



# Comparative analysis of antioxidant activity and capacity in apple varieties: Insights from stopped flow DPPH<sup>•</sup> kinetics, mass spectrometry and electrochemistry

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## ABSTRACT

The aim of this research was to investigate the antioxidant kinetics of different apple varieties, a red-flesh variety ('R201'), a non-browning ('Majda'), and a 'Golden Delicious'. Kinetic approaches for antioxidant activity provide more detailed information than conventional assays by examining both the quantity and velocity of active molecules in their reaction with radicals. In this study, DPPH<sup>•</sup> stopped flow method was applied to study the antioxidant activity and capacity of the three apples varieties, allowing the determination of the reaction rates. The results show that the antioxidant activity of 'R201' was not significantly different from 'Golden Delicious'. Instead, the activity of the non-browning variety was 20 times higher than the others according to the DPPH<sup>•</sup> kinetic approach, despite having the lowest phenolic content. To further understand their reactivity, the antioxidant molecules were identified using HPLC-HRMS/MS coupled with a coulometric array detector, which validated the DPPH<sup>•</sup> kinetics. This analysis also found differences in the phenolic profile of the three varieties, attributed to the enhanced antioxidant activity of 'Majda' to its high content of ascorbic acid. Overall, the research highlights that the antioxidant behavior of apples primarily depends on the velocity of the antioxidants rather than the amount of phenolic compounds.

## 1. Introduction

Apples (*Malus domestica*) are one of the most widely consumed fruits worldwide and their success relies on their sensory and nutritional properties (Chagné et al., 2019; Verma et al., 2019). Apples are a well-known source of bioactive compounds, such as vitamin C and phenolic compounds, such as hydroxycinnamic acids, flavonols, dihydrochalcones, and flavan-3-ols (Macià et al., 2022; Nkuimi et al., 2020). 'Golden Delicious' is one of the most popular varieties in South Tyrol, and its phenolic profile has been extensively characterized (Jakopic et al., 2012; Mayr, 1995), although the concentration of these compounds differs among varieties, with a different magnitude in wild accession (Vittani et al., 2023; Busatto et al., 2019; Farneti et al., 2015). In recent years, the market has drawn attention to new varieties that are

more interesting for esthetic appearance and nutritional values, i.e., red-fleshed varieties, such as 'R201', or for non-browning properties, i.e., 'Majda' (Ceci et al., 2021; Zhao et al., 2021; Čebulj, 2021). High levels of polyphenols can lead to enzymatic browning through reactions with the polyphenol oxidase enzyme (PPO) (Zhang et al., 2022). The non-browning features of 'Majda' apples are attributed to low chlorogenic acid levels and high amounts of non-phenolic antioxidants, such as ascorbic acid and glutathione (Noctor & Foyer, 1998; Njus et al., 2020). These antioxidants prevent apples from browning by reducing the oxidized forms of polyphenols, i.e., quinones, and by scavenging reactive oxygen species (Čebulj, 2021). While some studies have explored 'Majda's non-browning qualities, limited information is available on its antioxidant activity (Čebulj et al., 2021, 2023).

Many rapid spectrophotometric assays commonly used to study the

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antioxidant activity and capacity of complex matrices often yield non-comprehensive results because of the lack of understanding of the reaction mechanism, matrix, and solvent effects (Antolovich et al., 2002; Arnao, 2000; Apak, 2016). The DPPH<sup>•</sup> assay is one of the most widely used approaches to describe the antioxidant properties of foods. The common protocol is based on the recording of the absorbance of DPPH<sup>•</sup> at 515 nm after 30 min or 1 h reaction. A limitation of this assay is that it does not provide kinetic information about the rate at which different antioxidants produce their antioxidant effect (Angeli et al., 2021). Recently, a DPPH<sup>•</sup> stopped-flow kinetic method was developed, allowing the determination of both the antioxidant activity that indicates the reaction velocity, and the capacity that considers the reaction stoichiometry (Angeli et al., 2023). Therefore, this method can represent a useful tool to describe the antioxidant properties of natural extracts in a more comprehensive way than the classical approach. Furthermore, analytical techniques, such as mass spectrometry and coulometry, can identify redox compounds, helping to better understand the antioxidant mechanism. Specifically, the main advantage of the coulometric array detector (CAD) is its ability to measure the antioxidant capacity exclusively for compounds exhibiting redox behavior. This approach based on chromatography coupled with a triple detector (DAD-HRMS/MS-CAD) elucidates which compounds are responsible for the antioxidant activity revealed by the kinetic-based DPPH<sup>•</sup> method (Ding et al., 2022).

The aim of this research was to assess the antioxidant activity and capacity of a white-flesh apple variety together with a red-flesh and a non-browning flesh type of apple using different analytical approaches, such as DPPH<sup>•</sup> kinetics and HPLC with a triple detector system (DAD-HRMS/MS-CAD). In this way, it is possible to establish whether the antioxidant properties of apples depend only on the quantity of the antioxidants, or also on their velocity to scavenge reactive oxygen species. The faster the antioxidant reaction, the more efficient the scavenging mechanism. Our hypothesis is that a small quantity of high-reactive molecules is more important for antioxidant activity, than high number of slow-reacting antioxidants. Our work could represent a valuable source for food industries that want to select new varieties according not only to the amount of polyphenols, and the reactivity of the antioxidant compounds naturally present in the fruit.

## 2. Materials and methods

### 2.1. Materials

The fruits of 'Golden Delicious' and 'R201' were harvested in the experimental orchard of the Fondazione Edmund Mach, Trento, Italy (46°18'86.7" N, 11°10'63.1 E, altitude 204 m), while the apples of 'Majda' were harvested in Brdo pri Lukovici, Slovenia (46°10'04.8" N, 14°40'55.2" E, altitude 368 m). The trees of each cultivar were grafted onto M9 rootstock, trained with a slender spindle system for canopy management and maintained following the same standard horticultural practice. At the time of the experiment, each tree was in a full bearing stage, and fruit were sampled at the commercial harvest date established according to the Starch Pattern Index (SPI) chosen between 6 and 7 (CTIFL scale). Pulp isolated from 10 homogeneous apples/cultivar in terms of dimension and visual appearance, was collected from the equatorial part of the fruit and immediately frozen, pooled, and homogenized with a blender (IKA, Staufen, Germany) in presence of liquid nitrogen. The samples were then stored at -80 °C for two weeks until further analysis.

