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Research article

Development of a preliminary extraction protocol for phenol compounds during table grape grafting formation

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Abstract: The development of rootstocks that are less sensitive to abiotic and biotic stresses can help mitigate the negative effects of climate change on crop productivity, soil health, and water use. Though, the phenomenon of graft incompatibility is a significant limitation to the spread of new rootstock genotypes. Numerous studies have focused on this issue, highlighting the role of certain phenolic molecules as predictive markers of incompatibility. Given the lack of specific research on table grape, , this study proposes a protocol for the extraction of polyphenols from its woody tissues, which is a fundamental prerequisite for further analysis on secondary metabolites involved in graft incompatibility. The proposed eco-friendly method coupled a traditional maceration using solvent with a green technique utilizing an ultrasound-assisted extractor. The following parameters were compared: (1) sample weight (0.1 g, 0.5 g, and 1 g), (2) time of ultrasound-assisted extraction (10 min, 20 min, and 30 min), and (3) solvent volume for maceration (10 mL, 15 mL, and 20 mL). Four phenol classes were considered based on previous works on *Vitis* spp.: cinnamic acids, flavonols, benzoic acids, and catechins. The characterization of polyphenolic biomarkers was carried out via HPLC. 1 g of plant material, 30 minutes of sonification, and 20 mL of organic solvent was the combination of factors that resulted in the most efficient fingerprint, both quantitatively (267.68 ± 3.91 mg/100 g fresh weight [FW]) and qualitatively, with the four classes analyzed significantly represented. This is the first work to come up with an extraction protocol for phenol compounds in table grape woody tissue based on both ecological and routine techniques.

1. Introduction

Although investigations on rootstocks have been very limited throughout the 20th century, the interest has significantly increased over the last decades. The choice of rootstock can impart resistance to abiotic conditions related to weather (e.g., water scarcity and thermal stress) and soil (e.g., salinity, toxicity of heavy metals, stress related to soil pH, and nutrient deficiency) [1]. Rootstocks can provide several benefits, including the ability to create a well-developed and dense root system, which increases the plant's capacity to extract water from the soil. The vigor of the plant can also be positively influenced by rootstocks because it affects light interception and carbon assimilation [2].

Moreover, rootstocks can confer plant resistance to soil-borne pests and pathogens, as demonstrated by the phylloxera-resistant American rootstocks [3]. Many horticultural species belonging to the *Solanaceae* family are now grafted using rootstocks resistant to soil pathogens (*Fusarium* spp., *Verticillium* spp.), nematodes, bacteria, and extreme temperatures [4]. In the last decades, the University of Milan has been focusing on the selection of new rootstocks for grapevine cultivation, able to mitigate the effects of climate change. The series M-rootstocks were released in 2014, chosen for their tolerance to iron-limited conditions, resistance to salinity, and good tolerance to drought [5]. These new genotypes could represent a valuable crop strategy to face the current viticulture challenges for wine and table grapes.

Currently, selecting plant materials that can ensure high yield and quality standards while being able to withstand the impact of climate change is one of the most promising crop adaptation strategies. Research is actively addressing these challenges to guide genetic improvement and the breeding of new resilient rootstocks. However, the main challenge with using and spreading newly selected rootstock genotypes is the issue of incompatibility, which can occur during grafting propagation [6].

During the past decades, extensive research has focused on the issue of graft incompatibility on different species such as grapevine (*Vitis* spp.), olive (*Olea europea*), chestnut (*Castanea* spp.), peach (*Prunus* spp.), melon (*Cucumis melo*), and litchi (*Litchi chinensis*) [7–12], and different approaches have been undertaken to identify effective predictive techniques. Researchers worked on the main horticultural species, while few and outdated studies are reported for minor crops.

Recently, the comprehension of tissue regeneration at the graft junction has been improved, especially working with model species [13]. However, the mechanisms underlying graft incompatibility are not yet fully understood [14,15]. Many factors may interfere with the results interpretation, such as the technique employed, degree of graft incompatibility, the presence of pathogens within the vascular tissues, and environmental conditions (irregular temperature and/or humidity) [16]. The identification of valuable transcript or metabolite markers of compatibility or incompatibility is challenging because of the high number of control samples and grafted individuals to be tested [17].

One promising research path in addressing graft incompatibility has been the investigation of secondary metabolites, with a particular focus on phenol compounds. Studies have shown that these compounds may play a crucial role in the formation of graft unions because of their involvement in cell division, development, and differentiation [18]. Research has demonstrated a relationship between the accumulation of polyphenols and the degree of compatibility in many fruit species [19–23].

