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Research article

Unlocking the potential of biostimulants in sustainable agriculture: Effect of wood distillate on the nutritional profiling of apples

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ABSTRACT

In this work, we report the investigation of the effect of exposure of apple trees to the bioeffector wood distillate (WD), a plant biostimulant used for improving the nutritional profiling of crop plants. We measured the effect by evaluating the biochemical and nutritional profile of both pulps and skin of fruits. WD (0.2 %, v/v) was applied once a week by foliar application, from May 2023 until September 2023. The results indicate that the WD-treated apples have a significant increase in several analyzed parameters (*i.e.*, phenols, flavonoids, tannins, total antioxidant power, sugars, pectin, free amino acids, and mineral element content), especially in the pulp. These data were also confirmed by NMR and LC-ESI-MS techniques. This study pointed out that WD could be a handy tool for the cultivation of fruit trees.

1. Introduction

Organic farming (OF) is an agricultural method regulated for the first time in Europe in the early 1990s. OF is employed to limit the harmful consequences caused by the usage of synthetic chemicals, on which conventional agriculture was, and is still based (European Commission, 1997). To date, the efficacy of sustainable food systems and as a profitable and environmentally friendly method is still a matter of debate [1,2]. Indeed, the main criticism associated with OF is that it provides lower yields than traditional farming. The meta-analysis conducted by Knapp and van der Heijden [3] revealed that OF results in significantly lower temporal stability per unit of yield (approximately 15 %) than conventional agriculture. Therefore, it is imperative to find natural solutions to close this gap. The use of natural substances in farming procedures is becoming increasingly popular, aiming at reducing both the gap in crop yields and to obtain plants and fruits endowed with a higher nutraceutical potential [4]. Among the various strategies being proposed for boosting crop growing, the most promising is certainly the use of biostimulants [5]. A biostimulant is defined as *'any substances or materials, except for nutrients and pesticides, which, when applied to plants, seeds or growing media in specific formulations, can modify physiological*

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processes in plants to provide potential benefits to growth, development or stress response' [6].

Biostimulants could help to improve the resilience and sustainability of agriculture, offering an alternative to chemical fertilizers which are becoming increasingly unpopular among customers. Among the marketed biostimulants, one of the most interesting and promising is undoubtedly wood distillate (WD), also known as pyroligneous acid [7,8]. WD is obtained by steam countercurrent, using only the water contained in the wood sap, extracted at different temperature gradients, leaving the reactor at a maximum of 75 °C [9]. WD is further passed through a filter to remove any residue and is left to decant for at least three months to obtain an amber-coloured fluid [9]. This product is highly dependent upon the operational parameters employed during the process and on the used plant species. This process yields WD, activated carbon, and gases. Compared to petrochemical methods, the production of WD through biomass pyrolysis has a lower environmental impact, with reduced global warming potential, energy demand, acidification, antimony depletion, and ozone depletion [10]. The refinement process of WD involves various methods, including ultra-low freezing and thawing, charcoal coupled with activated carbon adsorption, and atmospheric and vacuum distillation. These refined WDs have effectively preserved fruits like strawberries and cherry tomatoes by reducing weight loss, decay incidence, and loss of vitamin C and soluble solids [11]. The pyrolysis temperature significantly affects the yield and properties of the products. For instance, increasing the temperature decreases the yield of charcoal but increases its specific surface area and carbon content [12]. In this scenario, the WDs showed strong antimicrobial activity, particularly against fungi, due to their high content of ketones [12]. Additionally, the heating value of the produced gases increases with higher pyrolysis temperatures, making the process economically and environmentally beneficial for producing biochar, WD, and gases [11,13].

From a technological standpoint, agriculture represents the primary application of WD where, depending on the dose, it may serve as a biostimulant [14] and as an herbicide [15,16].

The chemical composition of WD is highly complex, encompassing >300 molecules, such as phenols, flavonoids, tannins, alcohols, and esters [17]. The biostimulant action of WD is due to this mix of molecules that stimulate plant growth and quality. Numerous studies showed that WD has positive effects on a wide variety of crop species [14,18,19].

In addition to stimulating action on crops, WD was also found to be non-toxic on off-target organisms that are affected during the product's mode of application (*i.e.*, ferti-irrigation and foliar spray applications), thus also proving to be a safe product [20]. WD also joined the list of products useable in OF in Italy (Italian ministerial decree, 2018).

Apples (*Malus domestica (Suckow)* Borkh.) are the third most-produced fruit in the world and Italy is the world's sixth largest producer with 2.15 Mt in 2022 (FAOSTAT, 2023). This food is consumed in the human diet in a broad range of types (*i.e.*, fresh, dried, juice, and jam). From a nutritional perspective, apples are a low-fat and low-protein food (ca. 1 %), while sugars account for ca. 13 % of the apple, where fructose represents approximately for 8 % (U.S. Department of Agriculture Food Data Central, 2023). Micronutrients (*i.e.*, potassium, phosphorus, calcium, and magnesium), and antioxidant compounds (*i.e.*, vitamins, polyphenols, flavonoids, and tannins, U.S. Department of Agriculture Food Data Central, 2023) are also present. Another distinctive feature of apples is the presence of pectin, a gelling agent taken from pomace, which is mainly used by the food sector (*i.e.* preparation of jams and marmalades) [21].

Apple characteristics vary from cultivar to cultivar. In Italy, there are numerous cultivars, with the regions of South Tyrol, Trentino, and Piedmont among the most important cultivation areas for high-quality apples in Europe [22]. Among the most marketed and highest quality apples there is the "*Red Apple of Cuneo*" which is characterized by four different cultivars (*i.e.*, Red Delicious, Gala, Fuji, and Braeburn) from the Cuneo province (Piedmont, Italy), and the product is registered as Protected Geographical Indication (P.G.I.) as an Italian excellence since March 2013 (European Commission, 2013).

An increasing amount of evidence highlights the use of organic fertilizers to produce apples with significantly higher sugar content and, in general, higher nutritional quality [23].

Of note, OF can promote human and environmental health thanks to healthy foods. OF peculiarities can impact fruit quality and can be shifted to other agricultural contexts. However, OF has some implications that should be taken into consideration, such as heavy metal and microbial contamination. Despite some issues, there is a proof that merging of quality, yield, and sustainability in agriculture is feasible [24].

Ensuring high fruit quality is crucial for both nutrition and human health, and it requires immediate attention in today's agricultural practices. Although OF typically yields less than conventional methods, it can produce higher quality fruit for certain crops. This is largely due to the absence of chemical fertilizers and pesticides, improved pollination, and fewer protection treatments, all of which can enhance the production of antioxidant compounds. While OF does not always result in healthier food compared to conventional methods, valuable insights from organic practices can be applied to develop new sustainable farming models. Leveraging natural resources and effectively transferring knowledge will be key to improve the quality of fruits in future agricultural systems.

For apples, organic/inorganic fertilizer combined with a water-saving system was identified as a sustainable approach to increase apple productivity and soil fertility in rain-irrigated hill orchards [25]. In some cases, pesticides are considered a source of toxicity in the conventional apple production model, underlining the relevance of biological pest control. Although OF favours the use of some authorized pesticides, these did not have a strong impact.

do Amarante *et al.* [26] found that apples from OF, had a more yellowish background skin colour, higher levels of blush on the skin and soluble solids content, greater density, firmer flesh, and more severe russeting compared to apples from conventional orchards. These organic apples also exhibited lower titratable acidity in the 'Royal Gala' variety, as well as a higher incidence of moldy core and a lower incidence of watercore in the 'Fuji' variety, compared to those from conventional orchards. A non-trained sensory panel did not detect any significant differences in taste, flavor, or texture attributes between apples from the two production systems for both cultivars [27].