All solvents used in this study were of mass spectrometry grade and all the reagents were of analytical grade purity. Acetone, methanol, acetic acid, gallic acid, Folin-Ciocalteu phenol reagent, Trolox, catechin, epicatechin, glutathione, ascorbic and chlorogenic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>), 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH), Na<sub>2</sub>CO<sub>3</sub> were purchased from Sigma Aldrich (St. Louis, MO, USA) at the highest grade possible.

### 2.2. Extraction of phenolic compounds

The extraction procedure was performed following the method of Wolfe et al. (2003) with slight modifications. Briefly, 1 g of fresh sample was weighed and transferred to a 15 mL falcon tube. To dissolve the sample, 10 mL of 80% acetone was added. The mixture was homogenized at 25 °C for 10 min using a digital shaker (VWR, Milan, IT) at a speed of 200 rpm and then centrifuged (10 min, 10,000 rpm, 4 °C, max 24,700×g) using a centrifuge (SL 16R, ThermoFisher Scientific, Waltham, MA, USA). The resulting supernatant was transferred to a 50 mL falcon tube, and the extraction process was repeated. In the second extraction, 5 mL of 80% acetone was added to the residue, followed by a third extraction with 3 mL of 80% acetone. All individual extracts were combined and then evaporated using an evaporation system (MultiVap 8, LabTech, Sorisole (BG), Italy) under nitrogen pressure (10 psi). The evaporated extracts were reconstituted with 5 mL of methanol (100%) and stored at -80 °C for one week until further analysis. The extraction was carried out in triplicate.

### 2.3. Total phenolic content

To measure the total phenolic content (TPC) of apple extracts, the method of Singleton & Rossi, (1965) was used with slight modifications. In short, 130 µL of extract, 130 µL of Folin reagent, and 130 µL of a saturated solution of Na<sub>2</sub>CO<sub>3</sub> were added to 1 mL of distilled water. The absorbance was measured after 2 h of storage/incubation/keeping in the dark using an Infinite M Nano<sup>+</sup> spectrophotometer (Tecan Italia, Milan, IT) at 765 nm. The measurements were performed in triplicate. The results were expressed as µg kg<sup>-1</sup> of gallic acid equivalents (GAE) against a gallic acid calibration curve (R<sup>2</sup> = 0.995).

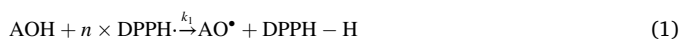
### 2.4. ORAC assay

The ORAC assay was performed according to the work of Prior (2015) with slight modifications. In detail, all samples, including the standard solution of Trolox, were prepared in a phosphate buffer solution (75 mM, pH 7). The apple extracts were standardized for the TPC value at 30 µM GAE to compare their antioxidant activity. The microplate was then prepared by adding 50 µL of standardized extracts (30 µM GAE) or Trolox standard solution (Trolox concentration ranges from 0 to 60 µM) and 50 µL of fluorescein (800 nM) to each well. Finally, 100 µL of freshly prepared AAPH solution (100 mM) was manually added to each well to initiate the reaction. Fluorescence intensity signals were recorded with an Infinite M Nano+ spectrophotometer (Tecan Italia, Milan, IT) at an excitation wavelength of 485 nm and emission wavelength of 520 nm. Measurements were recorded every 30 s until a relative fluorescence intensity of 5% was reached. The antioxidant capacity was calculated by integrating the area under the curve subtracted from the blank. The results were expressed as the micromolar equivalent of Trolox (TE) per g of extract (fresh weight).

### 2.5. DPPH<sup>•</sup> kinetics for antioxidant activity

The method followed the work of Angeli et al. (2023) with slight modifications. The decay of DPPH<sup>•</sup> absorption was measured at 515 nm using a Cary 60 UV-VIS spectrophotometer (Agilent Technology, Santa Clara, CA, USA) equipped with an RX2000 stopped-flow system and an RX pneumatic drive accessory (Applied Photophysics, Leatherhead, UK). A 0.1 mM DPPH<sup>•</sup> solution in methanol had a maximum absorbance of  $1.1 \pm 0.4$  ( $\epsilon$  in methanol =  $11,200 \pm 400 \text{ M}^{-1} \text{ cm}^{-1}$ ). The values for the rate constants ( $k_1$  and  $k_2$ ) and the stoichiometric factor ( $n$ ) were determined following the procedure described by Angeli et al. (2021). To compare the antioxidant activity of complex mixtures, the sample extracts were preliminarily diluted to reach an equivalent concentration of phenolic compounds equal to  $6.0 \times 10^{-5} \text{ M}$  of GAE, as determined by the Folin Ciocalteu assay. This step is crucial to standardize the assay and

compare the radical scavenging activity of the mixture of antioxidants. The two syringes of the stopped-flow system were filled one with the DPPH<sup>•</sup> solution (0.2 mM) and the other with the standardized apple extract ( $6.0 \times 10^{-5}$  M of GAE). The lines were flushed so that the cuvette at the end of the instrument was loaded with the reaction mixture without any bubbles. Just as the RX pneumatic drive accessory was pressed, the spectrophotometer started recording the absorption signals, and the syringes pushed the solutions in the system. The two reagents were stirred immediately before entering the cuvette, providing a delay of 8 ms. The DPPH<sup>•</sup> concentration was deduced from Beer–Lambert's law. The results of the DPPH<sup>•</sup> kinetic method was expressed with two rate constants, respectively, the  $k_1$  of the main reaction described in eq. (1), and the second  $k_2$  for the secondary reactions occurring between the oxidized antioxidants and the free radical DPPH<sup>•</sup>, as reported in eq. (2) and discussed elsewhere (Foti et al., 2004; Angeli et al., 2021). Finally, the stoichiometric factor,  $n$ , indicated the number of radical molecules that were scavenged by one antioxidant molecule.