The processes of identification and quantification of phenol molecules from vegetal samples are

performed through high-performance liquid chromatography (HPLC), preceded by a solvent extraction. Because of the complexity in the composition of the different phenol classes, there are no universal extraction procedures, and the extract composition depends on the technique and the solvent used [24]. Usually, solvents employed can be either organic or aqueous. Among them, the most commonly used ones are water, acidified water, methanol, propanol, ethanol, acetone, and their mixtures [25].

Usenik et al. proposed an extraction protocol on apricot (*Prunus armeniaca*) consisting of a 10-day maceration via acetone:water (80:20 v/v) [22]. The same protocol was performed on grapevine by slightly modifying the days of maceration and volume of solvent [7]. Phenol compounds on eucalyptus (*Eucalyptus gunnii*) were extracted via methanol:ethanol (1:1 v/v) solvent [26].

Many are the parameters that may influence the extraction yield of phenol compounds, such as the type of solvent, extraction time and temperature, liquid/solid ratio, sample matrix, size of the particles, and plant species were analyzed [27]. Although conventional techniques are widely used for phenolic compounds extraction, they suffer some setbacks that cannot be overlooked, namely the consumption of large amounts of hazardous organic solvents, the accumulation of residues, and the use of high-energy inputs [25].

In recent years, new extraction techniques for secondary metabolites have been developed in order to improve extract quality and efficiency and to reduce solvent consumption and extraction time. These techniques use different mechanisms such as ultrasound (ultrasound-assisted extraction, UAE), microwave energy (microwave-assisted extraction, MAE), supercritical fluids (supercritical fluid extraction, SFE), and elevated pressures (accelerated solvent extraction, ASE) [28].

The UAE method is a sustainable alternative to traditional extraction methods as it requires low solvent and energy inputs. This technique simplifies manipulation and reproducibility, as it can be performed at atmospheric pressure and ambient temperature and gives higher purity to final products. Finally, it can be employed in small and large-scale settings [29]. The UAE exploits the acoustic cavitation that damages cell walls of the vegetal samples and favors the release of bioactive compounds [28]. In the last decade, this green technique has been successfully employed for the extraction of many compounds from fruits, vegetables, and plant materials [30,31]. A recent study on *Punica granatum* [32] compared the extraction of antioxidants from the peel using UAE method and conventional solvent maceration. The results reported higher antioxidant yields (+24%) and reduced extraction time (−90%) in the case of the UAE method. Another work carried out on tomatoes (*Solanum lycopersicum*) demonstrated that the use of the UAE technique made it possible to increase the extraction yield of carotenoids by 143%, without causing any degradation of these compounds [33]. Finally, this environmentally friendly technique was used to extract biologically active molecules, such as polyphenols and other secondary metabolites, from *Castanea* spp. and *Vitis vinifera* by-products, with the purpose to analyze their nutraceutical composition. Chestnut wastes deriving from bud-derivates showed a great ecological value, suggesting their circular re-use as an alternative to composting or incineration [34]. On *Vitis vinifera*, authors compared the UAE technique to the traditional extraction by maceration. The innovative ultrasound bud-extracts presented a quali-quantitative polyphenolic composition that was similar to or better than the traditional bud-preparations, even if produced in a faster and more sustainable way [30].

The aim of the present work has therefore been to define a sustainable and effective extraction method for the evaluation of phenol compounds in table grape woody tissues.

The implementation of an extraction protocol for secondary metabolites in table grape woody tissues, based on both ecological and economical techniques, is an essential initial step towards researching graft incompatibility and ultimately enabling the use of new, sustainable rootstocks.

Though much research has been conducted on grapevine analyzing the expression and accumulation of phenolics during grafting, no literature is available for table grape as of today. Starting from extraction protocols already proposed in literature, a methodological study was conducted by combining a conventional extraction method with the UAE technique in order to reduce the use of organic solvents and extraction time and increase the extraction yield of phenol compounds.

2.   Materials and methods

2.1. Plant material

The plant material used in this experiment was grapevines of the table grape cultivar "Regal", one of the most commonly grown cultivars in the Piemonte region, grafted on the "420A" rootstock. While field experience suggests compatibility between these genotypes, no formal studies have been conducted yet to confirm this. For our experiment, the scion materials were provided by Cooperativa Monvisofruit soc. Agricola, while Vignevolute s.s.a. supplied the 420A rootstock.

