviations: see Fig. 1; 4pCoQA: 4-*p*-coumaroylquinic acid. Outliers and means are represented with circles and crosses, respectively.

Source file: GraphPadPrism 5.01

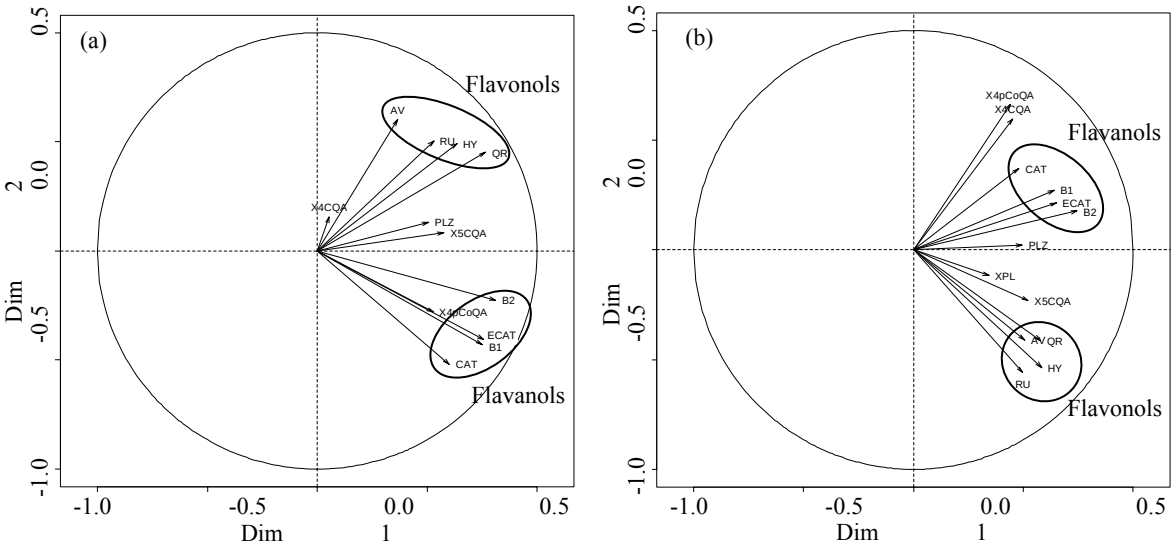

Figure 4. PCA of phenolic compounds quantified in juices prepared in 2009 (a) and 2010 (b). Abbreviations: see Fig. 1; X4CQA: 4-caffeoylquinic acid; X4pCoQA: 4-*p*-coumaroylquinic acid; X5CQA: 5-caffeoylquinic acid.

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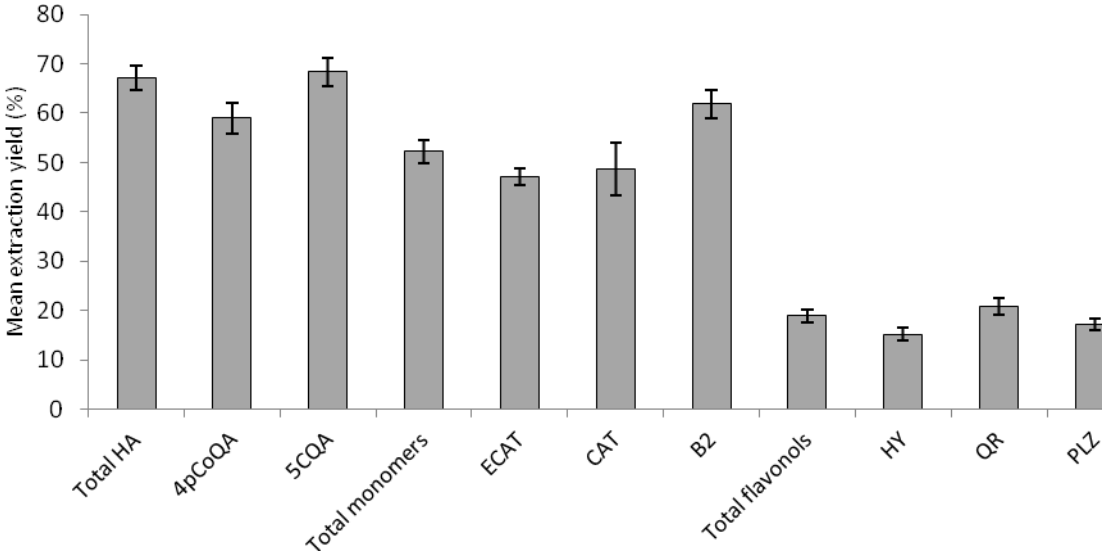

Figure 5. Mean extractability level (in %) of individual phenolic compounds as well as total hydroxycinnamic acids (HA), flavanol monomers, and flavonols determined within the 124 common individuals analyzed both for fruits and juices in 2009. Abbreviations: see Fig. 1.

Source file: excel 2007