Where AOH is the antioxidant,  $n$  the stoichiometric factor, AO<sup>•</sup> the oxidized form of the antioxidant, and DPPH-H is the reduced form of DPPH<sup>•</sup>. Because the reaction was carried out in methanol, the mechanism was defined as sequential proton loss electron transfer (SPLET), resulting in very fast reactions (Foti, 2015; Foti et al., 2004). Methanol was chosen because of its suitability for the analysis of food extracts. To calculate the rate constants and stoichiometry of the reactions, the transient decay of DPPH<sup>•</sup> was fitted in a differential evolution program that minimizes the difference between the experimental data and the fitted values using Copasi software version 4.38 (Angeli et al., 2023; Hoops et al., 2006). The measurements were performed in triplicate.

## 2.6. Classical DPPH<sup>•</sup> assay

For the classical DPPH<sup>•</sup> assay, the method of Ding et al. (2022) was used with slight modifications. A 2.5 mM methanolic stock solution of DPPH<sup>•</sup> was prepared and diluted to 200 μM (working solution). The extract (100 μL) was vortexed with the working solution (100 μL) and added to a 96-well microplate. The absorbance was measured after 1 h of storage/incubation in the dark using an Infinite M Nano<sup>+</sup> spectrophotometer (Tecan Italia, Milan, IT) at 515 nm. The analysis was repeated in triplicate, and the results were expressed as % inhibition of DPPH<sup>•</sup>, using eq. (3):

$$\% \text{ Inhibition} = \frac{t_0 - t_{60}}{t_0} \times 100 \quad (3)$$

where  $t_0$  is the absorbance of DPPH<sup>•</sup> and  $t_{60}$  is the absorbance of the sample after 60 min.

## 2.7. LC-DAD-MS-CAD for bioactive compounds identification

The apple extracts were filtered using 0.45 PTFE membrane filters (LLG, GmbH, Meckenheim, Germany), diluted 1:5 in milliQ water + 0.5% acetic acid (Phase A), and analyzed using HPLC coupled to high-resolution mass spectrometry (HRMS). The method of Ding et al. (2022) was used with slight modifications. Briefly, the UHPLC-MS/MS analysis was performed using an UltiMate 3000 UHPLC system (ThermoFisher Scientific, Waltham, MA, USA) coupled with a QExactive Orbitrap (ThermoFisher Scientific, Waltham, MA, USA) mass spectrometer. Separation of the analyte was performed using a Kinetex® 2.6 μm Biphenyl column (100 × 2.1 mm, Phenomenex, Torrance, CA, USA). MilliQ water with 0.5% v/v acetic acid was used as phase A, and methanol with 0.5% v/v acetic acid was used as phase B. The injection

volume was 20 μL. The elution gradient was set as follows: 0–20 min 10% phase B, 20–37 min 36% B, 37–44 min 90% B, 44–52 min 90% B, 52–53 min 10% B, 53–60 min 10% B with a flow rate of 0.3 mL min<sup>-1</sup>. The column was maintained at 30 °C. The operations were controlled using the Chromeleon™ 7 chromatography data system (version 7.2.10). For the full-MS analysis, the ESI source operated in both positive and negative ionization modes, with a capillary voltage of 4 kV, temperature of 320 °C, scan range 75–1000 m/z, resolution of 70,000 and AGC target of 5<sup>5</sup>. In the data-dependent settings the resolution was set at 17,500 and the AGC target at 1<sup>5</sup>, with a NCE of 30. A solution of Trolox 20 μM was used as internal standard to make a semi-quantification of peak areas. The analyses were performed in duplicates. The MS data and results were collected and analyzed using Xcalibur 3.1 and Compound Discoverer 3.1 software (ThermoFisher Scientific, Waltham, MA, USA).

The extracts were further analyzed with an Agilent 1260 Infinity HPLC system equipped with a binary pump and an autosampler with the temperature control system kept constant at 30 °C. Chromatographic analysis of the apple extracts was conducted under the same conditions as described above. The column was coupled with a diode array detector (DAD) and a coulometric array detector (CAD), in series. DAD was used to record the absorption spectra of the eluted molecules from 210 nm to 500 nm (±2 nm). Real time monitoring at 280 and 360 nm was used for screening bioactive compounds eluted from the chromatographic separation. For the coulometric detector, 16 porous graphite electrodes were connected in series to register the signals of the electroactive compounds. The channels were set at increasing potentials from +50 mV to +800 mV (vs. palladium electrode) with increments of 50 mV at a constant temperature of 25 °C. The applied potentials of each CAD channel were determined using a palladium pseudo-reference electrode. The palladium reference electrode had a –360 mV shift difference compared to the Ag/AgCl electrode, which was tested with the reference compound ferrocene methanol (Ding et al., 2022). CAD data were collected using CoulArray® datastation 3.10 software (ESA Bioscience, Inc., Chelmsford, MA, USA) and processed using R software (3.6.2), package MALDIquant (Gibb & Strimmer, 2012). The results of the quantification were expressed according to the Faraday's law (eq. (4)) as mg/g of fresh weight.

$$\frac{Q}{V} = n \times F \times [C]_0 \quad (4)$$

where Q is the total charge in Coulomb, V is the volume injected in the HPLC system,  $n$  is the stoichiometry, which is assumed to be 2.0 for most phenols, and  $[C]_0$  is the concentration expressed in molarity, which was converted in mg/g using the molecular weight of the specific compounds and the dilution factors (Ding et al., 2022).

## 2.8. Statistical analysis

Statistical analyses were performed using the software XLStat 2022 (Addinsoft, 40, rue Damrémont, 75018 Paris), including Principal Component Analysis (PCA) and one-way ANOVA plus Tukey post-hoc test. Results were considered statistically different when  $p < 0.05$ . The Shapiro-Wilk test was applied to check the normality of the data. Throughout this work, the results were expressed as mean and standard deviation. Graphics were drawn by using the software OriginPro 2022 (OriginLab Corporation, Northampton, MA, USA).

## 3. Results and discussion

### 3.1. Antioxidant activity of apple extracts

The antioxidant activity of apple extracts was assessed using the Folin-Ciocalteu method for TPC, the classical DPPH<sup>•</sup> assay, and a kinetic-based DPPH<sup>•</sup> method with a stopped-flow apparatus to determine the reaction rate. The results for the TPC, classical DPPH<sup>•</sup> assay,

and kinetic DPPH• method are presented in Table 1, and the optimal fitting of the experimental results for the DPPH• kinetic method is shown in Fig. 1.