In April 2022 plants were grafted at Vignevolute nursery, located in Montà d'Alba (Cuneo province). Scions and rootstocks were first subjected to thermotherapy, which consisted of a pre-treatment at 30 °C for 20 minutes, followed by a treatment of woody materials at 50 °C for 45 minutes to prevent future infections and contaminations, especially of phytoplasmas [35]. Then, the vines were grafted using a machine able to perform the whip and tongue graft. Next, the table grape vines were dipped into a solution made of *Trichoderma* spp., zeolite, and copper and then into paraffin to prevent dehydration at the grafting union.

Finally, the grafted vines were placed in forcing boxes filled with wet sawdust and stored for about four weeks at 30 °C and 80–90% relative humidity. These conditions are essential to induce callusing formation. In May, the table grape grafts were removed from the forcing boxes, dipped again into paraffin, and finally planted into 1.5 L pots. At this stage, known as callusing, plant material was sampled for the further experimental extraction of phenol compounds.

2.2. Plant material preparation

At callusing stage, the grafting union was removed from each grafted plant cutting 1 cm above and 1 cm below it. Samples were then stored refrigerated at −80 °C before being analyzed in the laboratory. The extraction of secondary metabolites was performed on inner and outer tissues separately. For this reason, the cortex and barks were separated from internal tissues. Then, they were ground in a mortar using liquid nitrogen to maintain the cold chain. Finally, the tissue powder was mixed, weighed, and stored at −80 °C until the following extraction, as reported in previous similar studies [7,9,36,37].

2.3. Extraction setup

In this study, different parameters were evaluated to define a protocol for the extraction of polyphenolic compounds produced by the plant tissues during grafting formation in table grape. Starting from previous studies [7,9] a blended approach was implemented, based on a preliminary overnight maceration through methanol solvent followed by an ultrasound-assisted extraction (UAE).

In particular, the amount of plant material resulting from the grafting union (0.1 g, 0.5 g, and 1 g), the time of ultrasonic extraction (10 min, 20 min, and 30 min), and the volume of solvent used for the extraction (10 mL, 15 mL, and 20 mL) were evaluated.

The experimental work started by considering the amount of plant material (step 1 in Table 1). Phenol extraction was performed by testing three different quantities of plant material composed of internal and external table grape milled tissues, leaving the extraction time and the solvent volume fixed at 30 minutes and 20 mL, respectively, as previously reported for chestnut [9]. Once the proper plant tissue quantity was defined, in step 2 three extraction times via ultrasound-assisted extractor were tested (10, 20, and 30 min), leaving the amount of plant material previously selected (1 g) and the solvent volume (20 mL) unchanged. Step 3 was concluded by evaluating the extraction of phenol compounds using different volumes of extraction solvent based on methanol, water, and HCl 95:4.5:0.5 v/v/v (10, 15, and 20 mL), without changing the plant material (1 g) and the extraction time (30 min), as resumed in Table 1.

Table 1. Experimental steps for the definition of the extraction protocol.

Step	ID code	Plant material (g)	Extraction time (min)	Extraction solvent (mL)
	0.1 g_30 min_20 mL	0.1	30	20
1	0.5 g_30 min_20 mL	0.5	30	20
	1.0 g_30 min_20 mL	1.0	30	20
	1.0 g_10 min_20 mL	1.0	10	20
2	1.0 g_20 min_20 mL	1.0	20	20
	1.0 g_30 min_20 mL	1.0	30	20
	1.0 g_30 min_10 mL	1.0	30	10
3	1.0 g_30 min_15 mL	1.0	30	15
	1.0 g_30 min_20 mL	1.0	30	20

Many phenolics have been considered as markers based on previous studies on *Vitis* spp.[7,20,37]: 2 catechins (catechin, epicatechin), 5 flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin), 2 benzoic acids (gallic and ellagic acids), and 4 cinnamic acids (caffeic, chlorogenic, coumaric, and ferulic acids). For each combination of parameters, selected amounts of the ground material were added to a specific quantity of extraction solvent based on methanol, water, and HCl overnight. Then, polyphenolic compounds were extracted by an ultrasonic bath for 10, 20, and 30 minutes at 23 kHz (Reus sarl, Drap—France). Finally, all the extracts were centrifuged at 4000 rpm for 10 minutes and then filtered through a 0,45 μm filter (polytetrafluoroethylene membrane-PTFE) before the HPLC-DAD analysis [30].