Considering also that organic apple production systems can reduce non-renewable energy intake and safeguarding the environment, we investigated the potential role of WD in this field. To the best of our knowledge, this is the first work providing new insights

on the effects of foliar application of WD on the biochemical and nutritional properties of apple fruits. By investigating these effects, this research contributes valuable insights into enhancing the sustainability and productivity of organic apple production systems.

2. Materials and methods

2.1. Site Description and experimental design

The experiment was conducted in 2023 in an apple orchard located in the municipality of Lagnasco, Cuneo (44,635360 N, 7,574987 E) in Piedmont, Italy. The orchard was located at a private company characterized by 12-year-old "*cv.* Red Apple of Cuneo, *var.* Red Delicious" apple trees. The size of the area used for the experiment was 4.2 ha, with a planting pattern of 4.3 × 1.2 m. The experiment consisted of two treatments: the first, the controls (named "C"), in trees grown exclusively with water; the second, the treated (named "WD"), in trees grown with foliar applications of 0.2 % WD (BioDea®, the chemical profile is reported by Celletti et al. [28]). For each of the two treatments, a total of six rows of apple trees were taken into account, for a total of approximately 2000 apple trees. To avoid cross-contamination the treatments of a part of the field were not taken into account, for a distance of approximately 60 m (Fig. 1).

Wood distillate treatments were carried out once per week, starting on May 20, 2023 until September 20, 2023, the date of apple harvest. Once the apples (approx. 200 apples x treatment) had been randomly picked from the tree divided into subsamples (approx. 40 apples x treatment), washed with deionized water to remove any external contamination, and finally were transported to the laboratory for the analysis (Fig. 2).

2.2. Phenols, flavonoids, tannins, and total antioxidant power content

The content of total phenols (TPC), total flavonoids content (TFC), and condensed tannins was determined in the extracts of apple pulp and skin, previously dried through a food drier, according to Wakeel et al. [29] with some modifications. Approximately 1 g of dry material was soaked in 10 mL of 80 % methanol (*v/v*) for extraction. After 30 min of orbital shaking (ASAL VDRL mod. 711, Cernusco s/N, Milan, Italy), the samples were incubated at 4 °C in the dark. The samples were filtered on a filter paper (Whatman filter paper no. 1) after 48 h of incubation, and the filtrates were utilized for TPC, TFC, and condensed tannin assays.

Determination of TPC was performed using the method reported in Al-Duais et al. [30] An amount of the filtrates (0.125 mL) was added to 2 mL of deionized H_2O, followed by the addition of 0.125 mL of the Folin-Ciocalteu's reagent. After 3 min in the dark, 1.250 mL of 7 % (w/v) Na_2CO_3 and 1 mL of deionized H_2O were added and shaken vigorously followed by 90 min incubation in the dark. Finally, the absorbance of the solutions was read at 760 nm with an Agilent UV–Vis 8453 spectrophotometer (Santa Clara, CA, USA). Quantification was performed using a calibration curve (5–300 μg mL^{-1}) using gallic acid as a standard (98 %, Thermo Fisher Scientific Inc., Rodano, Milano, Italy).

Determination of TFC was performed using a reported method [31]. An amount of the filtrates (0.250 mL) was added to 0.075 mL of 5 % (w/v) $NaNO_2$ and 5 min later with 0.075 mL of 10 % (w/v) $AlCl_3$. After shaking the samples and 5 min of incubation in the dark, 0.5 mL of 1 M NaOH solution were added. The samples were again left in the dark for 15 min and then the absorbances were taken at 415 nm with an Agilent UV–Vis 8453 spectrophotometer (Santa Clara, CA, USA). Quantification was performed using a calibration curve (12.5–150 μg mL^{-1}) using quercetin as a standard (≥95 %, Merck KGaA, Darmstadt, Germany).

Determination of tannins content was performed using the method reported by Broadhurst and Jones [32]. An amount of the filtrates (0.5 mL) was added to 3 mL of 4 % vanillin dissolved in pure methanol and 1.5 mL of pure HCl. After 20 min of incubation in the dark, the samples were read at 500 nm with an Agilent UV–Vis 8453 spectrophotometer (Santa Clara, CA, USA). Quantification was

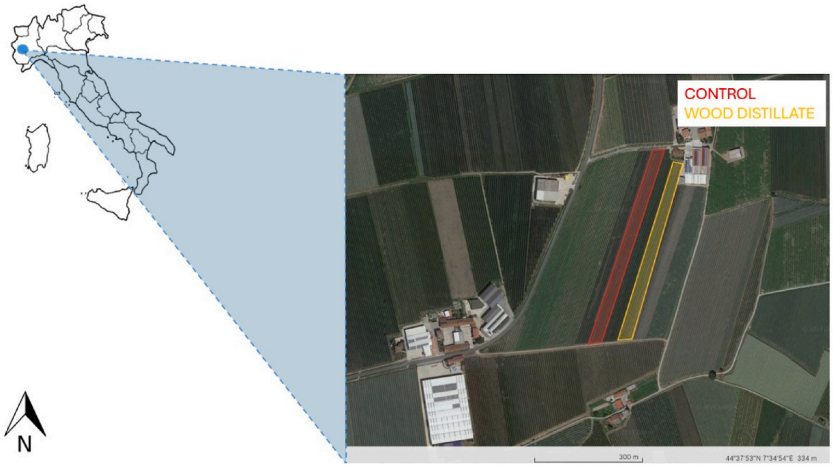

Fig. 1. Location and experimental design.

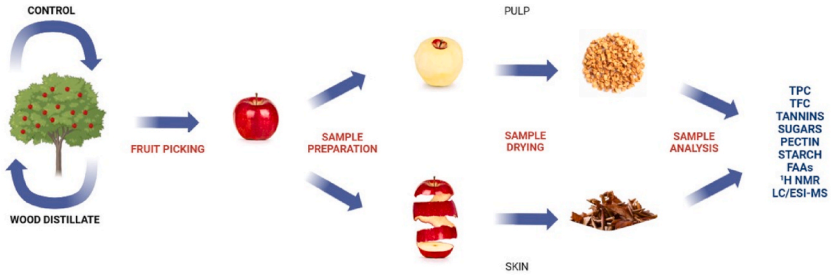

Fig. 2. Experimental scheme (created with BioRender.com).

performed using a calibration curve (12.5–900 μg mL^{-1}) using tannin acid as a standard (ACS reagent, Merck KGaA, Darmstadt, Germany).

