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Detailed LC-UV-MS quantification of native and oxidized phenolic compounds in experimental and commercial apple juices revealed highly contrasting compositions

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ABSTRACT

During apple juice production, phenolic compounds undergo enzymatic oxidation leading to newly formed molecules. More quantitative information is needed concerning these oxidized phenolic compounds (OP) that contribute to the organoleptic and nutritional properties of apple juices. Firstly, a new HPLC-UV-MS method was developed to simultaneously quantify native phenolic compounds and two families of OP (*i.e.* 5-*O*-caffeoylquinic acid dehydrodimers and epicatechin-5-*O*-caffeoylquinic acid heterodimers). Secondly, the method was applied to 54 commercial and experimental apple juices. Then, statistical cluster analysis (Euclidian Ward's method) was performed on all the phenolic compound data sets. To the best of our knowledge, it is the first time that two families of OP have been successfully quantified in a large series of apple juices revealing that they may represent up to 14% of the total phenolic compounds quantified (TPQ). Furthermore, the statistical classification of the juices based on their native and oxidized polyphenol compositions clearly underlined the great discrepancies in polyphenol composition observed in this panel of juices available on the French market. Interestingly, the polyphenol concentration in most of the juices made from cider apple varieties (experimental juices and local cider producers) was on average 8-fold higher than in most of the juices found in supermarkets.

1. Introduction

Apple is one of the main fruits produced worldwide (93 million tons in 2020) (FAO, 2021). A considerable amount of this production is processed into apple juice, which is the second most consumed fruit juice, just after orange juice (AIJN, 2018).

The composition of native phenolic compounds (NPC) present in apples and apple-derived products have been extensively studied (Guyot et al., 2003; Kahle et al., 2005; Pavun et al., 2018; Sanoner et al., 1999; Sun et al., 2002; Tsao et al., 2003). In contrast, as far as we know, no information is available regarding the concentrations of polyphenol oxidation products (OP) in juice resulting from the enzymatic oxidation of NPCs that occurs during apple processing. Indeed, during apple juice production, apple phenolics undergo enzymatic oxidation resulting in the possible transformation of some phenolic compounds. In the first

step (crushing and pressing), the cellular organization is disrupted and plastidial polyphenol oxidase (PPO) can come into contact with vacuolar phenolic substrates in the presence of oxygen (Nicolas et al., 1994). In apples, 5-*O*-caffeoylquinic acid (CQA), the preferential substrate of PPO, is enzymatically oxidized into its corresponding *o*-quinone. This reactive species is subsequently involved in several reaction pathways, especially coupled oxidation-reduction reactions and oxidative coupling reactions that may involve most of the NPCs.

In the case of apples, the principal NPCs are flavonoids (flavan-3-ols, flavonols, anthocyanins, and dihydrochalcones) and hydroxycinnamic acid esters (Guyot et al., 2002; Guyot and Poupard, 2011; Wojdyło et al., 2008) such as catechins ((-)-epicatechin and (+)-catechin), procyanidins, and 5-*O*-caffeoylquinic acid, a major hydroxycinnamic acid ester in apple (Guyot et al., 2002). Dihydrochalcones, which can be considered a specific phenolic class in apples, are also present in apple-derived

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products. Regarding flavonols and anthocyanins, they are mainly located in the peel and are present in low concentrations in apple juices. The structural features of native phenolic compounds found in apples are presented in Fig. 1.

As a consequence of oxidation, many OPs are present in apple juices and they likely contribute to the overall quality of apple-based beverages as they are responsible for the colour of juices (Le Deun et al., 2015; Févri er et al., 2017) or are involved in sensory qualities such as bitterness and astringency (Lea and Timberlake, 1974; Symoneaux et al., 2015). As OPs still have a phenolic structure, they also contribute to the antioxidant capacity of apple-derived products along with NPCs (Hotta et al., 2002; Wong-Paz et al., 2015).

Several OPs have been detected in apple juices using mass spectrometry (Bernillon et al., 2004; Guyot et al., 2008, 2003) and some have been synthesized and characterized in biomimetic model solutions (Castillo-Fraire et al., 2019; Guyot et al., 2007; Poupard et al., 2011; Wong-Paz et al., 2015). Concerning these studies, one of the major families of OPs was CQA dehydrodimers (MW, 706 Da) (Bernillon et al., 2004; Castillo-Fraire et al., 2019; Wong-Paz et al., 2015) but compounds resulting from the oxidative coupling between CQA and a catechin moiety (MW, 642 Da) were also formed (Wong-Paz et al., 2015).

In previous studies, these CQA dehydrodimers were classified as dihydrobenzofuran, dihydrobenzodioxan, or dihydronaphtalen types based on their phenolic skeleton (Fig. 1) (Castillo-Fraire et al., 2019). These structures were confirmed using ¹H and ¹³C one- and two-dimensional NMR spectrometry (Castillo-Fraire et al., 2022). In addition, we also highlighted that these CQA dehydrodimers can have a specific ability to complex *in vitro* with salivary proteins and might thus contribute to organoleptic qualities associated with astringency perception in apple-derived beverages (Castillo-Fraire et al., 2021).

However, more information is still needed concerning the qualitative and quantitative composition of OPs in apple juices to better understand their contribution to organoleptic and nutritional properties. Consequently, the first objective of this work was to develop and validate a new HPLC-UV-MS method that simultaneously quantifies NPCs and, for the first time, two families of OPs (5-*O*-caffeoylquinic acid dehydrodimers and epicatechin-5-*O*-caffeoylquinic acid heterodimers). Secondly, this method was used to quantify NPCs and OPs in 54 apple juices purchased from several supermarkets or local cider producers, or prepared experimentally on a pilot scale in our technology platform. Interestingly, the results allowed us to provide an overview of the phenolic composition of apple juices currently available on the French market revealing contrasting polyphenol compositions that largely depend on juice origin.

2. Material and methods

2.1. Chemicals and standards

Acetonitrile, methanol, and formic acid were purchased from Carlo Erba Reagents (France). (+)-catechin (CAT), (-)-epicatechin (EC), 5-*O*-caffeoylquinic acid (CQA), *p*-coumaroylquinic acid (PCQ), procyanidin B2 (PA-B2), and phloridzin (PLZ) were obtained from Sigma-Aldrich (St. Louis, MO). Quercetin-3-*O*-galactoside (QCE-3-*O*-GAL) was obtained from Extrasynth ese (France).