2.4. Chromatographic analysis

The characterization of polyphenolic biomarkers was carried out by a 1200 Agilent HPLC-UV-Vis Diode Array Detector (Agilent Technologies, Santa Clara, CA, USA). The single compounds were identified by the comparison and combination of their retention times and UV spectra with those of authentic standards in the same chromatographic conditions. The molecules were separated on a Kinetex C18 column (4.6 x 150 mm, 5 μm, Phenomenex, Torrance, CA, USA) [38]. The different HPLC methods and their specific parameters are reported and described in Supplementary Materials (Table S1).

2.5. Statistical analysis

A one-factor ANOVA test (SPSS 22.0) was performed on the data, and then a Tukey's HSD post hoc comparison test (n = 3; p < 0.05) was used to compare the mean values. Results were reported as mean value ± standard deviation (SD). Different letters were used to highlight significant statistical differences concerning the Tukey test (p< 0.05).

3. Results and discussion

Some scientific papers have proposed a protocol for the extraction of phenol compounds from *Vitis* spp. woody tissues. The procedures available were adapted starting from works on other fruit species with similar tissue composition, and the final protocol was set up for wine grapes [7]. Nevertheless, no studies have been carried out on the phenol expression in table grape woody tissues. In this study, a suitable and sustainable blended method to extract polyphenols has been proposed. This method evaluated different combinations of sample weight, time of UAE extraction, and solvent volume for maceration.

3.1. Step 1: amount of plant material

The experimental study focused on the weight of the plant samples as the primary variation factor. Phenol extraction is normally performed on tissues previously milled using liquid nitrogen. The process is time, energy, and sample-consuming for the operator, especially if tissues have more than one year or if they belong to hardwood species. Indeed, this operation cannot be mechanized since polyphenols are considered heat labile compounds and could undergo thermal degradation with the use of homogenizers [39]. Therefore, it is essential to find the minimum amount that allows for an efficient phenol extraction. Moreover, reduced quantities imply a limited consumption of solvent, which leads to lower waste production and minor environmental and economic costs [40].

Since no studies have addressed the issue for table grape tissues so far, the so-called liquid/solid ratio (expressed in mL of solvent per g of solid) must be investigated to define a protocol that maximizes the extraction yield and minimizes the solvent consumption and the sample preparation time.

Most extraction methods reported in literature for phenol compounds used a liquid/solid ratio in the range of 5–50 mL/g[41]. On other woody crops, the amount of plant material used in literature for phenol characterization varies depending on the species investigated and on the extraction technique (0.01 g for apricot [22], 0.1 g for pear [21], 0.5 g for Siberian elm [42], and 10 g for pear-quince [23]).

The current study examined three different weights of plant samples (0.1 g, 0.5 g, and 1 g), and the extraction efficiency was evaluated as the sum of the phenolic compounds extracted. Figure 1 shows the results of the extraction efficiency based on the quantity of plant material used for the extraction.
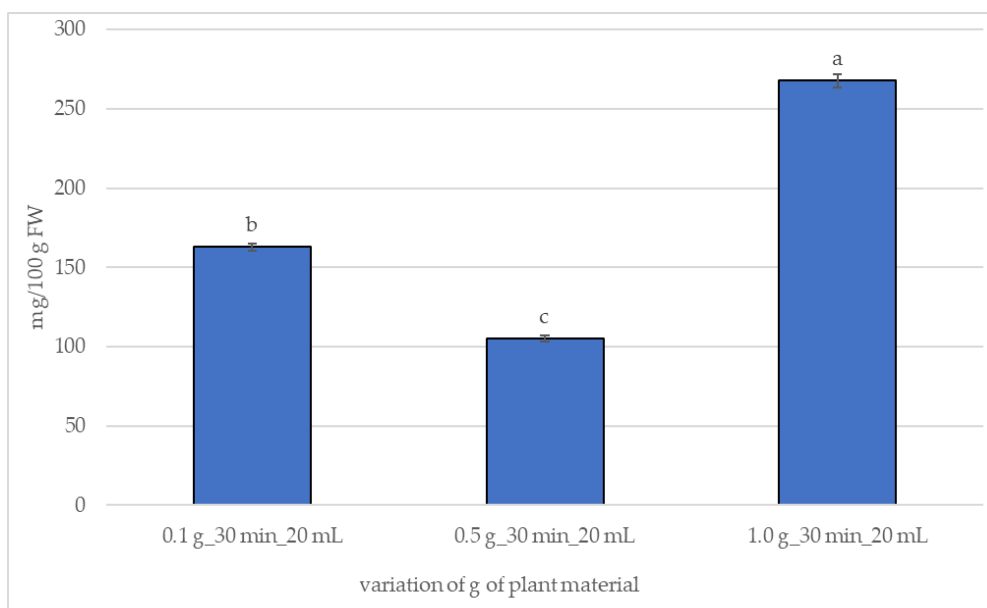
Figure 1. Influence of the different amounts of plant material on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW (FW = fresh weight). Mean value and standard deviation are given for each sample (n = 3). Different letters for each groups indicate significant statistical differences ($p < 0.05$).