According to the TPC, 'R201' had the highest number of phenols, nearly double that of 'Majda', followed by 'Golden Delicious'. This result was expected because of the presence of anthocyanins that are distinctive of this variety and that are a substrate of the reaction. The results of the classical DPPH• assay and the ORAC assay on the standardized extracts correlated with the TPC. 'R201' had  $2.91 \pm 0.25$  mg/g, 'Golden Delicious'  $1.82 \pm 0.14$  mg/g and 'Majda'  $1.53 \pm 0.12$  mg of TEAC/g of fresh weight. These results are in accordance with previous reports (Henríquez et al., 2010). Concerning the ORAC assay, 'R201' had  $5.0 \pm 0.19$  mg TE/g, 'Golden' had  $4.2 \pm 0.24$ , while 'Majda' had  $2.0 \pm 0.15$  mg TE/g of fresh weight. These results for ORAC are in agreement with those reported by Obara, Masumoto, Ono, Ozaki, and Shoji (2016). However, considering the values of % inhibition for the DPPH• assay on the standardized extracts, before accounting for the dilution factor, the classical DPPH• assay could not detect any significant differences among the samples ( $p > 0.05$ ), as reported in Table 1. This confirmed the limited ability of such an assay to discriminate samples based on their reactivity. Standardization of the TPC of the extracts is important to allow comparison of antioxidant activity in the kinetic method.

The kinetic-based DPPH• method showed significantly higher  $k$  values for 'Majda' than the other two varieties. Higher  $k$  values are related to a faster drop in the DPPH• concentration and therefore higher antioxidant activity of the extract. 'Majda' reported higher scavenging activity toward radicals, even though its TPC was significantly lower than the other two varieties. Accordingly, the  $k_1$  value for 'Majda' was 24 times higher than that of the other two varieties, while no significant differences ( $p < 0.05$ ) were detected between 'R201' and 'Golden Delicious'. This result highlighted that the anti-browning effect typical of 'Majda' sample might be related to the presence of very reactive antioxidants rather than a high amount of phenolic compounds. In addition, this effect can be related to the low amount of polyphenols that are a substrate of the PPO enzyme (Cebulj et al., 2021). Nevertheless, the antioxidant capacity, underlined by the stoichiometric value ( $n$ ), was higher in 'Golden Delicious', followed by 'R201' and 'Majda'. Even if similar results were previously observed in a comparison between 'Majda' and 'Golden Delicious', these types of results are new and allow a better discrimination of complex foods based on their overall reactivity (Cebulj et al., 2023).

### 3.2. Characterization of apple extracts by HPLC-HRMS/MS and quantification of the main antioxidant compounds

To further understand the contradictory results obtained from the antioxidant assays of the three apple cultivars, a characterization of the potential compounds responsible for the antioxidant activity was performed using HPLC-HRMS/MS. A total of 44 compounds were detected and putatively identified in the apple extracts. The proposed

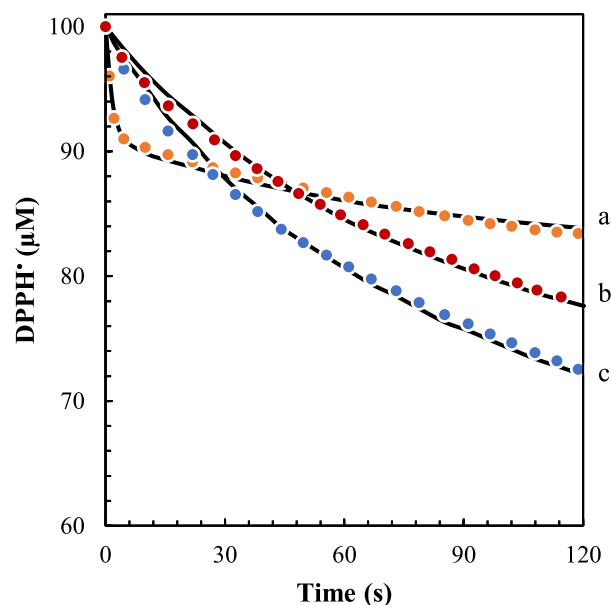
**Table 1**

Results of the total phenolic content (TPC) content, classical DPPH• assay and kinetic stopped-flow DPPH• method on the apple varieties.

Sample	TPC $\mu\text{g g}^{-1}$ GAE	Classical DPPH•		Kinetic DPPH•		
		% inhibition <sup>b</sup>	mg of TEAC/g	$k_1$ ( $\text{M}^{-1}$ $\text{s}^{-1}$ )	$k_2$ ( $\text{M}^{-1}$ $\text{s}^{-1}$ )	$n$
Majda	$404 \pm 9^{\text{a}}$	$63 \pm 8^{\text{a}}$	$1.53 \pm 0.12^{\text{a}}$	$5540 \pm 390^{\text{a}}$	$150 \pm 5^{\text{a}}$	$0.3^{\text{a}}$
R201	$811 \pm 69^{\text{b}}$	$63 \pm 5^{\text{a}}$	$2.91 \pm 0.25^{\text{b}}$	$215 \pm 9^{\text{b}}$	$51.3 \pm 2^{\text{b}}$	$0.6^{\text{b}}$
Golden D.	$502 \pm 59^{\text{c}}$	$63 \pm 3^{\text{a}}$	$1.82 \pm 0.14^{\text{c}}$	$231 \pm 9^{\text{b}}$	$54.7 \pm 2^{\text{b}}$	$0.8^{\text{c}}$

<sup>a</sup> Letters indicate the significant differences ( $p$ . value  $< 0.05$ ) according to ANOVA plus Tukey post-hoc test.

<sup>b</sup> % of inhibition standardized by the measured TPC.