CQA dehydrodimers (MW 706 Da) (Castillo-Fraire et al., 2019) and EC-CQA heterodimers (MW 642 Da) (Wong-Paz et al., 2015) were synthesized by enzymatic oxidation and purified as described previously (Wong-Paz et al., 2015). The structural features of these compounds are given in Fig. 1.

2.2. Preparation of standard solutions

Six phenolic reference compounds (PA_B2, EC, PLZ, CAT, CQA, PCQ, QCE-3-*O*-GAL) were accurately weighed and dissolved (together) in a solvent mix (methanol-water-formic acid, 50:49:1 v/v/v) at a final

concentration of approximately 50 µg/mL.

In the case of OPs, four CQA dehydrodimers of MW 706 Da (705-1.1, 705-2, 705-4, and 705-6) corresponding to different structures (Fig. 1) and one EC-CQA heterodimer of MW 642 Da (641-1) for which no additional detailed information on the chemical structure was given at this time, were accurately weighed and dissolved in purified water to prepare a stock solution containing each OP standard at a final concentration of 100 mg/L.

Before HPLC-UV-MS analyses, the standard working solutions used for the calibration curves were prepared by appropriate dilution of the stock solution in methanol-water-formic acid (50:49:1 v/v/v). The range of concentrations was 1 mg/L to 50 mg/L for NPCs and 0.1 mg/L to 10 mg/L for OPs. All stock solutions and their corresponding diluted solutions were stored at -25 °C under an argon atmosphere.

2.3. Sample Preparation

In this study, 13 experimental apple juices were prepared on a pilot scale in our technology platform, 12 juices were purchased directly from local cider producers (direct sale), and 29 apple juices were purchased from two supermarkets. The descriptions of the apple juices are provided in Table S1 (supplementary data). The experimental juices were prepared from French cider apple varieties under conditions that allowed oxidation, as described in a previous study (Le Deun et al., 2015). All samples were filtered using a 0.45 µm PTFE filter and diluted 5 times in the solvent mixture described previously. The analyses were performed in triplicate (independent dilutions) in random order.

2.4. HPLC-UV-MS instrument and analysis conditions

All samples were analyzed using a High-Performance Liquid Chromatography System composed of an SCM1000 degasser (Thermo Scientific, San Jose, CA, USA), an 1100 series binary high-pressure pump (Agilent Technologies, Santa Clara, CA, USA), and a surveyor auto-sampler (Thermo Finnigan, San Jose, CA, USA) with a cooling system set to 4 °C. HPLC was coupled with two detectors: a UV-visible diode array detector (UV6000 LP, Thermo Finnigan, San Jose, CA, USA) used in the 240-600 nm range and an ion trap mass spectrometer equipped with an electrospray ionization source (LCQ Deca, Thermo Finnigan, San Jose, CA, USA) used in negative mode. Mass spectrometry parameters were adapted from a previous publication (Wong-Paz et al., 2015). A reverse-phase Hibar HR Purospher STAR RP-18 end-capped chromatography column (150 mm × 2.1 mm, 3 µm, Merck) was used. The injection volume was 2 µL with a flow rate of 0.2 mL/min and a column temperature of 30 °C. The eluent was a gradient of 0.1% (v/v) formic acid (A) and acetonitrile acidified with 0.1% (v/v) formic acid (B). The elution gradient was applied as follows: initial, 3% B; 0 - 3 min, linear gradient to 7% B; 3 - 21 min, linear gradient to 13% B; 21 - 27 min, 13% B isocratic; 27 - 41 min, linear gradient to 20% B; 41 - 51 min, linear to 45% B. The column was then washed and reconditioned to 3% B for the next analysis.

A total of 32 phenolic compounds were analyzed of which eighteen were NPCs: three hydroxycinnamic acid esters (5-*O*-caffeoylquinic acid (CQA), caffeic acid (CAF), *p*-coumaroylquinic acid (PCQ)); two dihydrochalcones (phloridzin (PLZ), phloretin xyloglucoside (XPL)); six flavonols (avicularin (QCE-3-*O*-Ara), hyperoside (QCE-3-*O*-Gal), isoquercitrin (QCE-3-*O*-Glu), quercitrin (QCE-3-*O*-Rha), rutin (QCE-3-*O*-rha-glu), reynoutrin (QCE-3-*O*-Xyl)); two flavan-3-ol monomers ((+)-catechin (CAT), (-)-epicatechin (EC)); five flavan-3-ol oligomers corresponding to procyanidins B2 (dimer PA-B2), B1 (dimer PA-B1), B5 (dimer PA-B5), C1 (trimer PA-C1), and DP4 (major tetramer). Fourteen OPs were also analyzed: ten CQA dehydrodimers (705-1.1, 705-1, 705-2, 705-3, 705-4, 705-5, 705-6, 705-7, 705-8, 705-9) and four EC-CQA heterodimers (641-1, 641-2, 641-3, and 641-4). Typical HPLC-UV and HPLC-MS chromatograms of juice are shown in Fig. S2 (Supplementary data).