According to Figure 1, 1 g of plant material was found to be the best amount (267.68 ± 3.91 mg/100 g FW). The reduction in yield observed when increasing the amount of plant material from 0.1 g to 0.5 g can be due to many reasons. First, the intrinsic internal variability of plant materials such as scions and rootstocks used in this research, which have been collected from different mother plants. Moreover, the sampling variability related to the number of the biological replicates. Enlarging the number of samples can significantly help to reduce this variability. However, in some cases plant material might be limited, as for this study. Furthermore, the methods used for the chromatographic analysis (detailed in Table S1) are not compound-targeted (so called marker approach); they provide good extraction performance for a wider range of polyphenolic compounds (multi-component approach), with a certain degree of variability. In the present study, an overall assessment of the main compounds involved in grafting dynamics according to previous research was performed. For this reason, and by virtue of the preliminary nature of the work, analytical methods with a multi-component approach have been adopted [43]. Finally, also the positioning of scion over the rootstock could trigger minor differences in the biosynthesis of certain compounds.

Reduced quantities of the sample were not enough to detect all the phenol classes investigated (Figure 2). Cinnamic acids, which are almost one-third of the total phenolic compounds identified with 1 g, were not found with 0.1 and 0.5 g of plant material. The most represented was the class of flavonols (34.99%), followed by the catechins (31.06%).

In recent years cinnamic acids have been proven to be related to grafting process in *Vitis* spp. In particular, the concentration of sinapic acid increases in incompatible combinations, highlighting its role as a potentially suitable marker of graft incompatibility [7,20]. The behavior of chlorogenic acid, another cinnamic acid, is quite different. This acid was found in larger quantities at the grafting union

of compatible combinations, especially during callusing and rooting stages [20]. As its chemical structure is very similar to those of some lignin intermediates, chlorogenic acid could be considered an intermediate of the lignin biosynthetic pathway [44]. The employment of this compound to produce lignin monomers is linked to physiological or environmental signals.
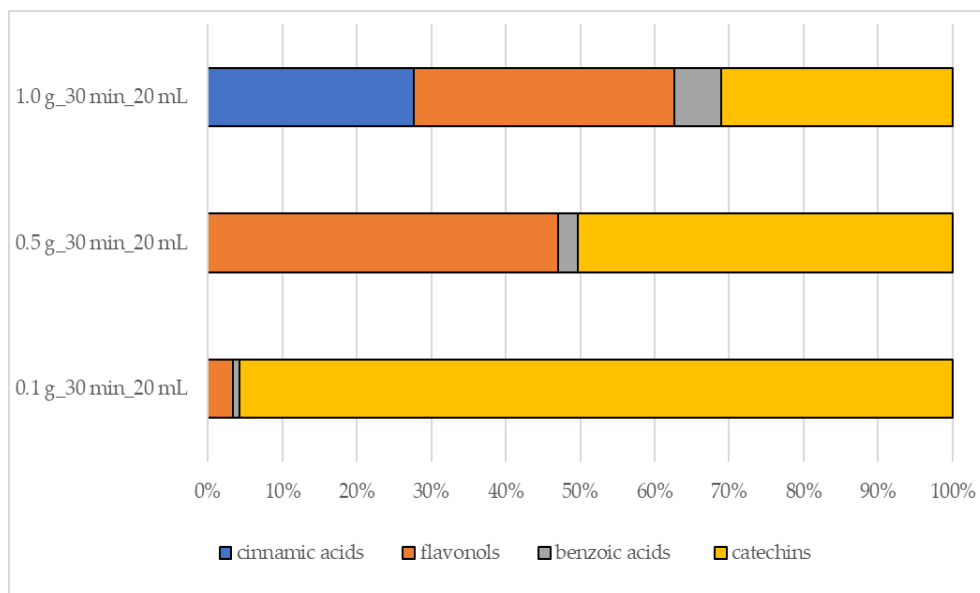

Figure 2. Phytochemical fingerprint according to the amount of plant material used for the extraction of phenol compounds.