**Fig. 1.** Resulting curves for the kinetic DPPH• assay on the three apple extracts. Experimental (bullet points) and fitting curves (line) of  $30 \mu\text{M}$  of GAE 'Majda' (a), 'R201' (b), and 'Golden Delicious' (c) against  $100 \mu\text{M}$  of DPPH• methanolic solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compounds, their formulas, experimental vs. theoretical  $m/z$ , retention times, and product ions are reported in Table S1. The putative identification was confirmed by comparing the product ions and fragmentation patterns with the available standards, and those found in the literature and online databases. Principal component analysis (PCA) was performed to observe the differences in the chemical composition of the samples. The resulting biplot of the PCA is shown in Fig. 2. F1 explains 47.97% of the total variance, whereas F2 explains 29.23%. The samples appeared separated and clustered according to the variety. In particular, 'R201' (R) was separated from 'Golden Delicious' and 'Majda' by both F1 and F2, because of the presence of anthocyanins and phloretin-2-xyloglucoside. 'Majda' (M) had a higher amount of ascorbic acid, glutathione, and several amino acids, such as methionine, tyrosine, and glutamine. The results were consistent with those of previous studies by Cebulj et al. (2021) and Persic et al. (2017). 'Golden Delicious' had a higher concentration of rutin, catechin and some amino acids.

Although phenolic profiling is crucial for understanding the composition of each variety, predicting which compounds play a role in the antioxidant mechanism remains challenging. Therefore, the samples were analyzed using a coulometric array detector (CAD), which selectively detected antioxidants with the highest electron transfer capacity. The cumulative sum chromatograms of the extracts of the three apple cultivars are shown in Fig. 3. The plot shows, for each cultivar, the sum of the sixteen current signals ( $\mu\text{A}$ ). The CAD signals for each variety are shown in Fig. S1-3. The redox profile of 'Majda', 'R201' and 'Golden Delicious' extracts reported eight main redox peaks, appearing in superimposing retention times. This supported the fact that, regardless of the cultivar, the apple extracts had a similar qualitative composition of antioxidants.

The chemical species responsible for this composition were next identified by HRMS. The fragmentation spectra corresponding to such eight redox peaks are shown in Fig. 3.

As illustrated in Fig. 3, the three varieties had the same composition in terms of the number of peaks, except for peak "c", that was not reported in 'R201', but their intensities were very different. The main



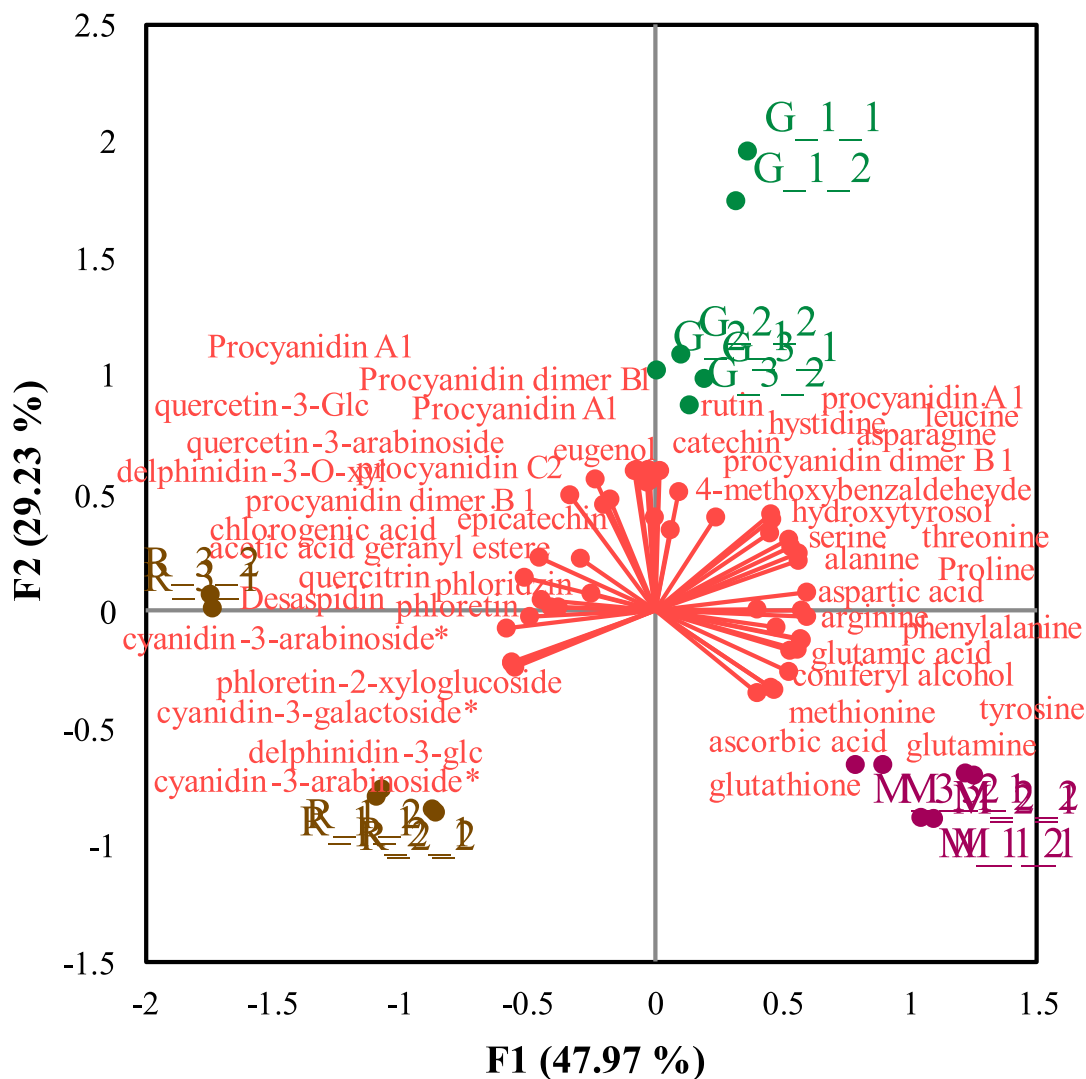
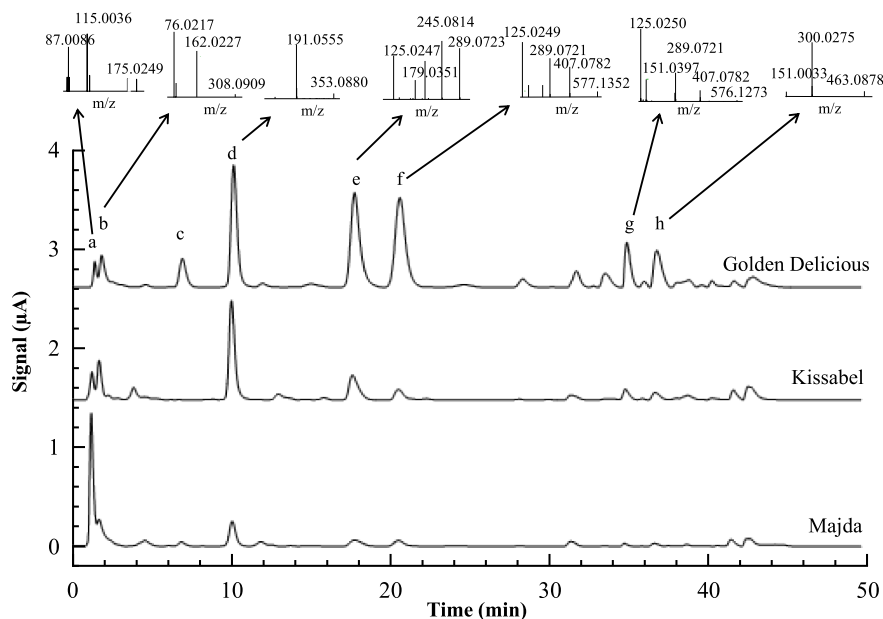