Determination of total antioxidant power was performed using the method reported by Fedeli et al. [33] Briefly, 0.5 g of samples were homogenized in 2 mL of 80 % (v/v) ethanol and centrifuged at 15,000 rpm for 5 min. A 200 μL aliquot of the supernatant was mixed with 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (prepared by dissolving 3.9 mg of DPPH in 100 mL of 80 % (v/v) methanol). Blank and control samples were also prepared. After incubation for 1 h in the dark, the absorbance of the samples was measured at 517 nm using a UV–Vis spectrophotometer (Agilent 8453, Santa Clara, CA, USA). The total antioxidant power was expressed as the percentage antiradical activity (ARA, %) using the formula in equation (1):

$$ARA(\%) = 100 \times \left[1 - \frac{sample\ absorbance}{control\ absorbance}\right]. \quad (1)$$

2.3. Sugars, pectin, and starch content

Determination of the content of sugars and pectin content was performed following the method reported by Fedeli et al. [34] with minor modifications. Approximately 0.5 g of samples were homogenized in 4 mL of deionized H_2O and then centrifuged at 15,000 rpm for 5 min. The supernatant was filtered using a 0.45 μm syringe filter and analyzed by HPLC (ArcHPLC, Waters) equipped with a Waters 2410 refractive index detector. Sugar separation was allowed using deionized water as mobile phase, eluted at 0.6 mL min^{-1}, and a Waters Sugar-Pak I ion-exchange column (6.5 × 300 mm) kept at 90 °C using an external temperature controller (Waters Column Heater Module). Sugar and pectin quantification was obtained through calibration curves prepared by dissolving analytical sugars (Merk) in deionized water at concentrations of 0.1–0.5 mg mL^{-1}.

For the determination of starch content approximately 0.05 g of samples were homogenized in 2 mL of DMSO. Then, 0.5 mL of 8 M HCl was added, and the samples were dried at 60 °C for 30 min. After cooling, 0.5 mL of 8 M NaOH and 7 mL of deionized H_2O were added. The samples were then centrifuged at 4000 rpm for 5 min, after which 0.5 mL of the supernatant was added to 2.5 mL of Lugol's solution (0.05 M HCl, 0.03 % I_2, and 0.06 % KI). After 15 min, samples were read at 605 nm using a UV–Vis spectrophotometer (8453, Agilent, Santa Clara, CA, United States). Quantification was performed using a calibration curve (10–400 mg mL^{-1}) using pure starch as a standard (Sigma-Aldrich) [19].

2.4. Free amino acids' content

Determination of free amino acids (FAAs) was performed following the method reported by Fedeli et al. [35] Approximately 1 g of the samples were homogenized in 2 mL of deionized H_2O and then centrifuged at 4000 rpm. According to the AccQ Tag protocol (Waters, Milford, MA, USA), 10 μL of each reconstituted sample was derivatized with amino acids, using the fluorescent reagent AQC (Waters, Milford, MA, USA) and 0.02 M borate buffer. FAAs were separated and quantified using a high-pressure liquid chromatography system (HPLC- LC1, Waters, Milford, MA, USA) equipped with a C18 column (250 × 4.6 mm, 5 μm, Agilent, Santa Clara, CA, USA), thermostated at 20 °C, and a scanning fluorescence detector (470, Waters, Milford, MA, USA), (excitation at 250 nm, detection at 395 nm). The mobile phases used were: (A) 22.9 % (w/v) sodium acetate/deionized H_2O, 7.7 % (v/v) phosphoric acid/deionized H_2O, and 4.1 % (v/v) triethylamine/deionized H_2O; (B) 60 % (v/v) acetonitrile/deionized H_2O, with a specific time for gradient (Table S1).

The concentration of each amino acid (Asp: aspartic acid; Ser: serine; Gln: glutamic acid; Gly + Hys: glycine + histidine; Arg + Thr: arginine + threonine; Tau: taurine; β-Ala: β-alanine; Ala: alanine; Pro: proline; γ-Aba: γ-aminobutyric acid; β-ABA+α-ABA: β-aminobutyric acid + α-aminobutyric acid; Cys: cysteine; Tyr: tyrosine; Val: valine; Met: methionine; Orn: ornithine; Lys: lysine; Ile: isoleucine; Leu: leucine; Phe: phenylalanine) was estimated by matching the area under the peak of the chromatogram to the standard (WAT088122, Waters, Milford, MA, USA), using Clarity software (DataApex).

2.5. Mineral elements

The content of mineral elements was determined following the method reported by Fedeli et al. [14] A microwave-digestion system (Milestone Ethos 900, Bergamo, Italy) was used to dissolve 150 mg of dried samples in 3 mL of 70 % HNO_3 and 0.5 mL of 30 % H_2O_2 at

280 °C and 55 bar. Zn, Pb, Cu, Fe, and Cd contents were measured using ICP-MS (PerkinElmer NexION 350, MA, USA). Using the certified reference material NCS DC 73350 "*Poplar leaves*", which showed recoveries in the range of 95–111 %, the analytical quality was confirmed. The five replicates' coefficient of variation was used to estimate the analysis's precision, which was consistently >98 %.

Apple pulps and skins (5 g) were treated with EtOH:H_2O (50:50, v/v, 50 mL) to obtain the corresponding extracts: WD pulp extract (**WDP_e**), WD skin extract (**WDS_e**), control pulp extract (**CP_e**) and control skin extract (**CS_e**). The extracts obtained were then quantified: **WDP_e**, 329 mg (yield 6.58 %); **WDS_e**, 318 mg (yield 6.36 %); **CP_e**, 394 mg (yield 7.88 %); **CS_e**, 402 mg (yield 8.04 %).

2.6. Ultrasound-assisted extraction

Skins (S) and pulps (P) derived from conventional (C) or WD treatment (WD) were extracted by means of ultrasound-assisted extraction, carried out using a published procedure [36]. Freeze-dried sample of apple (5 g) was treated with EtOH:H_2O (50:50, v/v) in a solid/liquid ratio of 1:10 (w/v) at 45 °C for 45 min in an ultrasonic bath. The extracts were then filtered, the solvent evaporated, and the extracts quantified: WD pulp extract (**WDP_e**, 329 mg, yield 6.58 %), WD skin extract (**WDS_e**, 318 mg, yield 6.36 %), control pulp extract (**CP_e**, 394 mg, yield, 7.88 %) and control skin extract (**CS_e**, 402 mg, yield 8.04 %).

2.7. Liquid chromatography-mass Spectrometry (LC-MS)

The LC-MS system used to analyze the composition of extracts was an Agilent 1260 Infinity II. ESI source parameters were set to a capillary voltage of 2 kV, sampling cone of 20 eV, source temperature of 150 °C, and source offset of 10 °C. The desolvation temperature was set at 450 °C, the cone gas flow at 0 L/h and the desolvation gas flow at 900 L/h. Measurement was performed in MSe high-resolution negative mode using an acquisition mass range from 100 *m/z* to 1500 *m/z* and a scan rate of 0.5 s, where fragmentation was carried out using Independent Data Acquisition for all eluting compounds with collision energy ramp from 20 V to 30 V storing at the high energy function. Instrument calibration was applied in the mass range of the measurement with sodium formate. Separation was carried out on a InfinityLab Poroshell 120 Aq-C18 column (4.6 × 100 mm, 2.7 μM, Agilent technologies, USA). Solvents used in the mobile phase were water with 0.1 % formic acid (A), and acetonitrile with 0.1 % formic acid (B). Then, 10 μL of sample was injected with a flow rate of 0.5 mL min^{-1} at 25 °C. The chromatographic gradient started at 83 % A and 17 % B a, changing to 79 % A and21 % B at 4.8 min, then to 74 % A and 26 % B at 14.8 min, then to 0 % A and 100 % B at 48 min, holding it for 10 min. Then, the column was equilibrated for 5 min to initial conditions.