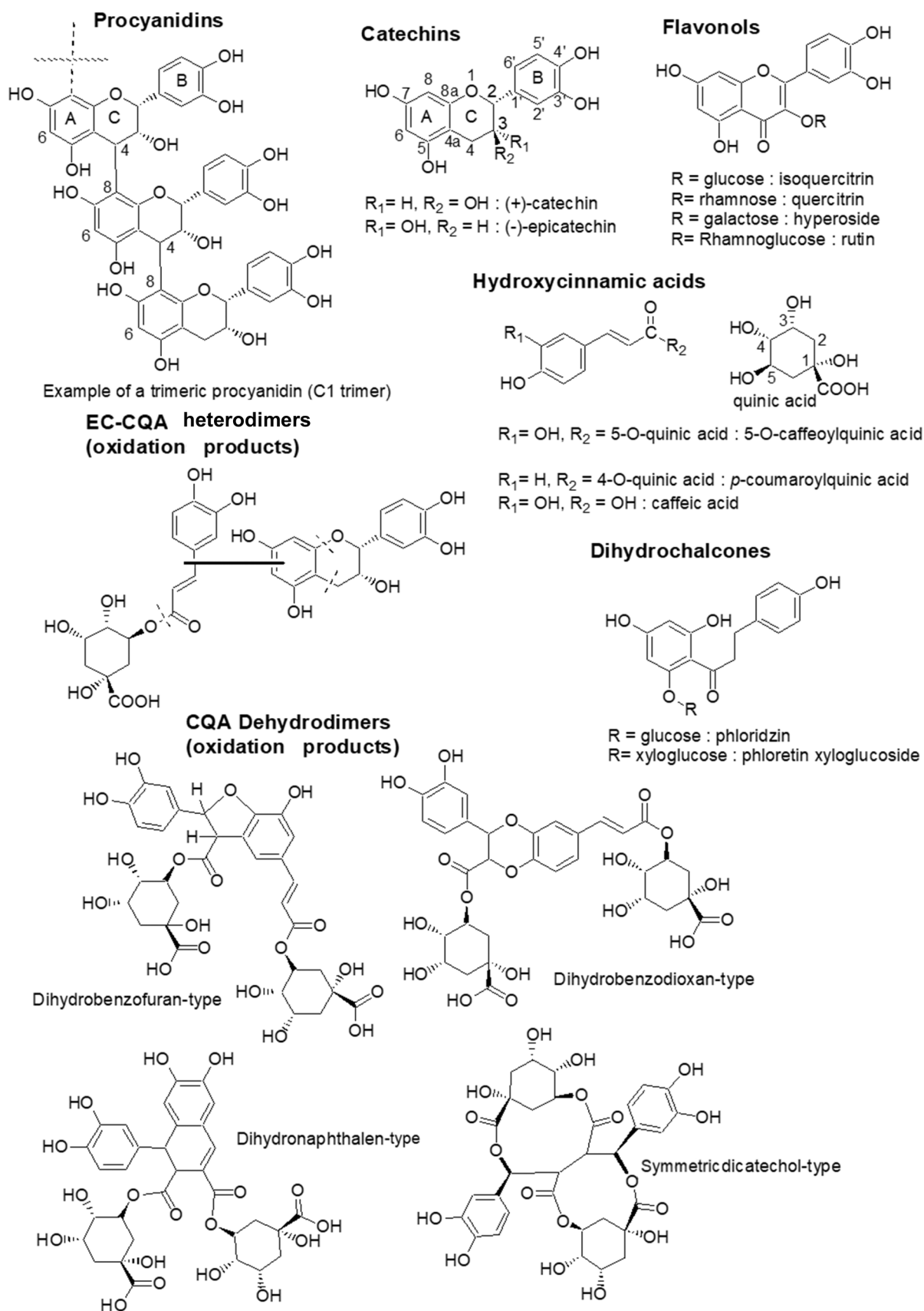


Fig. 1. Families of Native Phenolic Compounds (NPC) and Oxidation Products (OP) studied.

All the compounds (NPCs and OPs) were analyzed on the same HPLC run, except for total procyanidins (corresponding to whole condensed tannins). NPCs and OPs were quantified using UV and MS, respectively. Total procyanidins were quantified using UV in another HPLC run after depolymerization by phloroglucinolysis according to a method adapted from a previous study (Le Deun et al., 2015).

Data were collected and processed using Xcalibur software (version 1.2). Peak integration and calibration were performed with the UV signal at 280 nm for commercial phenolic compounds, except for CQA, PCQ, and CAF (320 nm), and flavonols (350 nm).

2.5. Method validation

HPLC-MS (Single Ion Monitoring) method was validated for quantification of OPs with the calibration curves of four CQA dehydrodimers (705-1.1, 705-2, 705-4, and 705-6) and one EC-CQA heterodimer (641-1). Three CQA dehydrodimers (705-1.1, 705-4, and 705-6) were used as quantification standards for the other six dehydrodimers. Based on their structural similarities, 705-1.1 was the standard for 705-1, 705-4 for 705-3, and 705-6 for 705-5, 705-7, 705-8, and 705-9. Concerning EC-CQA heterodimers, one compound (641-1) was used as the standard for its quantification and the other three heterodimers found in the juices. Three replicates of different concentrations of the stock solution in the range of 0.1 µg/mL to 10 µg/mL were analyzed and the mean linear regression of the peak area versus the quantity of each analyte injected (ng) was used as a calibration curve (Table 1).

The HPLC-UV method has already been validated for NPCs in a previous study (Le Deun et al., 2015). This validation concerned CAT, EC, CQA, PCQ, PA-B2, QCE-3-O-Gal, and PLZ (data not shown). Based on structural similarities, some NPCs were used as standards for the quantification of others. CQA was the standard for CAF, PLZ for XPL, QCE-3-O-Gal for QCE-3-O-Ara, QCE-3-O-Glu, QCE-3-O-Rha, QCE-3-O-rha-glu, and QCE-3-O-Xyl, PA-B2 for PA-B1 and PA-B5, and EC was the standard for PA-C1 and DP4.

For OP standards, the slopes, linear ranges, correlation coefficients of the calibration curves, the lowest limit of detection (LOD), the lowest limit of quantitation (LOQ), and precision (%RSD) data were calculated. LOD and LOQ corresponded to the concentrations at the signal-to-noise ratios (S/N) of ~3 and 10, respectively.

To check the stability of the equipment, in particular, the possible loss of MS sensitivity related to fouling of the electrospray source, a control sample of OPs (2.5 µg/mL) was injected after each series of 16 injections. The precision of the MS method was evaluated and expressed as the relative standard deviation (%RSD).

2.6. Statistical analysis

Hierarchical classification of the samples was performed on all phenolic data sets (detailed NPC and OP profiles) according to Euclidian Ward's method using Statgraphics Plus software (version 5.1). This

analysis is based on the degree of similarity between each item: the more similar the phenolic content between two juices, the closer they are on the dendrogram. Phenolic families selected as parameters for this classification were hydroxycinnamic acid esters, dihydrochalcones, catechins, procyanidins, flavonols, CQA dehydrodimers, and EC-CQA heterodimers.

3. Results and discussion

3.1. HPLC-MS method validation

The HPLC-MS (Single Ion Monitoring) method was validated for the quantification of ten CQA dehydrodimers and four EC-CQA heterodimers. The slopes, linear ranges, correlation coefficients of the calibration curves, LOD, LOQ, and precision are summarized in Table 1. The precision of the method was expressed as the relative standard deviation (%RSD). The number of degrees of freedom was 12.

The RSDs of the CQA dehydrodimers used as standards varied from 2.4% to 3.4%. The RSD of the EC-CQA heterodimer was lower (1.9%) than those of the CQA dehydrodimers. The calibration data indicate the linearity of MS detection ($r^2 > 0.997$) for all OP standards in the range of 0.1 mg/L to 10 mg/L.

LOD values for the four CQA dehydrodimer standards were below 0.04 mg/L. EC-CQA heterodimer presented the lowest LOD value (0.02 mg/L). LOQ values for CQA dehydrodimers and EC-CQA heterodimer ranged from 0.10 mg/L to 0.14 mg/L (Table 1).