Fig. 2. PCA of the distribution of the detected compounds in samples of 'Majda' (M), 'R201' (R), and 'Golden Delicious' (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compounds responsible for the antioxidant activity of the extracts were identified as follows: (a) ascorbic acid ( $[M-H]^-$  of 175.0249 m/z), (b) glutathione ( $[M+H]^+$  of 308.0909 m/z), (d) chlorogenic acid ( $[M-H]^-$  of 353.0880 m/z), and (e) epicatechin ( $[M-H]^-$  of 289.0716 m/z). Identification was confirmed by comparing the retention time and fragmentation of individual standards. Letters "c", "f", and "g" were putatively assigned to procyanidin B and A isomers, respectively, on the basis of their fragmentation patterns from MS/MS data, as supported by previous studies (Kiselova-Kaneva et al., 2022) and PubChem database. Finally, peak (h) was assigned to quercetin-3-glucoside ( $[M-H]^-$  at 463.0878 m/z). The base peak at 300.0273 m/z and the fragment at 151.0033 m/z were confirmed with the fragmentation pattern of the aglycone analytical standard, the Foodb database, and the Mass Bank of North America. Detailed information about the main identified antioxidant compounds, including MS data reported in Table 2.

Although the three apple cultivars showed similar composition of natural antioxidants, quantitative analysis revealed large differences in their content. Based on the sum of the signals 'Golden Delicious' provided the highest total charge ( $140 \pm 6 \mu\text{C}$ ), followed by 'R201' ( $56.3 \pm 2 \mu\text{C}$ ) and 'Majda' ( $42.5 \pm 2 \mu\text{C}$ ). A possible reason for the high total

phenol content of 'R201' was its high level of anthocyanins (Ceci et al., 2021). Nevertheless, despite the high content of total phenols, 'R201' showed a lower total charge than 'Golden Delicious', indicating a lower antioxidant capacity. Interestingly, 'Majda' samples showed the lowest total phenol content and fewer antioxidant compounds in the HPLC-CAD chromatogram. 'Golden Delicious' samples had higher amounts of all the antioxidants found, except for ascorbic acid and glutathione, which were significantly higher in 'Majda', as reported in Table 2. In detail, the concentration of ascorbic acid was almost 5 times higher than that of the other varieties, as previously reported (Cebulj et al., 2021). This result was consistent with the result of the DPPH $^{\bullet}$  kinetic method, which reported higher antioxidant capacity for 'Golden Delicious'. The antioxidant profile of 'R201' was similar to that of Golden Delicious, although no redox peaks for anthocyanins were found in the CAD analysis. This result was consistent with the non-reactivity of anthocyanins found by Della Vedova et al. (2022) and our kinetic results. Moreover, it explained the similarity in the antioxidant activity between 'R201' and 'Golden Delicious' found with the DPPH $^{\bullet}$  kinetics and further justified the higher capacity of 'Golden Delicious'. The content of chlorogenic acid, which together with epicatechin is the main substrate of the PPO enzyme, was



**Fig. 3.** CAD cumulative sum chromatograms (A) and peak assignment of ‘Golden Delicious’, ‘R201’, and ‘Majda’ with corresponding fragmentation spectra. Where a = ascorbic acid; b = glutathione; c = procyanidin dimer B\*; d = chlorogenic acid; e = epicatechin; f = procyanidin dimer B\*; g = procyanidin A\*; h = quercetin-3-glucoside.

**Table 2**

RT, charge exchanged ( $\mu\text{C}$ ) and concentration for each of the compounds proposed in the three apple varieties. In some cases, the quantification was not possible (n.q.) and in one case the peak was not detected (n.d.).