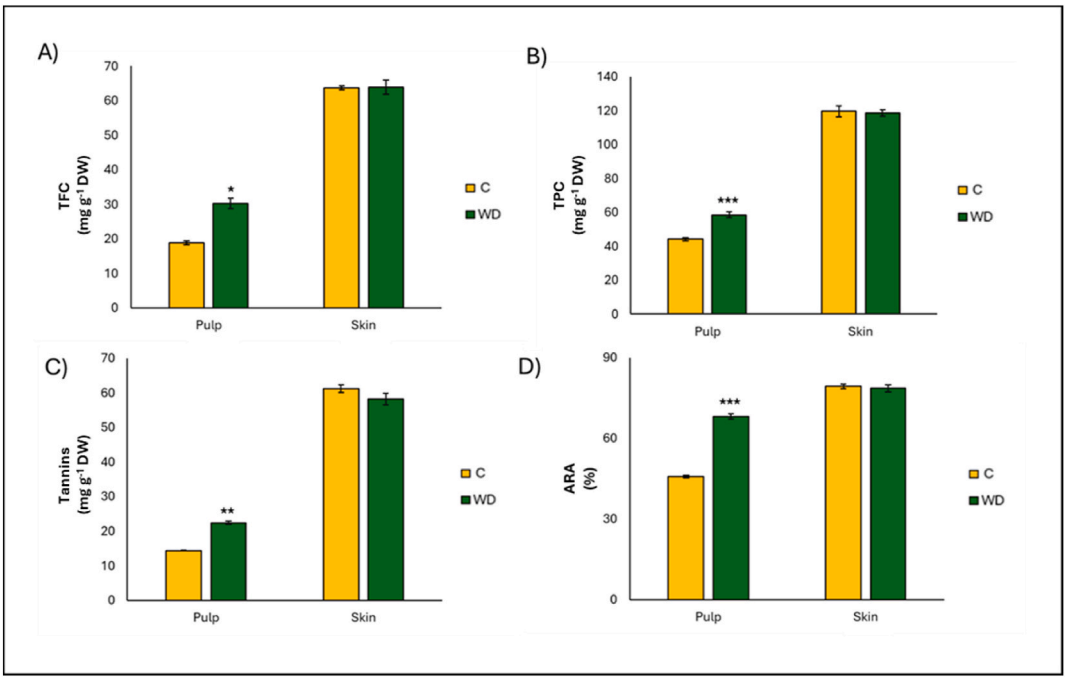

Fig. 3. **A**) Total phenols content (TPC), **B**) Total flavonoids content (TFC), **C**) Tannins, **D**) Total antioxidant power (ARA)in pulp and skin of apples expressed as mean ± standard error. "P" refers to apple pulp; "S" refers to apple skin, "C" refers to apples without the treatment of wood distillate; "WD" refers to apples with the treatment of wood distillate. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

2.8. Mono (^{1}H) Nuclear Magnetic Resonance experiments (^{1}H NMR)

Each dry extract (2 mg) was dissolved in 0.6 mL of DMSO-d_6 containing benzoic acid (1 mg) as concentration reference. All solvents and standards were purchased from Merck (Milan, Italy). All spectra were recorded at 298 K on a Bruker AVANCE III spectrometer operating at the proton frequency of 400.13 MHz and equipped with a Bruker multinuclear z-gradient inverse probehead. ^{1}H spectra were acquired employing a spectral width of 6000 Hz, and 64,000 data points for an acquisition time of 5.5 s. The recycle delay was set to achieve a 15 s total acquisition time to avoid relaxation effects. Quantification of the metabolites was performed by comparison of the signal integral to the reference integral, and quantities were expressed in milligrams per litre of extract.

2.9. Statistical analysis

The normality of data was verified through the Shapiro–Wilk test ($p < 0.05$). All results are presented with mean ± standard error. Statistical differences between control and WD-treated samples were evaluated by the student t-test ($p < 0.05$). Statistical analysis was performed using the R software (R Core Team, 2024).

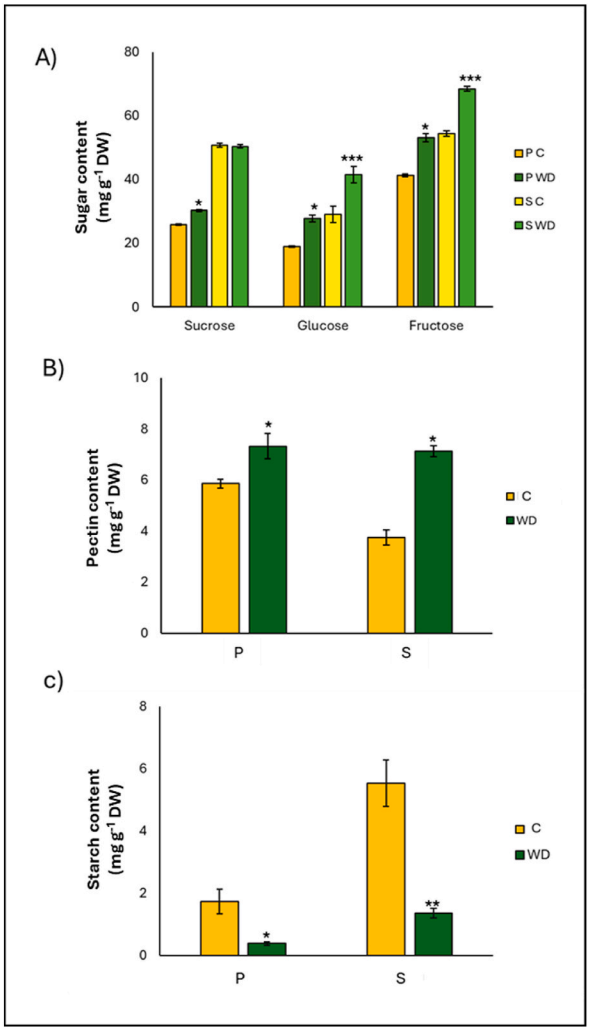

Fig. 4. **A)** Sugar content (sucrose, glucose, fructose), **B)** Pectin content, **C)** Starch content in pulp and skin of apples expressed as mean ± standard error. "P" refers to apple pulp; "S" refers to apple skin, "C" refers to apples without the treatment of wood distillate; "WD" refers to apples with the treatment of wood distillate. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3. Results and discussion

3.1. Antioxidant profile of WD-treated apple skin and pulp

This study investigated for the first time the effect of WD on apple fruit quality. Our results showed that the increase in antioxidant compounds (TPC: +32 %; TFC: +60 %; tannins: +55 %; ARA: +49 %), retrieved in the treated samples were statistically significant only for the pulp while for the skin the values were comparable to those of the untreated samples (Fig. 3A–D). This result strongly suggests that the effect of WD is not directly due to the spray applications, but it may trigger several mechanisms in the formation of secondary metabolites. The increase found is mainly attributed to the chemical characteristics of WD that trigger stress in the plant, determining an increase in antioxidant compounds to counteract the stress itself [37].

The effects of WD are only related to other species since this is the first study investigating its effects on apple fruits. Findings revealed that WD at concentrations of 0.2 % and 0.4 % increased the fruit's TPC levels in tomato plants. Additionally, only the 0.4 % WD increased total antioxidant power [38]. In contrast, in strawberry fruits, there was no overall increase in TPC, although the levels of chlorogenic acid doubled compared to the control [39].