The grafting process can be considerably stressful for plants, therefore the re-routing of the chlorogenic acid towards lignin during grafting formation can be expected, especially with compatible unions. The concentration of this molecule was not detected using 0.1 and 0.5 g of plant material, while 1 g allowed to obtain a significant value of 34.76 ± 1.55 mg/100 g FW. Given that the chromatographic methods employed are non-specific for single compounds, increasing the plant material significantly helped to increase the yield accuracy and to detect the chlorogenic acid.

Two other cinnamic acids have been linked to graft incompatibility in recent works. Ferulic and coumaric acids have been found in higher concentrations in incompatible unions of *Uapaca kirkiana* [45], while ferulic acid was more abundant in *Vitis* spp. [7] incompatible grafts. Finally, research on graft incompatibility between Sato-zakura cherry cultivars and *Prunus avium* rootstock pointed out the role of coumaric and ferulic acids as biomarkers for possible delayed incompatibility [46]. In the present work, no traces of ferulic acid were detected for all the amounts of plant material tested, while coumaric acid was found in low concentration only with 1 g, following a pattern previously noted for the chlorogenic acid.

Due to the lack of previous studies on table grape and based on the findings on other fruit species, this preliminary research focused on the expression of the main classes of phenol compounds involved in the grafting process, rather than delving into the dynamics of individual markers. In light of this, the amount of plant material that yielded the broadest and most comprehensive phenol fingerprint across the four investigated classes was determined to be 1 g.

3.2. Step 2: time of extraction using UAE

Among the conventional extraction techniques of phenol compounds, maceration has been one of the most used. It consists of blending pulverized plant material into appropriate solvent, which can extract valuable compounds by a proper selection of solvent polarity [47]. The main limitations of maceration are the large amounts of time and solvent required. Nowadays, thanks to the availability of more practicable and eco-friendly techniques this method has been gradually abandoned [25].

In this step, the amount of sample material and the solvent volume were left constant at 1 g and 20 mL, respectively. Then, three extraction times were tested for the UAE (10, 20, and 30 min). Moreover, the operator would be less exposed to noise, which is a physical hazard. These benefits add to the environmental advantages of ultrasounds if compared to other techniques. These benefits include less use of non-renewable resources and less energy consumption [48].

Figure 3 reports the sum of phenol compounds extracted with different UAE times. Reducing the time from 30 to 20 and 10 minutes statistically lowered the extraction yield by 36.28% and 23.21%, respectively.
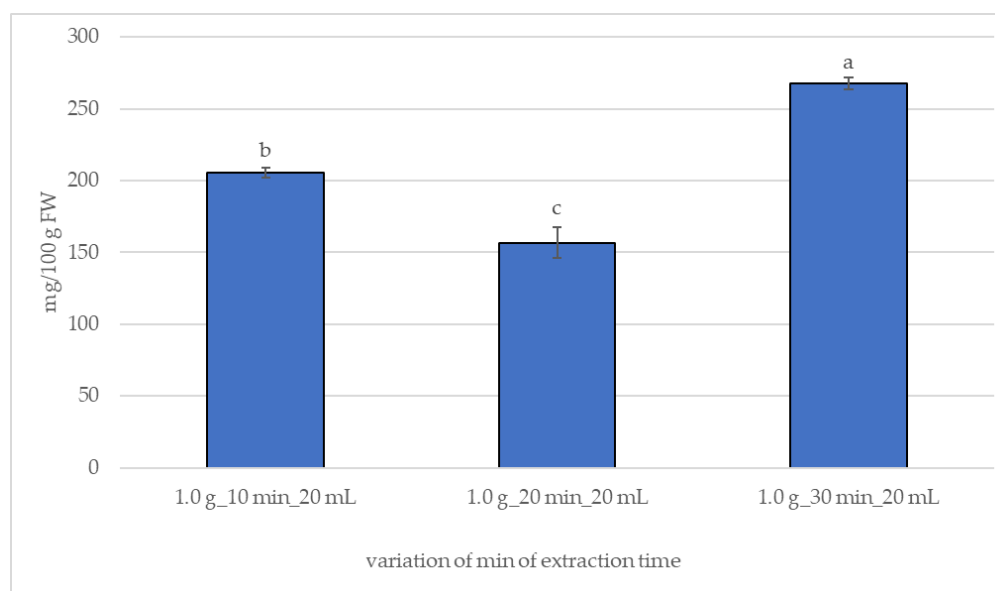

Figure 3. Influence of the different extraction times on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW. Mean value and standard deviation are given for each sample (n = 3). Different letters for each groups indicate significant statistical differences ($p < 0.05$).