Peak	RT minute	ESI	Exp. m/z	Theor. m/z	Error ppm	Fragments	Formula	Putative identification	Majda		Golden		R201		$E_{1/2}$ mV
									C	g/g	C	g/g	C	g/g	
A	1.4	-	175.0248	175.0246	1.143	115.0036, 87.0087, 71.0138	$\text{C}_6\text{H}_8\text{O}_6$	ascorbic acid <sup>a</sup>	21.8 $\pm 0.9$	49.8 $\pm 2.2$	4.42 $\pm 0.2$	10.1 $\pm 0.3$	5.6 $\pm 0.2$	12.7 $\pm 0.5$	+100
B	1.7	+	308.0911	308.0909	0.649	179.0486, 162.022, 130.0501, 84.0444, 76.0216	$\text{C}_{10}\text{H}_{17}\text{N}_3\text{O}_6\text{S}$	glutathione <sup>a</sup>	9 $\pm$ 0.4	35.8 $\pm 1.6$	6.82 $\pm 0.3$	27.2 $\pm 1.2$	7.7 $\pm 0.3$	30.6 $\pm 1.3$	+750
C	6.9	-	577.1352	577.1342	1.646	273.0764, 125.0241	$\text{C}_{30}\text{H}_{26}\text{O}_{12}$	procyanidin B isomer	n.q.	n.q.	8.18 $\pm 0.3$	61.3 $\pm 2.7$	n.d.	n.d.	+450
D	10	-	353.0878	353.0877	-0.283	191.0562	$\text{C}_{16}\text{H}_{18}\text{O}_9$	chlorogenic acid <sup>a</sup>	7.03 $\pm 0.3$	32.3 $\pm 1.45$	32.5 $\pm 1.4$	149 $\pm 6.7$	25.4 $\pm 1.1$	117 $\pm 5.1$	+200
E	17.6	-	289.0717	289.0716	-0.346	179.0315, 139.0395, 121.0290, 70.4601	$\text{C}_{15}\text{H}_{14}\text{O}_6$	epicatechin <sup>a</sup>	2.5 $\pm 0.1$	9.38 $\pm 0.4$	32.1 $\pm 1.3$	121 $\pm 5.4$	9.21 $\pm 0.4$	34.6 $\pm 1.5$	+500
F	20.4	-	577.1352	577.1342	1.733	273.0764, 125.0241	$\text{C}_{30}\text{H}_{26}\text{O}_{12}$	procyanidin B isomer	2.19 $\pm 0.1$	16.4 $\pm 0.7$	32 $\pm$ 1.3	240 $\pm 11$	3.35 $\pm 0.1$	25.1 $\pm 1.1$	+450
G	34.9	-	576.1273	576.1264	1.562	289.0712, 151.0401, 125.0243	$\text{C}_{30}\text{H}_{24}\text{O}_{12}$	procyanidin A isomer	n.q.	n.q.	11 $\pm$ 0.5	82.3 $\pm 3.7$	2.57 $\pm 0.1$	19.2 $\pm 0.8$	+450
H	36.9	-	433.0776	463.0878	0.462	300.0275 151.0033	$\text{C}_{20}\text{H}_{18}\text{O}_{11}$	quercetin-3- glucoside	n.q.	n.q.	13.1 $\pm 0.4$	78.4 $\pm 3.5$	2.44 $\pm 0.1$	14.6 $\pm 0.6$	+450

<sup>a</sup> Confirmed with analytical standard injection.

almost 5 times higher in ‘Golden Delicious’ and ‘R201’ than in Majda. Our quantification was in accordance with other results reported in the literature. In detail, [Butkeviciute et al. \(2022\)](#) reported a content in chlorogenic acid that ranged from  $109.4 \pm 10.4 \mu\text{g/g}$  to  $780.4 \pm 31.7 \mu\text{g/g}$ , while a study by [Bars-Cortina et al. \(2016\)](#) showed that chlorogenic acid in red-fleshed and white-fleshed apple samples ranged from  $52.3 \mu\text{g/g}$  to  $306.0 \mu\text{g/g}$ . ‘Golden Delicious’ had a content in epicatechin that was 12 times higher than ‘Majda’, and three times higher than ‘R201’. Our results are confirmed by data reported by [Butkeviciute et al. \(2022\)](#). These values reinforce the hypothesis that the non-browning

properties of ‘Majda’ are related to a high concentration of ascorbic acid combined with a low amount of polyphenols, which are substrates of the PPO enzyme ([Cebulj et al., 2023](#)).

### 3.3. Reactivity of single antioxidants using hydrodynamic voltammograms

One of the most distinctive advantages of HPLC coupled with a coulometric array detector is the possibility of plotting the hydrodynamic voltammogram (HDV) of each eluted compound. HDV is a

powerful tool that provides information on the half-wave potential ( $E_{1/2}$ ) of the studied compounds. This is the applied potential corresponding to 50% of the total signal obtained for electrochemical oxidation. This means that the lower the  $E_{1/2}$  value, the easier it is for that compound to donate electrons, and, thus, the higher its antioxidant potential. Thus, based on the  $E_{1/2}$  values, it was possible to screen the most important antioxidants among the compounds eluted by HPLC. Fig. 4-A shows the HDV plots of the antioxidants, whose identification was confirmed by matching the mass fragmentation with that of the corresponding individual standards.

The antioxidant with the lowest  $E_{1/2}$  (thus, the highest antioxidant potential) was ascorbic acid (+100 mV vs Ag/AgCl electrode), followed by chlorogenic acid (+200 mV), procyanidins and quercetin-3-glucoside (+450 mV), epicatechin (+500 mV), and glutathione (+750 mV). The high amount of vitamin C in 'Majda', which is one of the most powerful antioxidants and the one with the lowest half-wave potential, might compensate for the lower amount in other phenolic compounds. Indeed, the high amount of ascorbic acid quantified with CAD correlated with the high  $k_1$  of 'Majda' in the DPPH<sup>•</sup> kinetic approach. This was also confirmed by the rate constants of the single standard compounds of ascorbic acid, chlorogenic acid, epicatechin and glutathione, as shown in Fig. 4-B. The reaction between 10  $\mu\text{M}$  of vitamin C and 100  $\mu\text{M}$  of DPPH<sup>•</sup> led to a  $k_1$  of  $21,100 \pm 540 \text{ M}^{-1}\text{s}^{-1}$ , that was significantly faster ( $p < 0.05$ ) than the reaction of chlorogenic acid ( $k_1 = 819 \pm 9 \text{ M}^{-1}\text{s}^{-1}$ ), and epicatechin ( $k_1 = 778 \pm 43 \text{ M}^{-1}\text{s}^{-1}$ ) at the same conditions correlating with the HDV. The slow kinetics of glutathione correlated with the high value of  $E_{1/2}$  because the reaction between 200  $\mu\text{M}$  of glutathione and 100  $\mu\text{M}$  of DPPH<sup>•</sup> led to a  $k_1$  of  $67.3 \pm 2.7 \text{ M}^{-1}\text{s}^{-1}$ .