The increased amount of these compounds in the pulp is of considerable interest since these molecules are responsible for the nutraceutical properties of apples in terms of antioxidant, anti-inflammatory, and cardioprotective properties [40]. Apple pulp contains polyphenols, which act as powerful antioxidants, protecting cells from ROS damage. This antioxidant effect is linked to a lower risk of life-threatening diseases (*i.e.*, heart disease and certain types of cancer). Furthermore, they might help regulate blood sugar levels, an effect from which people who have diabetes or are at risk of developing it could benefit [41].

Flavonoids can help reducing inflammation in the body [42]. This is especially significant as chronic inflammation has been linked to a variety of diseases, including cardiovascular, cancer and autoimmune disorders [43]. Tannins can help reduce blood cholesterol levels and promote arterial health, providing important support to the cardiovascular system [44]. Nevertheless, these effects are often hampered by the low bioavailability of these compounds [45,46].

Generally, our findings showed that the content of antioxidant compounds (TFC, TPC, tannins, and ARA) of the tested apples are higher in the skin than in the pulp, for both treated and not-treated apples (Fig. 3A–D). For these reasons, the skin is often reused in the cosmetic and pharmaceutical industries, to produce creams and other dermatological products, as well as to produce food supplements and drugs [47].

Our results showed that there is an inverse correlation between the content of sugars and starch (Fig. 4A–C). A significant increase in sugar content in both **WDP** (sucrose: +48 %; glucose: +46 %, fructose: 32 %) and **WDS** (glucose: +43 %, fructose: +26 %) was found (Fig. 4A). whereas a statistically significant reduction in the starch content was found in both **WDP** and **WDS** (−78 %, and −87 %, respectively) (Fig. 4C).

During the early developmental stages of the apples, starch is the predominant element in the pulp composition. However, as ripening progresses, a gradual conversion of starch into sugars occurs. This process is regulated by specific enzymes, such as starch hydrolase, which catalyze the transformation of starch into simple sugars, such as glucose and fructose. This conversion is responsible for the increased sugar content and sweetness of the fruit during the ripening process. It is therefore possible to state that WD can influence the conversion of starch to sugars, decreasing the starch content in both pulp and skin and consequently increasing the sugar content in the pulp.

Limited data are available regarding the impact of WD on the sugar content of crop species. However, it has been demonstrated that a 0.5 % WD application enhances the soluble sugar concentration in tomatoes [48]. Moreover, Fedeli et al. [14] reported that WD application increased the soluble sugar content in tomatoes, with glucose levels rising by 32.9 %, fructose levels by 24.4 %, and the total sugar content by 27.8 %.

Regarding the pectin content, our results showed a statistically significant increase in both **WDP** and **WDS** apples (+20 %, and +87 %, respectively) (Fig. 4B).

Pectins play a crucial role in the structure and texture of apples, giving them a crispy consistency and contributing to their stability.

Table 1
Mineral element content in pulp and skin of apples expressed as mean ± standard error. "*C*" refers to apples without the treatment of WD; "*WD*" refers to apples with the treatment of WD. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

	Pulp		Skin	
	Mineral element (mg kg^{-1} DW)			
	C	WD	C	WD
Ca	2324 ± 30**	2484 ± 4	2821 ± 28	2819 ± 68
Mg	583 ± 15	608 ± 3	1180 ± 20	1145 ± 16
K	8226 ± 97***	6864 ± 64	6287 ± 149**	4905 ± 72
Fe	30 ± 0.8	32.5 ± 2.9	44.8 ± 1.4*	56 ± 3
Mn	1.24 ± 0.19	1.08 ± 0.04	5.1 ± 0.2	5.45 ± 0.24
Cu	4.49 ± 0.25	5.22 ± 0.04	4.5 ± 0.5	3.99 ± 0.23
Zn	2.1 ± 0.20	3.23 ± 0.69	2.2 ± 0.2	2.15 ± 0.28
P	727 ± 16**	624 ± 7	598 ± 9**	434 ± 19
S	652 ± 89	706 ± 77	838 ± 63	931 ± 33

An augmentation of pectins can further enhance these characteristics, making apples firmer and fresher for an extended period [49]. Moreover, pectins are important to produce food products such as jams, jellies, and preserves. A high concentration of pectin in apples can be advantageous for the food industry, enabling the production of high-quality products with optimal texture and increased stability during storage.

From a nutritional point of view, WD-treated apples showed an increase in **WDP** Ca content (+7 %) and a decrease in K (−17 %/-22 %) and P (−14 %/-27 %) in **WDP** and **WDS**, respectively (Table 1). Overall, the mineral content was highly variable, but measured values are consistent with the normal ranges reported in literature [50]. Focusing on the reduction of P, similar results were found in previous studies on tomato fruits [14]. In those investigations, the use of 0.2 % and 0.4 % concentrations of WD resulted in a comparable decrease in P levels. This reduction may be related to the low pH of WD [51]. Nevertheless, the correlation between the application of WD and the observed P reduction emphasizes the interplay between treatment conditions and elemental composition in various crop species. This sheds light on the mechanisms that are active within these biological systems, providing valuable insights for future research and agricultural practices.

The decrease in K content in WD-treated apples can be explained through several mechanisms related to the composition and effects of WD on nutrient dynamics and plant physiology. Firstly, WD contains a variety of organic compounds, including phenols, acids, and aldehydes [13]. Some of these compounds can interfere with K uptake by altering soil pH or interacting with potassium ions, making them less available for root absorption. The application of WD can change the soil pH. If the WD acidifies the soil, it can reduce K availability, as potassium is most accessible to plants in a neutral to slightly acidic pH [52]. Moreover, WD may contain various ions and compounds that compete with potassium for uptake by plant roots. An increase in other cations such as Na^+ or H^+ can compete with K^+ absorption, leading to a decrease in its content within the plants [52]. The compounds present in WD might also affect root health and function. If root growth or root hair development is compromised, the overall ability of the plant to absorb K can be reduced, resulting in lower levels in the fruit. Additionally, WD can impact the nutrient transport mechanisms within the plant, changing the expression of specific transport proteins or the energy status of plant cells can affect how K is transported from the roots to the fruits [53].

The content of FAAs showed different responses in both the pulp and skin. A statistically significant increase emerged for **WDP** apples regarding the content of Asp (+17 %), Arg + Thr (+67 %), Tau (+96 %), β-Ala (+57 %), Pro (+120 %), and a decrease in Gln (−44 %), Val (−32 %), Met (−36 %), and Ile (−64 %). On the other hand, **WDS** showed a statistically significant increase in the content of Met (+100 %), and a decrease in Asp (−40 %), Gln (−49 %), Orn (−46 %), Lys (−40 %), and Phe (−45 %) (Table 2).

To the best of our knowledge, only one study investigated the effects of WD on the FAAs content. The comparison of these results with Fedeli et al. [37] on chickpea seeds reveals complexities. A significant increase in Val, Ile, Tyr, Asp, and Ala, with smaller increases in Glu, Leu, Thr, Ser, and Pro was observed for apple, while a slight decreases in Lys, Phe, and Met was observed in chickpea.

In contrast, WD treatment effects on **WDP** and **WDS** showed diverse responses. Proline had a notably higher increase in **WDP** compared to chickpea seeds, while Asp increase was more pronounced in the chickpea seeds. **WDS** exhibited a decrease in Asp, in contrast with other datasets. Val and Ile decreased in **WDP** but increased significantly in chickpea seeds, indicating variability in treatment effects across different plant tissues. Met increased in WDS but decreased in both **WDP** and chickpea seeds, highlighting varied responses.