The shorter extraction time (10 min) was not enough to detect the phenol class of the cinnamic acids (Figure 4), showing a trend already observed during the first experimental step, when the lower quantity of plant material (0.1 g) did not allow for their detection. Decreasing from 30 to 20 minutes resulted in an extraction loss of 78.78% for cinnamic acids (74.09 ± 1.18 vs 18.69 ± 1.12 mg/100 g FW), and 52.02% for flavonols (93.65 ± 0.96 vs 44.93 ± 1.40 mg/100 g FW).
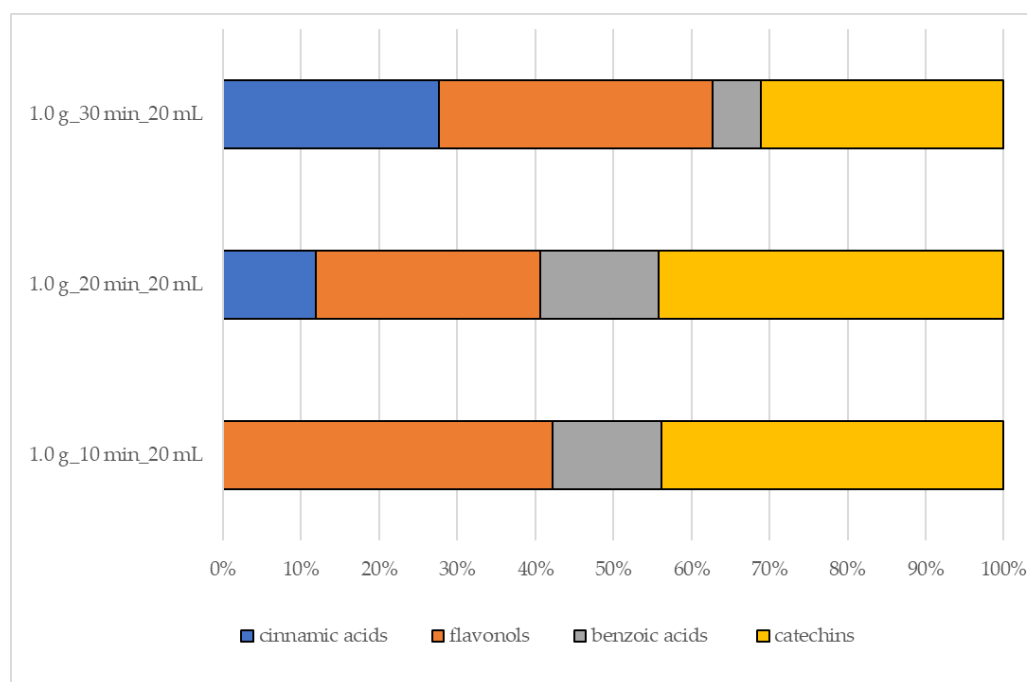
Figure 4. Phytochemical fingerprint according to the extraction times tested for the extraction of phenol compounds.

Gallic acid, catechins, sinapic and ferulic acids, and epicatechins were found in higher concentrations in tissues of incompatible grafts of different species [7,21,22]. Accordingly, low concentrations of these molecules may be interpreted as a sign of affinity. Among the benzoic acids, gallic and ellagic acids were investigated because of their role in grafting dynamics. In particular, gallic acid turned out to be a good marker of graft incompatibility for several species such as *Prunus armeniaca*, *Vitis* spp., and *Castanea* spp. [9,22,37]. The concentration of this compound was found to be higher at the graft union at the early stages of development, such as callusing, while decreasing during the growing season.

Grafting propagation represents a stress for plants, especially if two incompatible genotypes are combined. Gallic acid is normally biosynthesized under stress condition since it is active in protecting plant cells from oxidative stress [49]. At present, little research has been done on its antioxidant effects in the reaction to stress. However, an increased production of this molecule could be related to incompatibility between partners, as an attempt to overcome the grafting stress. Ellagic acid has a similar behavior as it decreases during grafting establishment, though differences between compatible and incompatible combinations were significant only on *E. gunnii* [26].