3.2. Analyses of apple juices

Fifty-four apple juices were analyzed in triplicate using HPLC-UV-MS to quantify NPCs and OPs namely CQA dehydrodimers and EC-CQA heterodimers. Among them, 13 apple juices were experimental juices prepared on a pilot scale in our technology platform, 12 were bought directly from cider producers in western France (mainly Brittany and Normandy), and 29 were purchased from supermarkets. Of the 54 juices, 15 were identified as organic juices, 47 were pure juices and 7 were made from apple juice concentrates, while 33 were clear juices and 21 were cloudy.

3.2.1. Descriptive analysis of the qualitative and quantitative distribution of native phenolic compounds (NPC) and phenolic oxidation products (OP) in the whole apple juice sample set

Fig. 2 presents the overall distributions and the main descriptive parameters (minimum, maximum, average, and median) for the concentrations of the four main families of NPC (*i.e.* hydroxycinnamic acid esters, dihydrochalcones, catechins, and total procyanidins), two families of OP (*i.e.* CQA dehydrodimers and EC-CQA heterodimers), total phenolic compounds quantified (TPQ) corresponding to the sum of NPCs and OPs, and the percentage of OPs (in relation to TPQ).

The hydroxycinnamic acid (HCA) family was composed of CQA,

Table 1

Method validation for simultaneous quantification of oxidized phenolic compounds in apple juices: CQA dehydrodimers (705) and EC-CQA heterodimers (641). Degrees of freedom (dof) = 12.

Compounds used as standards	Other compounds quantified with the same standard	Regression equation	R ²	Linear range (mg /L)	LOD (mg /L)	LOQ (mg /L)	RSD (%)
705-1.1	705-1	$y = 1.08E+13x + 3.78E+06$	0.9988	0.1-10	0.04	0.12	2.55
705-2	-	$y = 8.70E+12x + 3.90E+06$	0.9984	0.1-10	0.04	0.14	2.72
705-4	705-3	$y = 1.47E+13x + 7.80E+06$	0.9988	0.1-10	0.03	0.11	2.37
705-6	705-5, 705-7, 705-8, 705-9	$y = 1.23E+13x + 6.14E+06$	0.9981	0.1-10	0.03	0.10	3.39
641-1	641-2, 641-3, 641-4	$y = 1.69E+13x + 1.14E+07$	0.9977	0.1-10	0.02	0.10	1.85

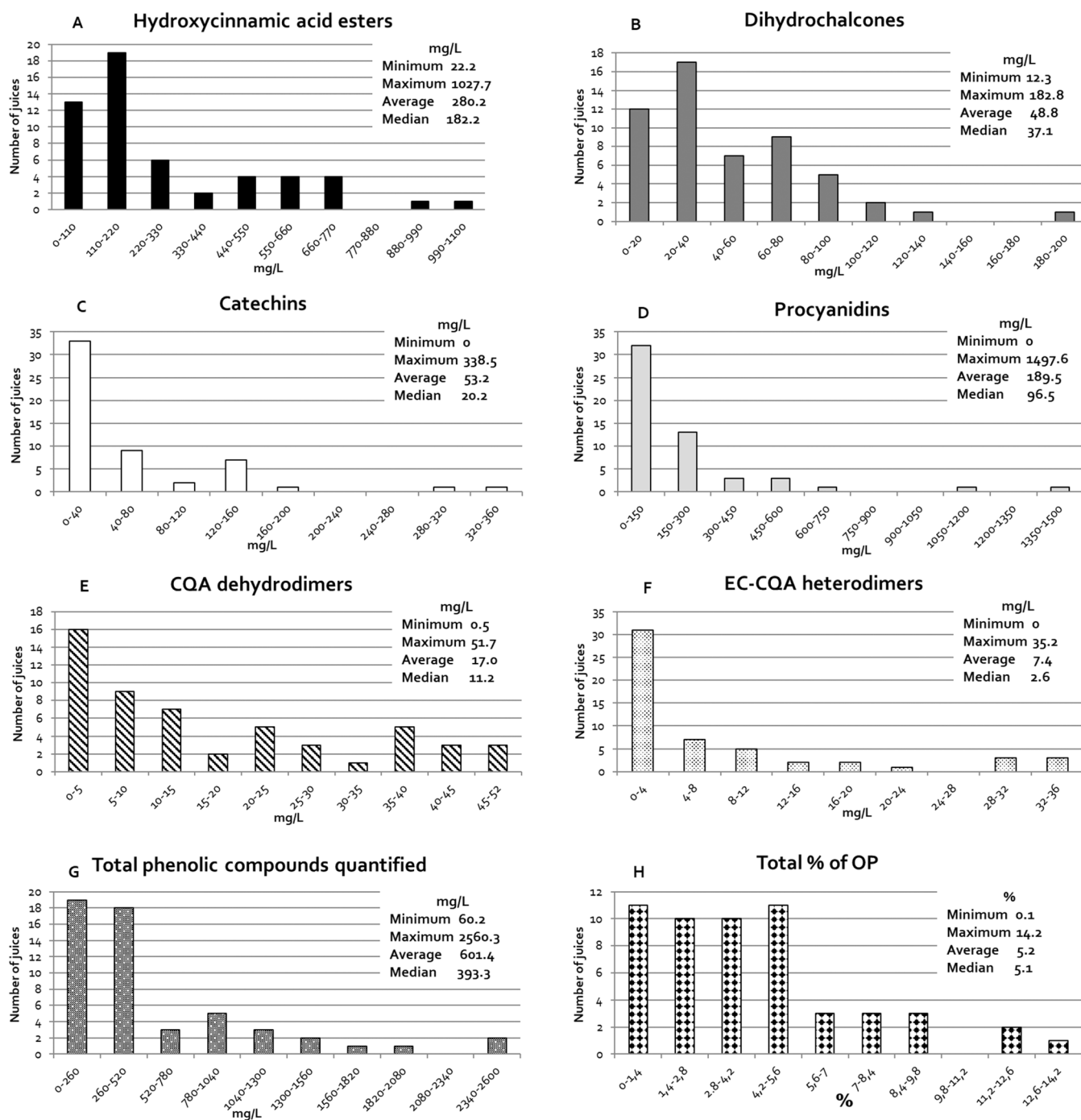


Fig. 2. Quantitative distribution of native phenolic compounds and oxidation products in 54 apple juices. **A:** hydroxycinnamic acid esters; **B:** dihydrochalcones; **C:** Catechins; **D:** Procyanidins; **E:** CQA dehydromers; **F:** EC-CQA heterodimers, **G:** total phenolic compounds quantified (TPQ), **H:** total % of OP (including CQA dehydromers and EC-CQA heterodimers) based on TPQ.