Table 2
Free amino acid (FAAs) content in pulp and skin of apples expressed as mean ± standard error. "*C*" refers to apples without the treatment of wood distillate; "*WD*" refers to apples with the treatment of wood distillate. * = $p < 0.05$.

	Pulp		Skin	
	FAAs (μg g^{-1} DW)			
	C	WD	C	WD
Asp	327 ± 5*	382 ± 7	166 ± 8*	99.1 ± 12.6
Ser	435 ± 26	439 ± 3	271 ± 7	250 ± 21
Gln	28.7 ± 4.1*	16.1 ± 0.8	67.1 ± 6.4*	34.3 ± 3.7
Gly + Hys	13.3 ± 1.6	10.9 ± 0.9	8.8 ± 0.7	10.6 ± 0.7
Arg + Thr	29.1 ± 5.8*	48.7 ± 4.1	29.1 ± 0.3	30.1 ± 3.7
Tau	16.4 ± 3.2*	32.1 ± 1.8	49.9 ± 5.1	42.1 ± 5.2
β-Ala	71.1 ± 13.9*	112 ± 4	48.8 ± 0.5	60.6 ± 9.8
Ala	50.2 ± 2.2	55.7 ± 5.6	46.8 ± 0.2	44.8 ± 4.9
Pro	24.8 ± 2.6*	54.8 ± 6.7	83.4 ± 3.2	83.1 ± 10.7
γ-Aba	77.2 ± 13.4	100 ± 9.	77.9 ± 5.7	83.5 ± 13.8
β-ABA+α-ABA	10.4 ± 2.7	11.3 ± 1.2	33.3 ± 0.5	28.4 ± 3.6
Cys	15.2 ± 5.7	8.7 ± 0.7	58.4 ± 8.5	44.9 ± 12.2
Tyr	13.2 ± 6.5	12.6 ± 2.6	16.2 ± 1.8	10.2 ± 1.8
Val	12.3 ± 3.7*	8.4 ± 0.3	18.3 ± 1.2	19.3 ± 1.2
Met	10.4 ± 5.2*	6.6 ± 0.6	7.9 ± 2.5*	15.9 ± 0.9
Orn	10.2 ± 4.8	12.6 ± 0.4	6.1 ± 0.8*	3.3 ± 0.9
Lys	10.1 ± 5.5	13.4 ± 1.4	9.7 ± 1.4*	5.8 ± 0.5
Ile	14.6 ± 5.8*	5.3 ± 0.4	8.9 ± 1.1	11.7 ± 2.5
Leu	18.9 ± 2.1	16.7 ± 0.9	131 ± 2	156 ± 13.6
Phe	12.7 ± 5.6	9.8 ± 0.4	9.8 ± 2.1*	5.4 ± 0.3

Overall, FAAs respond differently to WD treatments depending on the plant species and tissue, necessitating comprehensive studies to understand these complex biochemical responses. The variations observed in the content of Tau and Pro in WDP are noteworthy. These amino acids are involved in protecting plant cells from osmotic stress and other environmental stresses [54].

The increase in these two FAAs is further confirmed by the statistically significant reduction observed in the content of Gln, a non-essential amino acid involved in various metabolic processes within plants [55]. Indeed, the reduction of Gln content may indicate a redirection of metabolic resources towards pathways involved in stress mitigation, such as the biosynthesis of Pro and other stress-responsive metabolites. In addition to the observed variation in Tau and Pro, it is important to highlight the potential implications of alterations in other amino acids, including Arg, Thr, and β-Ala, which are crucial components of the plant's defence mechanisms against environmental stresses [56]. Arg is known for its role in enhancing plant resilience to various biotic and abiotic stresses [57], while Thr acts as a precursor for the biosynthesis of important metabolites involved in stress responses [58].

Besides, β-Ala has been linked to the synthesis of polyamines, which are known to play key roles in plant growth and stress tolerance [56].

These findings once again support the possible eustress mechanism triggered by WD, which generally increases stress, but ultimately results in an overall increase in antioxidant compounds, as observed in this study and others already present in the scientific literature [59].

3.2. Antioxidant profile and tannins content of the extracts

To fully analyze the apples' chemical composition, their hydroalcoholic extracts were screened in terms of antioxidant activity to compare their profiles with those of pulps and skins. All the extracts were tested for their antioxidant profile in terms of TPC, TFC. The results are reported in Fig. 5. Our results showed that no significant differences were shown for the antioxidant compounds (TPC, TFC, tannins, and ARA) for both pulp and skin between WD and control samples, except for the tannin content in the **WDP_e** that showed a significant increase with respect to **CP_e** (+52 %) (Fig. 5A–D). These findings suggest that the extraction reduces the significant differences between the **WDP** and **CP** samples, where significant differences were recorded in all the antioxidant compounds tested (TPC, TFC, tannins, and ARA) (Fig. 5A–D).

3.3. Metabolomic profile

To assess the chemical composition of **WD** and **C** apples, we carried out ^{1}H NMR analyses of both pulp and skin extracts (**WDP_e**, **CP_e**, **WDS_e**, **CS_e**). Pulp and skin extracts have similar NMR profiles, as shown in the representative spectra of **WDP_e** (Fig. 6A) and **WDS_e** (Fig. 6B).

A total number of 21 molecules were identified through ^{1}H NMR analysis. Fig. 6 indicates the diagnostic peaks that were used for

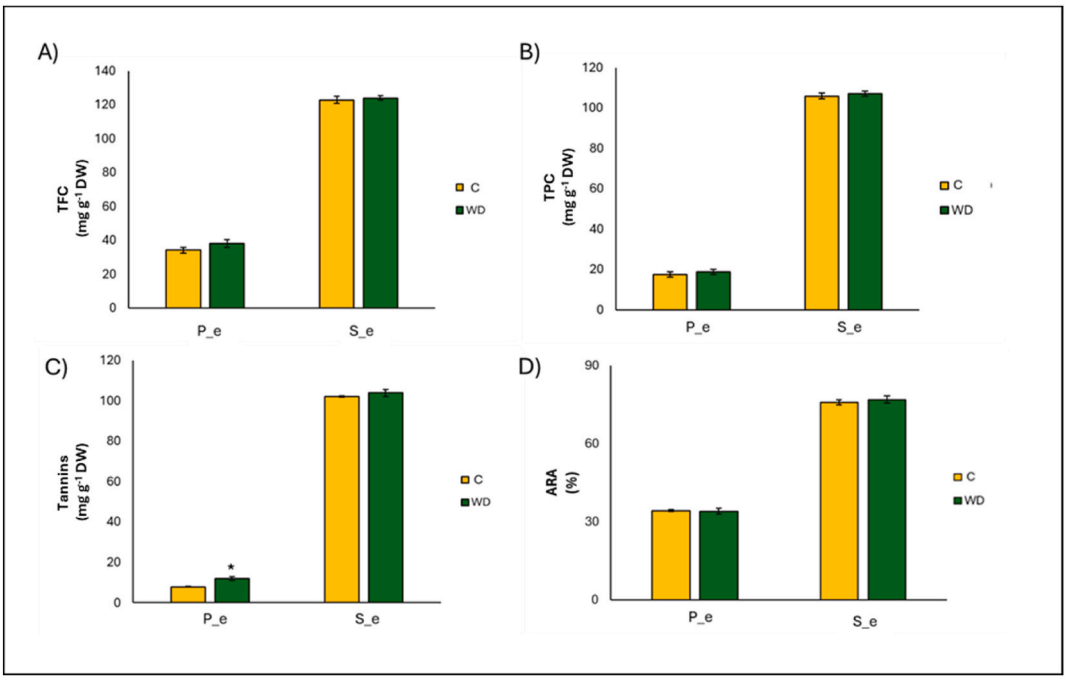

Fig. 5. **A**) Total phenols content (TPC), **B**) Total flavonoids content (TFC), **C**) Tannins, **D**) Total antioxidant power (ARA) in pulp and skin of apples extract expressed as mean ± standard error. "P_e" refers to apple pulp extract; "S_e" refers to apple skin extract, "C" refers to apples without the treatment of wood distillate; "WD" refers to apples with the treatment of wood distillate. * = $p < 0.05$.