Accordingly, it is very likely that the non-browning activity was due to the high level of ascorbic acid (Moon et al., 2020; L. Zhou et al., 2020). Moreover, the lower levels of chlorogenic acid and epicatechin could also play an important role in the non-browning mechanism, because they are among the most preferred substrates by polyphenol oxidase (Weurman & Swain, 1953; X. Zhou et al., 2021). These results confirmed the hypothesis that the antioxidant mechanism was related to a small quantity of reactive molecules, i.e., ascorbic acid, rather than a high amount of phenolic compounds.

Such findings suggested that a combined approach including kinetic-based methods, electrochemistry, and mass spectrometry allowed a

comprehensive study of the antioxidant properties of natural extracts. DPPH<sup>•</sup> kinetic method represents a valuable tool for predicting the antioxidant activity in apple extracts. Indeed, the DPPH<sup>•</sup> kinetic approach valorized the high antioxidant activity of the non-browning variety, which was validated using the coularray detector and mass spectrometry. The higher antioxidant activity of the non-browning variety was explained by the presence of fast antioxidants, such as ascorbic acid rather than the total amount of phenolic compounds. Ascorbic acid showed superior antioxidant activity than chlorogenic, epicatechin, or glutathione, highlighting the importance of having fast antioxidants in an extract rather than many slow-reacting antioxidants.

Overall, this research significantly advances our understanding of the antioxidant properties of natural extracts, based on a case study of three apple varieties. Our study revealed that not only high levels of bioactive compounds are crucial for enhancing the functionality of this fruit, but also high concentrations of potent antioxidants that rapidly react with reactive oxygen species. Hence, understanding the reactivity of compounds in terms of reaction velocity contributes to a more precise characterization of different apple varieties based on their antioxidant properties. This knowledge will contribute to the advancement of the selection and breeding of apple varieties with enhanced antioxidant activity and potential health benefits.

#### 4. Conclusion

In this study, the antioxidant properties of three apple varieties were successfully described using a kinetic-based approach supported by electrochemistry and mass spectrometry techniques. The findings demonstrated that relying on a single method was insufficient for comprehensive assessment of the antioxidant properties of apple extracts. By integrating kinetic analyses with high-resolution mass spectrometry and coulometry, we gained valuable insights into the composition and reactivity of apples. Furthermore, our investigation highlighted the superior antioxidant activity of certain compounds, such as ascorbic acid, compared with those of chlorogenic acid, epicatechin, or glutathione. We demonstrated that the DPPH<sup>•</sup> kinetic method offers advantages over the classical assay in assessing both the antioxidant activity and capacity of apple extracts. These insights contribute to the understanding of the complex nature of antioxidant systems and pave

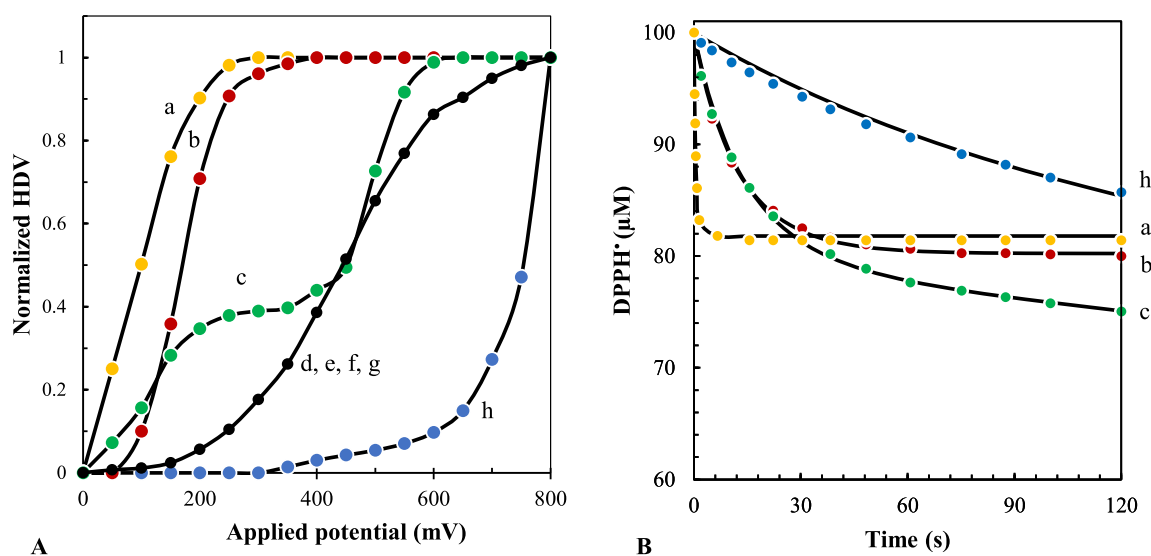


Fig. 4. A: Normalized HDV representative of ascorbic acid (a), chlorogenic acid (b), epicatechin (c), procyanidin B isomers, procyanidin A, quercetin-3-glucoside (d, e, f, g), and glutathione (h). B: Experimental and fitted curves of 200  $\mu\text{M}$  of glutathione (h), 10  $\mu\text{M}$  of ascorbic acid (a), chlorogenic acid (b), and epicatechin (c) against 100  $\mu\text{M}$  of DPPH<sup>•</sup> methanolic solution.

the way for further advancements in antioxidant research, such as interactions among compounds, and in the selection of new apple varieties with enhanced nutritional, functional, and esthetic properties.

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## CRedit authorship contribution statement

**Lucrezia Angeli:** Writing – original draft, Investigation, Data curation, Conceptualization. **Francesca Populin:** Resources, Project administration, Conceptualization. **Ksenia Morozova:** Writing – review & editing, Supervision, Conceptualization. **Yubin Ding:** Methodology, Investigation. **Umme Asma:** Methodology, Data curation. **Sara Bolchini:** Software, Data curation. **Anka Cebulj:** Resources. **Nicola Busatto:** Resources, Conceptualization. **Fabrizio Costa:** Resources, Project administration, Conceptualization. **Giovanna Ferrentino:** Writing – review & editing, Supervision. **Matteo Scampicchio:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

## 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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## Appendix A. Supplementary data

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