PCQ, and CAF. These NPCs constituted one of the most concentrated classes with an average of 280 mg/L and a maximum of 1028 mg/L (Fig. 2A). Around sixty percent of the juices showed HCA concentrations lower than 220 mg/L (32 apple juices). These results are consistent with previous studies on apple juice phenolic composition (Kahle et al., 2005; Oszmianski et al., 2007; Verdu et al., 2014).

The average concentration of dihydrochalcones (DHC) such as PLZ and XPL, in the apple juices, was 49 mg/L and the maximum was 183 mg/L (Fig. 2B). The maximum DHC concentrations were in accordance with results previously published for apple juices made from cider apple varieties (Guyot et al., 2003; Kahle et al., 2005; Verdu et al., 2014)

whereas the lower values were close to those made from “dessert apple” varieties (Candrawinata et al., 2013; Oszmianski et al., 2007; Vrhovsek et al., 2004). DHC concentrations were lower than 40 mg/L in 54% of the juices (29 juices).

Concerning flavan-3-ols (Fig. 2 C and D), the maximum values for monomers (catechins, 338 mg/L) and procyanidins (1498 mg/L) were consistent with previous studies on apple juices made from cider apple varieties (Guyot et al., 2003; Kahle et al., 2005; Verdu et al., 2014). In the case of catechins (*i.e.* flavan-3-ol monomers), concentrations were below 40 mg/L in 33 juices, 24 of which were supermarket juices. Catechin concentrations in some of these apple juices were under the

LOQ (10 apple juices). Catechins are known to be involved in the redox coupling process, being either directly oxidized by PPO or oxidized to their *o*-quinone by the CQA *o*-quinone that is then reduced to CQA according to an oxidation-reduction mechanism (Poupard et al., 2008). This high susceptibility of catechins to oxidation could explain their low concentrations in certain apple juices. Regarding procyanidins, they are one of the most abundant classes. However, 45 juices showed concentrations below 300 mg/L, almost five times less than the maximum value quantified (circa 1500 mg/L). It is important to underline that 60% of these “low procyanidin level” juices were purchased in supermarkets.

In the case of OPs, ten CQA dehydrodimers and four EC-CQA heterodimers were considered (Fig. 2E and F, respectively). On average, CQA dehydrodimers (17 mg/L) were found in higher concentrations in apple juices than EC-CQA heterodimers (7.4 mg/L). Moreover, the maximum value for CQA dehydrodimers (51.7 mg/L) was higher than that of EC-CQA heterodimers (35.2 mg/L). For the latter, a large majority of juices (31 juices) showed concentrations below 4 mg/L. In contrast, CQA dehydrodimers were more widely distributed over the concentration ranges (Fig. 2E). In fact, 16 apple juices presented CQA dehydrodimer concentrations below 5 mg/L (Fig. 2E), and 12 of them were below 4 mg/L.

A total of 16 apple juices stand out (Fig. 2E) for their relatively high CQA dehydrodimer concentrations, which were between 5 mg/L and 15 mg/L. However, these concentrations are relatively low when compared to the total polyphenols quantified. Indeed, Fig. 2G shows the total phenolic compounds quantified (TPQ) using our HPLC method with an average of 601 mg/L. The lowest concentration of TPQ in apple juices was 60.2 mg/L, which represents 10% of the average. However, some apple juices could reach higher concentrations with a maximum of 2560 mg/L (for experimental apple juices). We must underline that for all the juices, the total OPs quantified represented a minor part (Fig. 2H) of the TPQ (Fig. 2G), accounting for less than 2.8% in 21 juices. On average, OPs accounted for 5.2% of the TPQ. However, among the oxidation products, only two series of OPs (*i.e.* CQA dehydrodimers and EC-CQA heterodimers) were quantified in our study. Other OPs, in particular those resulting from the oxidation of procyanidins, likely

make up a large part of the phenolic oxidation products, in apple juices. Interestingly, in one case, quantified OPs represented 14% of the TPQ. Detailed NPC and OP contents are displayed in Table S1 (Supplementary data).

Our data are in accordance with previous studies showing that apple juices from cider apple cultivars present a higher concentration of NPCs than those produced from “dessert apples” (Guyot et al., 2003; Kahle et al., 2005; Markowski et al., 2015; Oszmianski et al., 2007; Verdu et al., 2014).

The statistical boxplot graphs supplement the descriptive variability of phenolic compounds in the set of juices, in particular the magnitude of variation in terms of OPs. Considering the OPs individually (Fig. 3), many points are far from the interval, some might even appear to be outliers. This underlines that the distribution of the values does not comply with normal laws. This lack of normality can probably be explained by the large heterogeneity of the sample set in view of the origins of the selected juices.

The boxplot representation clearly shows that major OPs were CQA dehydrodimers 705-3 and 705-4, and EC-CQA heterodimer 641-2. Products 705-1, 705-1.1, and 641-1 were also significantly present but in lower concentrations. Other CQA dehydrodimers (705-2 and 705-5 to 705-9) were also detected and quantified but in very low concentrations.

Interestingly, the average distribution of the individual CQA dehydrodimers present in the juices analyzed (Fig. 3) was different from the distribution of those synthesized in a biomimetic apple juice model solution (Castillo-Fraire et al., 2019). Indeed, in this previous study, CQA dehydrodimers were produced enzymatically by incubating pure CQA in the presence of a crude apple PPO extract. In these synthetic conditions, two pairs of CQA dehydrodimers were formed as the main OPs and approximately in the same proportions: dehydrodimers 705-3 and 705-4 (dihydrobenzofuran-type) and dehydrodimers 705-5 and 705-6 (dihydrobenzodioxan-type) (Castillo-Fraire et al., 2019). However, it is especially the dihydrobenzofuran-type structures that are present in the real apple juices whereas 705-5 and 705-6 are present in much lower concentrations. In addition, CQA dehydrodimers 705-1 and 705-1.1