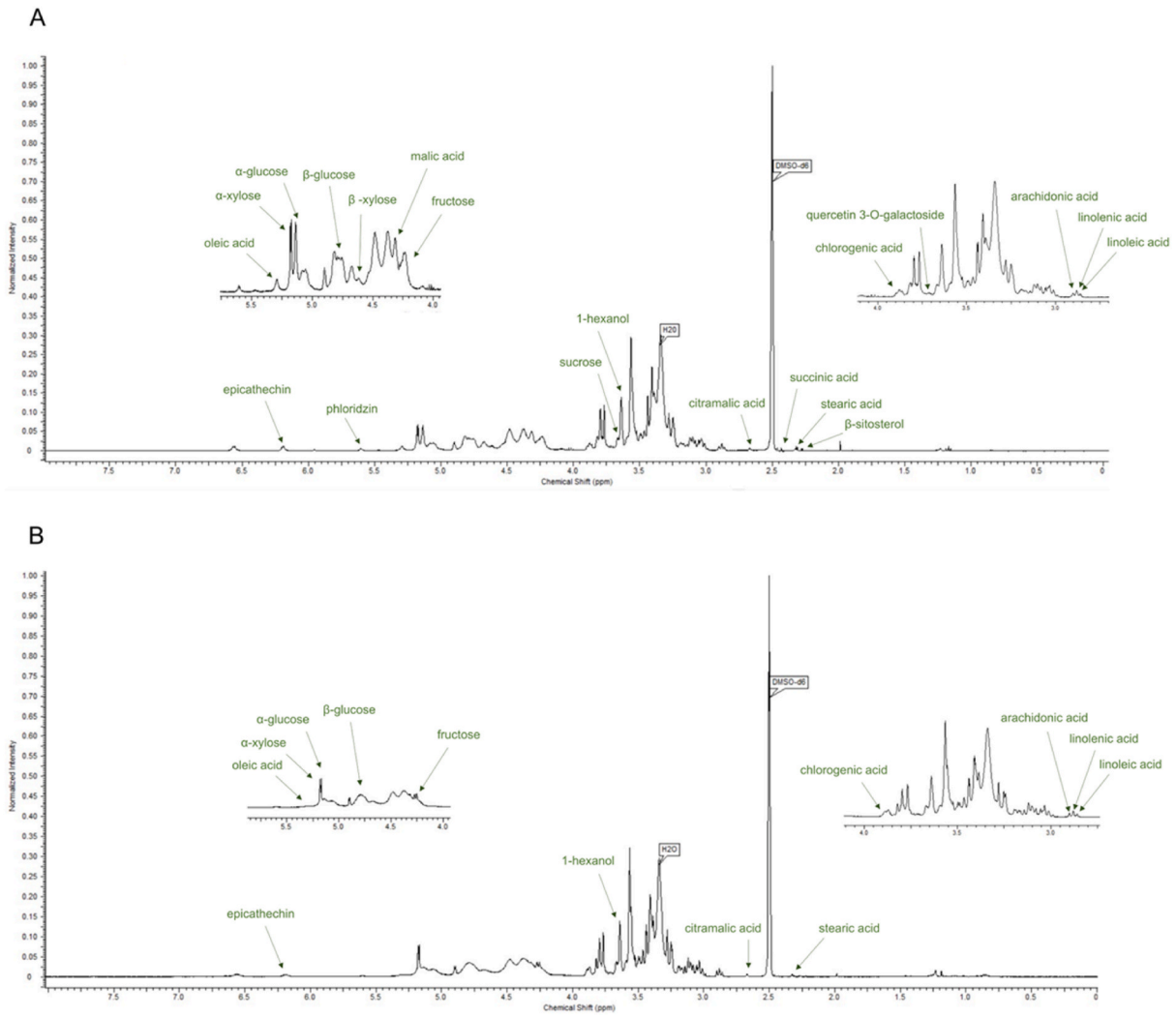

Fig. 6. Representative ^{1}H NMR spectra of **WDP_e** (**A**) and **WDS_e** (**B**).

the discrimination and attribution of substances, using the different chemical shifts and literature data [60,61].

In particular, citramalic acid was identified in all four analyzed samples thanks to its multiplet at 2.66 ppm (CH_2).

Malic acid was identified in high amounts only in **WDP_e** while slight traces were identified in **CS_e**. Of interest, succinic acid was quantifiable only in **WDP_e**, due to the presence of interference peaks present in the **WDS_e** and **CP_e** spectra, near the region of diagnostic peak (i.e. α,β-CH_2, singlet, 2.43 ppm). Among the sugars, α-glucose and β-glucose were found in all the samples, while fructose and sucrose were not visible in some of them (Fig. 5 and Table 2).

Different fatty acids were also identified in the samples, such as stearic, arachidonic, oleic, linoleic, and linolenic acids. All of them were highly present in the WD-treated apples.

Among the polyphenol-like compounds, epicatechin was identified in all the extracts by the doublet of CH-8 (6.19 ppm) with high amount present in **WDP_e**. Phloridzin was found only in the pulp extracts **WDP_e** and and **CP_e**. Finally, Quercetin-3-O-galactoside was also identified and found in good amount especially in **WDP_e** (Table 3).

To fully complete the chemical characterization, the extracts have been subjected to LC-DAD-ESI-MS/MS method. In order to identify the expected secondary metabolites, *i.e.* polyphenols and their glycosides, anthocyanidins and sugars, different absorbance wavelengths have been selected (192, 280, 320, 400 nm).

Analyses were performed in both positive and negative ion modes for the detection of all the above-mentioned compounds.

Preliminary analyses were carried out using full scan, data-dependent MS/MS scanning from *m/z* 100–1400. Identities of the target molecules were determined by matching their molecular ions (*m/z*) and MS/MS product ions obtained by LC-ESI-MS/MS and literature data [62–66]. Table 4 reports the tentative attribution of products scanned by LC-ESI-MS/MS.

Interestingly, caffeoyl glycosides like caffeoyl glucose and hexosides I and II were identified in all the analyzed samples, while caffeoylquinic acid was significantly higher in the **CS_e** sample than the corresponding **WDS_e**.

Table 3
Resonance assignments with chemical shifts of identified metabolites and quantification of metabolites.