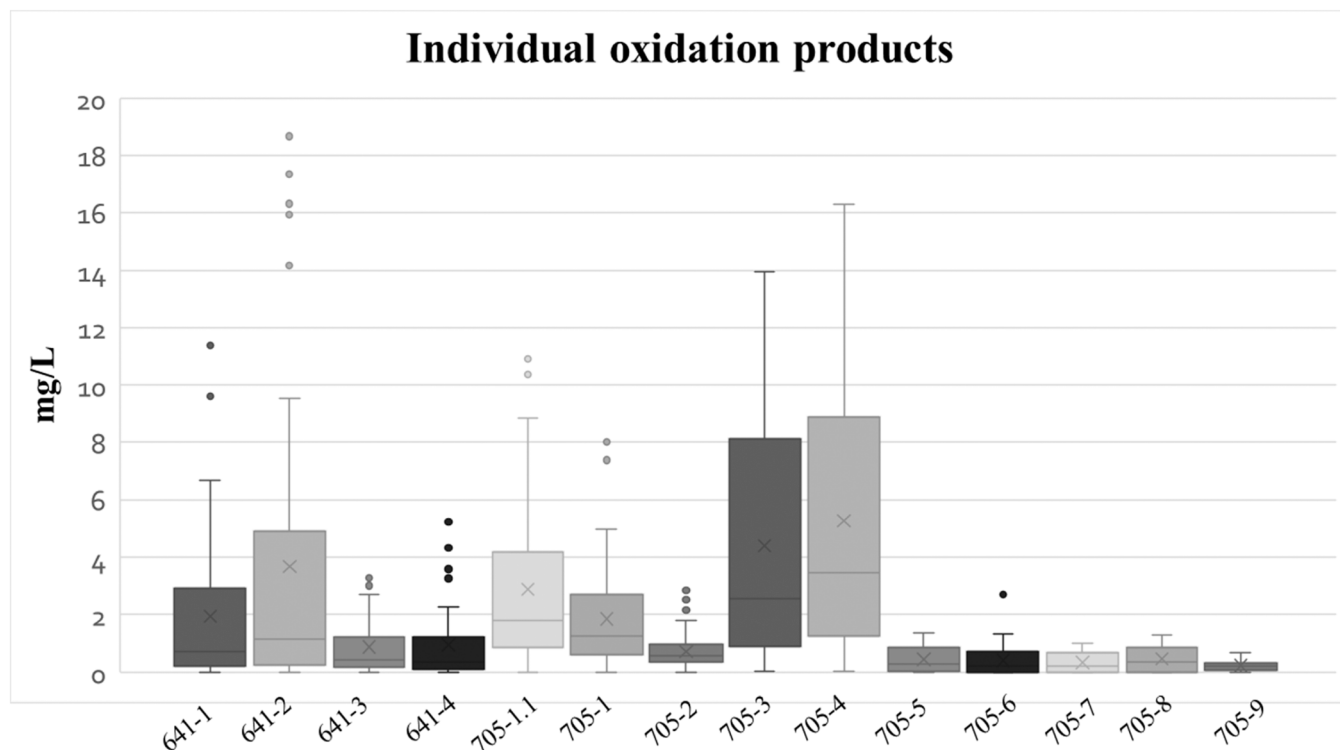


Fig. 3. Boxplot graphic of individual oxidation products (OP).

(dihydronephthalene structure) were present in apple juices at higher concentrations than those observed in the model solution (Castillo-Fraire et al., 2019). The CQA dehydrodimer 705-2, which has a very specific symmetric structure with two catechol groups (Castillo-Fraire et al., 2022), was also detected in the juices but at a low concentration compared to other CQA dehydrodimers (705-3, 705-4, 705-1, 705-1.1).

3.2.2. Statistical classification of the juices and discussion regarding apple categories produced in France

Different classes of apples are processed in France to produce apple juice, which can be classified into 3 categories:

Category A probably accounts for most of the production worldwide; these apples are commonly known as “dessert apples” or “eating apples”. The polyphenol content in juices is generally low (lower than 1 g/L) and the acidity variable (usually below 6 g/L of malic acid). Most “dessert apples” widely marketed (Golden Delicious, Fuji, Granny Smith, Gala, Ariane, etc.) are commonly used for apple juice production. These apples were selected for their storage tolerance and other criteria such as visual attractiveness, crunchiness, sugar/acidity balance, and lack of

browning when cut (correlated with low polyphenol content). Some of them result from a breeding program, so they are often considered “new varieties” as opposed to “old varieties”.

Category B exhibits an intermediate polyphenol content (from 1 g/L to 2 g/L) and acidity is usually higher. Most of them are local varieties grown for many years, sometimes known as “old varieties”. In some regions in France, especially in the west, these apples are often grown for juice or cider (i.e. alcoholic beverage resulting from juice fermentation). However, some old varieties in this category used as dessert apples (i.e. Reine des Reinettes or Reinette Grise du Canada) could be classified in this category given their polyphenol content.

Category C encompasses apple varieties with a high polyphenol content (more than 2 g/L) and low acidity. These local apples are not consumed raw as “dessert apples” given their bitter taste. In France, they are grown especially in the western region (Brittany and Normandy) and are mainly processed for cider production (alcoholic beverage), hence they are called “cider apples”. However, blended with other apple varieties, they could be more extensively used in juice production.

Apple juices are produced at different scales:

International or European-scale producers primarily use “dessert

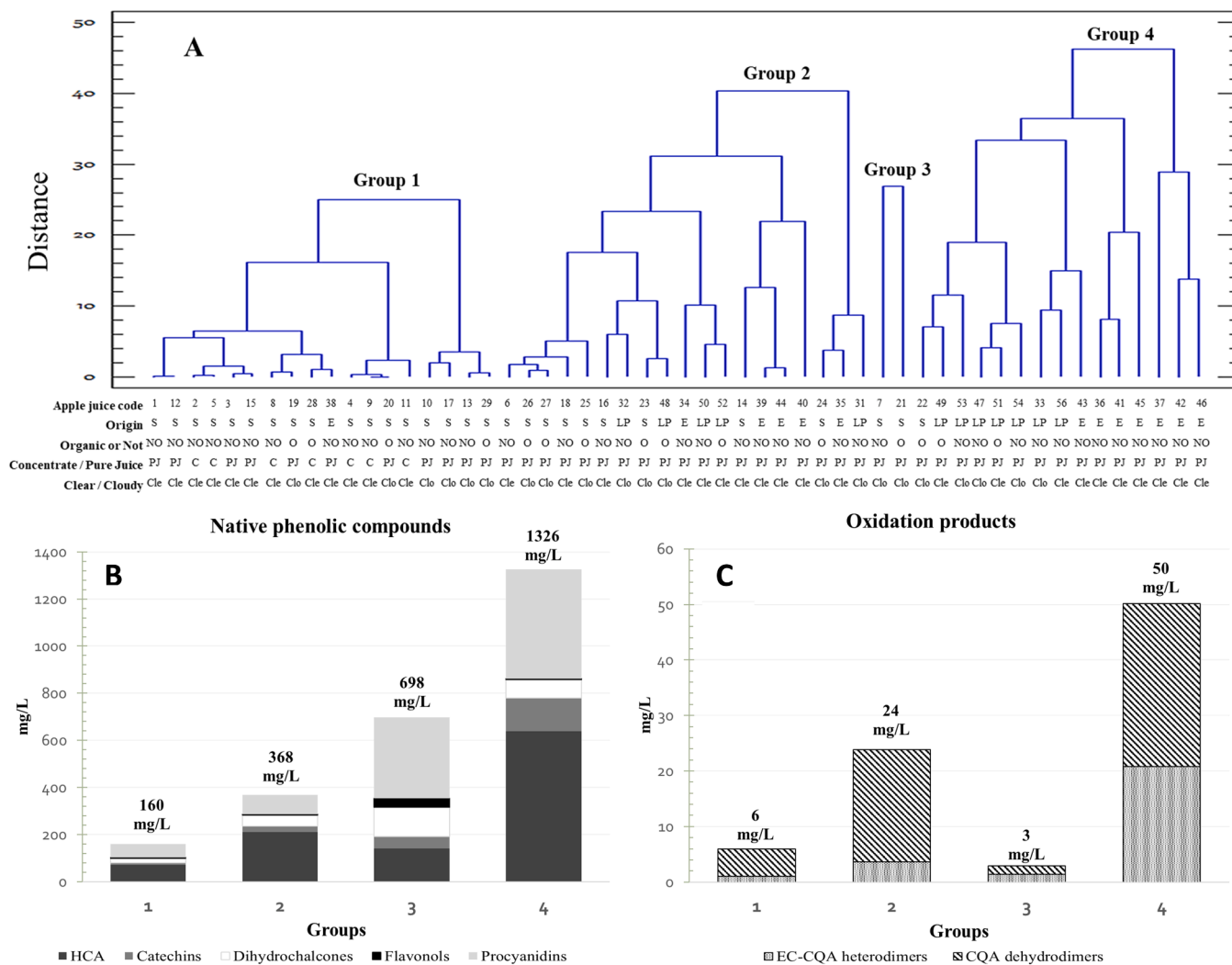


Fig. 4. Hierarchical classification of the samples performed on all phenolic data sets (detailed NPC and OP profiles) according to Euclidian Ward’s method. A: Dendrogram related to some qualitative criteria: Origin S = Supermarket, LP = Local Cider Producer, E = Experimental, Organic = O, Not organic = NO, Concentrate = C, Pure juice = PJ, Clear = Cle, Cloudy = Clo; B: Group distribution of native phenolic compound families: HCA, Catechins, Dihydrochalcones, Flavonols, Procyanidins; C: Group distribution of OP families: CQA dehydrodimers, EC-CQA heterodimers.

apples" from sorting apples when harvested or at the end of their storage period. These producers also buy fruit or apple juice concentrates on the international market. They only use raw materials from category A.

The French apple industry primarily processes local varieties from western regions for cider and juice production. Their juices are usually made from B and sometimes a blend of B and C apple categories.

Local French producers: in western France (Brittany and Normandy), producers process local apple varieties belonging to categories B and C, and their production is mainly sold locally. In other regions, some "dessert apple" producers also process part of their non-marketed fruits (category A) to make juice. Most of this fruit is from sorting after harvest (non-standard fruits) or after storage in a controlled atmosphere (fruits rejected due to their appearance).

Experimental juices: in the present study, experimental juices were non-marketed juices produced with local cider apple varieties using a pilot-scale process (50 kg) under conditions that allowed oxidation, as described in a previous study (Le Deun et al., 2015). They were all clear juices made from local apple varieties (categories B and C common in Brittany and Normandy).

To compare the phenolic profile of the sample set of juices analyzed, a statistical cluster analysis (Euclidian Ward's method) based on the total amount of each NPC family (hydroxycinnamic acid esters, catechins, procyanidins, dihydrochalcones, and flavonols) and each OP family (CQA homodimers and EC-CQA heterodimers) was performed. This method allowed us to classify the complete sample set of juices into four groups (Fig. 4A). This classification with regard to juice origin and apple categories that are likely used to make these juices, is discussed below.

Group 1 (1↔29) encompassed 18 juices containing the lowest concentrations of NPC (160 mg/L on average) and the second lowest concentrations of OP (6 mg/L on average) (Fig. 4 B and C). All these juices were bought in supermarkets, except one, juice 38, an experimental juice made from one particular apple variety (*Guillevic*) producing a sour juice used for making a pale yellow cider produced in a very limited geographic area. Notably, none of the juices purchased directly from cider producers belongs to this group, whereas all juices made from concentrates (seven juices) do. The low amounts of phenolic compounds found in the juices purchased in supermarkets could be explained by the apple varieties used that probably belong to category A, which comprises mainly dessert apples with a low polyphenol content. Moreover, most apple juice concentrates are made primarily from dessert apples. These results are in accordance with previous studies related to apple juices made from concentrates. Indeed, these juices have shown poor nutritional and sensory qualities and also exhibited low phenolic content compared to whole apples or pure apple juices (Adnan et al., 2018; Aguiar et al., 2012; Ashurst, 2009). Nevertheless, we noted that this group also contained a great proportion (close to 61%) of pure juices (processed without any dilution). Regarding oxidation products in juices in this group, CQA dehydromers were predominant, representing 83% of the total OPs.

Group 2 (6↔31) contained 19 juices of diverse origins including 9 juices bought in supermarkets, 5 juices bought directly from local cider producers, and 5 experimental juices. This group presented the second-lowest NPC values (368 mg/L on average) (Fig. 4B) and the second-highest OP values (24 mg/L on average) (Fig. 4C).

Interestingly, the experimental juices in this group were made from local varieties belonging to the "sour" cider apple type classified as category B (data not shown). This class of local apples is well known for producing acidic juice with an intermediate phenolic compound concentration (higher than "dessert apples" but lower than cider varieties classified in the bitter category). This is likely the reason why these experimental juices were found in group 2. Concerning the juices from supermarkets and those from local cider producers in this group, the information available on the bottle (data not shown) informed us that most of them were produced in western France, probably from the same kind of local sour apple varieties as the experimental juices in this group

(category B). Regarding OPs for group 2, CQA dehydromers represented 85% of the total OPs (Fig. 4C).

Group 3 (7 and 21) was the smallest group comprising only two atypical commercial juices. This group had the second-highest NPC concentrations quantified (698 mg/L on average) (Fig. 4B) but had the lowest OP values (2.9 mg/L on average) (Fig. 4C). The juices in this group had a phenolic compound composition quite distinctive from all the other juices. Their hydroxycinnamic acid ester, catechin, and procyanidin concentrations were relatively high but their main atypical characteristic was their high dihydrochalcone (125 mg/L on average) and flavonol content (38 mg/L on average) (Fig. 4B). The high dihydrochalcone and flavonol concentrations and extremely low OP values in these two apple juices might be due to a different processing method including heat treatment of the mash. Such treatment could potentially explain this atypical phenolic compound composition by increasing the diffusion of dihydrochalcones from the seeds and flavonols from the peel, as well as inducing PPO inactivation, thus stopping the enzymatic oxidation mechanism (Spanos et al., 1990).

Group 4 (22 ↔ 46) comprises 15 individuals including seven experimental juices, seven juices purchased from local cider producers, and, surprisingly, one bought in a supermarket (juice 22). However, considering the information on the bottle, it is quite evident that this juice originated from a local cider company. Noticeably, this group presented the highest NPC (1325 mg/L on average) and OP (50 mg/L on average) concentrations (Fig. 4 B and C). This result underlined the distinctive composition of the experimental juices made from French cider apple varieties (category C), most of which are very rich in phenolic compounds compared to dessert apples (category A) (Candra-winata et al., 2013; Jakobek and Barron, 2016; Verdu et al., 2014). Interestingly, average NPC concentrations in these juices were eight times higher compared to the average value in juices from group 1, which were almost all purchased in supermarkets. It must also be underlined that 58% of the juices purchased directly from local cider producers belong to group 4.

Group 4 also encompassed juices containing relatively high OP concentrations (50 mg/L on average) (Fig. 4C). EC-CQA heterodimers in this group accounted for 42% of the total OP quantified, whereas this proportion did not exceed 17% in the other groups (except for group 3). This can be explained by the high levels of flavan-3-ols (including monomers) found in cider apple varieties used to produce these experimental juices. Interestingly, the contribution of these OP (CQA dehydromers and EC-CQA heterodimers) to the antioxidant activity was studied using the DPPH method in a previous study (Wong-Paz et al. 2015). The results showed that EC-CQA heterodimers maintain a higher radical scavenging activity than CQA dehydromers (Wong-Paz et al. 2015).

3.2.3. Discussion regarding other segmentation criteria (Fig. 4A)

Organic/Bio label versus conventional juices.

All the juices were also characterized according to their production label by distinguishing "Bio" (a European Union label similar to organic juices) (15 juices) and conventional (39 juices). Among the "Bio" juices, 11 were purchased from supermarkets and 4 from local cider producers. These juices were distributed as follows: four in group 1, seven in group 2, one in group 3, and three in group 4. Therefore, within the sample set investigated in our study, we consider that the statistical classification did not reveal a clear trend with regard to polyphenol composition and concentration for this criterion.

Pure juices versus juices made from apple juice concentrate.

The juices purchased in the supermarket exhibited a low polyphenol content suggesting they were mainly produced using "dessert apples" (category A) and 24% of those juices were made from concentrate. Notably, all the juices made from concentrate (seven juices) were in group 1, which encompasses juices with a low polyphenol content, and none of them came from local cider producers. This highlights the fact that apple juice concentrates are mainly produced from dessert apples

(Category A) and that the process of juice concentration could also decrease the concentration of phenolic compounds (Dutra et al., 2021; Soural et al., 2022; Suzme et al., 2014).

Clear versus cloudy juices.

In total, 33 clear and 21 cloudy juices were analyzed. All the groups presented both types of juices, but group 3 comprised only two cloudy juices. Group 1 comprised 12 clear and 6 cloudy juices, group 2 included 11 clear and 8 cloudy juices, and group 4 contained 10 clear and 5 cloudy juices.

The effect of the apple varieties on phenolic compound content is at least as important as the technology used (Guyot et al., 2003; Markowski et al., 2015; Verdu et al., 2014). In general, when the same apple variety is used for both cloudy and clear juice production, the lowest amounts of phenolic compounds are found in clear juices compared with cloudy ones (Markowski et al., 2015; Oszmianski et al., 2007). However, given the difference in polyphenol content between category A apples and category B and C apples, apple varieties had a much greater impact on phenolic compound content in juices than the technology used for apple production.

4. Conclusion

In this study, an HPLC-MS method was developed for the simultaneous quantification of two families of OPs, specifically CQA dehydrodimers and EC-CQA heterodimers present in apple juices. This method was successfully validated based on LOD, LOQ, linearity, and precision (%RSD). Using this method, native phenolic compounds (NPCs) and oxidized phenolic compounds (OPs) were successfully quantified in 54 apple juices purchased from supermarkets, local cider producers, or prepared experimentally.

Regarding native phenolic compounds, the composition was in accordance with results previously published, revealing very large concentration discrepancies (Candrawinata et al., 2013; Delage, 1991; Guyot et al., 2003; Kahle et al., 2005; Oszmianski et al., 2007; Verdu et al., 2014). The two families of OPs explored in this work accounted on average for 5.2% of the total phenolic compounds quantified, and in some juices (mostly experimental), they represented up to 14%.

Where the juice was purchased was an objective criterion but was probably not the best way to classify the juice as different kinds of juices could be sold in supermarkets. The best explicative variable should be the class of varieties used to produce the juice. This information was not always available on the bottle and had to be hypothesized from the kind of producer and the region where the apples were processed. Experimental juices for which the apple varieties were known helped to confirm these hypotheses.

The results were consistent with our hypothesis: juices made from “dessert apples” (category A) had a low polyphenol content and were classified in group 1. Those made from cider apple varieties (category C) had a much higher polyphenol content, up to 8-fold higher, than the previous one and were classified in Group 4. Group 2 was the intermediate class between Group 1 and Group 4 and probably corresponded to juices made from sour local apple varieties (category B) often used in western regions of France. Group 3 seemed to be more exotic with only 2 juices based on a very specific composition that might be explained by a specific processing method.

This study expands our knowledge of the composition of NPC and OP in apple juices. However, more information is still needed concerning the qualitative and quantitative composition of other families of OP in apple juices to better understand their contribution to organoleptic and nutritional properties. In addition, the results clearly show the great discrepancies in polyphenol composition observed in this panel of juices available on the French market, either from supermarkets or local cider producers. Particular cases were observed but in general, most of the juices currently available in supermarkets were much less rich in polyphenols than those of local cider producers. The most obvious reason for this is that local cider producers make their apple juice from varieties

that are traditionally much richer in phenolic compounds.

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Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Author statement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jfca.2023.105450](https://doi.org/10.1016/j.jfca.2023.105450).

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