Compound	Assignment	^{1}H δ (ppm)	Multiplicity	Amount (mg 100 mL^{-1})			
				WDP_e	WDS_e	CP_e	CS_e
Citramalic acid	CH_2	2.66	m	3.26	2.37	3.70	2.81
Quinic acid	CH_2-1	1.89, 2.08	m	nq	nq	nd	nd
Malic acid	α-CH	4.31	dd	15.29	nq	nq	2.95
Succinic acid	α,β-CH_2	2.43	s	8.62	nq	nq	/
Fructose	CH-3	4.23	d	13.15	nq	nd	9.01
α-Glucose	CH-1	5.13	m	13.87	4.86	4.50	5.22
β-Glucose	CH-1	4.78	d	10.27	9.91	6.85	15.5
Sucrose	CH2-1′	3.66	m	16.77	15.4	13.30	nd
α-Xylose	CH-1	5.16	d	21.17	6.91	13.4	10.1
β-Xylose	CH-1	4.60	d	4.65	nq	nd	nd
Chlorogenic acid	CH-3′	3.88	m	10.27	14.17	8.86	22.3
Epicatechin	CH-8	6.19	d	6.68	4.06	3.19	2.03
Phloridzin	CH-1″	5.60	m	4.80	nq	1.31	nd
Stearic acid	CH_2-CO_2	2.31	t	7.11	3.41	4.55	4.27
Linoleic acid	= CH-CH_2-CH =	2.86	m	3.37	2.80	1.40	2.24
Linolenic acid	= CH-CH_2-CH =	2.88	m	4.73	3.34	3.06	2.78
Arachidonic acid	= CH-CH_2-CH =	2.90	m	3.65	2.74	2.13	2.13
Oleic acid	CH=CH	5.30	m	4.38	2.26	nd	nd
β-sitosterol	CH_2-4	2.28	m	3.53	nq	0.60	0.40
Quercetin-3-O-galactoside	CH_2-6″	3.71	m	4.64	nq	2.10	4.40
1 Hexanol	O-CH_2-1	3.63	t	15.43	7.66	15.02	9.40

nq: not quantifiable; nd: not detected.

Table 4
Profile of the metabolites identified by LC-ESI-MS/MS.

Proposed compound	Molecular formula	Molecular weight	Observed *m/z*	MS/MS Product Ions	WDP_e	WDS_e	CP_e	CS_e
Syringic acid-4-O-glucoside	$C_{15}H_{18}O_8$	360.1056	360.5		–	+	–	–
Caffeoyl glucose	$C_{16}H_{20}O_9$	342.0951	341.0		+	+	+	+
3-p-Coumaroylquinic acid	$C_{16}H_{18}O_8$	338.1002		190.5	+	–	+	+
Ferulic acid-4-O-glucoside or Feruloyl glucose	$C_{13}H_{16}O_{10}$	356.1107	355.4		–	+	–	–
Caffeoyl hexoside (I of II) or Caffeoyl hexoside (II of II)	$C_{15}H_{17}O_9$	342.3	341.0		+	+	+	+
Malic acid	$C_4H_6O_5$	134.087	133.0		+	+	+	–
Sucrose	$C_{12}H_{22}O_{11}$	342.297	340.9		+	–	+	+
p-coumaroyl-hexoside	$C_{15}H_{18}O_8$	326.301	325.5		–	+	–	–
Procyanidin tetramer I, II, III	$C_{60}H_{50}O_{24}$		1153	125	–	+	–	–
Quercetin-3-O-xyloside or Quercetin-3-O-arabinopyranoside	$C_{20}H_{18}O_{11}$	434.3	432.9		–	+	–	–
Quercetin-di-hexoside	$C_{30}H_{25}O_{13}$	626.52	625.4		–	+	–	–
Procyanidin type B trimer	$C_{45}H_{37}O1_8$	866.2058		713.3, 739.9	+	–	–	–
Procyanidin type B tetramer II, III	$C_{60}H_{49}O_{24}$	1154.269		1001.9	+	–	–	–
qKaempferol hexoside	$C_{21}H_{20}O_{11}$	448.38		285	–	–	+	–
Phloridzin	$C_{21}H_{24}O_{10}$	436.413		166.4	–	–	+	–
Caffeoylquinic acid	$C_{16}H_{17}O_9$	354.31		352.8	–	–	–	+

+: present; -: not present.

Of note, the presence of peaks ascribable to procyanidin derivatives like type B trimer and tetramer II and III could be detected only in WD-treated samples. On the other hand, the tetrameric forms were identified in **WDS_e**. **CP_e** is the only extract showing the presence of kaempferol hexoside and phloridzin. Feruloyl glucose and syringic acid-4-O-glucoside were observed only in **WDS_e**. The redundant presence of procyanidin derivatives in apple skin is generally associated with a high antioxidant profile, in agreement with studies indicating higher antioxidant values related to the presence of this type of procyanidin oligomers [65]. The detailed chemical characterization of the extracts gives a further contribution highlighting some information previously published for their exploitation of these extracts as available sources of a multitude of compounds including sugars and particularly polyphenols, which in turn can be of interest for further research due to their potential biological activities [67–70].

4. Conclusion

In this study, we have reported for the first time the effect of WD foliar application on apple fruits. The results highlighted that apples derived from WD-treated trees show increased amounts of sugars (sucrose: +48 %; glucose: +46 %, fructose: +32 %), minerals (Ca: +7 %), and antioxidants (TPC: +32 %; TFC: +60 %; tannins content: +55 %; total antioxidant power content: +49 %), especially

in the pulp, thus suggesting the use of biostimulants in organic agriculture, due to their beneficial effects on nutritional parameters.

Overall, these findings open the way to further exploration of biostimulant use in enhancing fruit quality and sustainability in OF practices. Future research could investigate the long-term effects of WD application on various apple cultivars and other fruit crops, evaluating not only the nutritional benefits but also potential impacts on yield, disease resistance, and overall plant health. These insights could contribute to the development of more effective and environmentally friendly agricultural practices, promoting healthier food production systems and reducing reliance on synthetic chemicals.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Riccardo Fedeli: Writing – original draft, Investigation, Conceptualization. **Maria Dichiara:** Writing – review & editing, Investigation, Data curation. **Gabriele Carullo:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Valeria Tudino:** Writing – review & editing, Investigation, Formal analysis. **Sandra Gemma:** Validation, Software, Formal analysis. **Stefania Butini:** Validation, Formal analysis. **Giuseppe Campiani:** Writing – review & editing, Supervision, Formal analysis. **Stefano Loppi:** Writing – review & editing, Supervision, Resources, Data curation.

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.

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Abbreviations Used

Ala	Alanine
Asp	Aspartic acid
Ca	Calcium
Cu	Copper
Cys	Cysteine
Fe	Iron
Gln	Glutamic acid
Gly + Hys	Glycine + histidine
Arg + Thr	Arginine + threonine
Ile	Isoleucine
K	Potassium
Leu	Leucine
Lys	Lysine
Met	Methionine
Mg	Magnesium
Mn	Manganese
Orn	Ornithine
P	Phosphorous
Phe	Phenylalanine
Pro	Proline
S	Sulfur
Ser	Serine
Tau	Taurine
β-Ala	β-alanine
Tyr	Tyrosine
Val	Valine

Zn Zinc
β-ABA+α-ABA β-aminobutyric acid + α-aminobutyric acid
γ-ABA γ-aminobutyric acid

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37599.

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