Though phenol markers increase in the case of incompatible grafts, for at least one compound a different behavior was observed. In fact, the biosynthesis of chlorogenic acid seems to be stimulated in the case of compatible unions, as was reported on *Vitis* [20]. The extraction time of 30 minutes enabled the extraction of the most abundant amount of cinnamic acids. Among them, the most expressed was chlorogenic acid (46.92%), supporting the compatibility of the experimental combination. The remaining part of this class of compounds was represented by caffeic acid (40.69%), and coumaric acid (12.39%). In previous research on *Vitis*, caffeic acid was found in very low quantities only at the callusing stage of both compatible and incompatible grafts. Therefore, its

biosynthesis may not be related to disaffinity [20]. Coumaric acid's involvement in graft dynamics has still not been clarified on grapevine. Considering the low quantity measured on table grape tissues during this study, it was not considered as a proper predictive marker.

The most effective extraction time according to the results was 30 minutes, as it was possible to obtain a thorough screening of the four classes of phenol compounds considered.

3.3. Step 3: volume of extraction solvent

The extraction solvent used was made of methanol, water, and HCL (95:4.5:0.5, v/v/v). Methanol is an organic solvent among the most widely used for polyphenol extraction, especially from plant matrices [50]. The combination of UAE and maceration was evaluated on table grape aimed at reducing the volume of methanol-based solvent making the extraction process more sustainable [51].

A recent work on *Crocus sativus* has shown the effectiveness of this combined method on petal extracts for the assessment of total phenolic content [52]. In this study on saffron, this hybrid approach was compared to a traditional maceration at 45 °C under magnetic stirring, using two different extraction solvents. The combined method via sonification produced an extract with a statistically higher total phenol content if compared to the method via maceration.

In the present study, 1 g of sample material was macerated overnight at room temperature testing different solvent volumes and further placed in an ultrasonic bath filled with distilled water. The sonification process took 30 minutes, which was the best extraction time as demonstrated in the second step. The phenol extraction efficiency was evaluated by comparing 10, 15, and 20 mL of organic solvent (Figure 5).
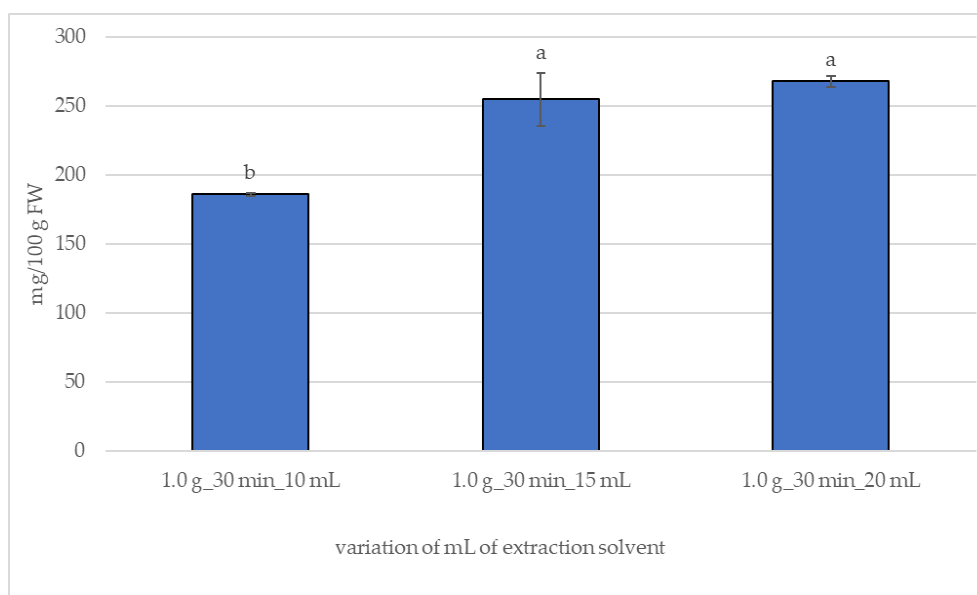

Figure 5. Influence of the different volumes of solvent on the extraction efficiency, expressed as the sum of the phenol classes considered for the analysis obtained by HPLC fingerprint. The results are reported as mg/100 g FW. Mean value and standard deviation are given for each sample (n = 3). Different letters for each groups indicate significant statistical differences ($p < 0.05$).

The significantly lower extraction yield was obtained using 10 mL of organic solvent (185.79 ± 1.30 mg/100 g FW). No statistical differences in the amount of total phenol compounds were found using 15 mL (254.67 ± 19.25 mg/100 g FW) or 20 mL (267.68 ± 3.91 mg/100 g FW). According to the proposed experimental protocol, the effective liquid/solid ratio for table grape is between 15 and 20 mL/g.
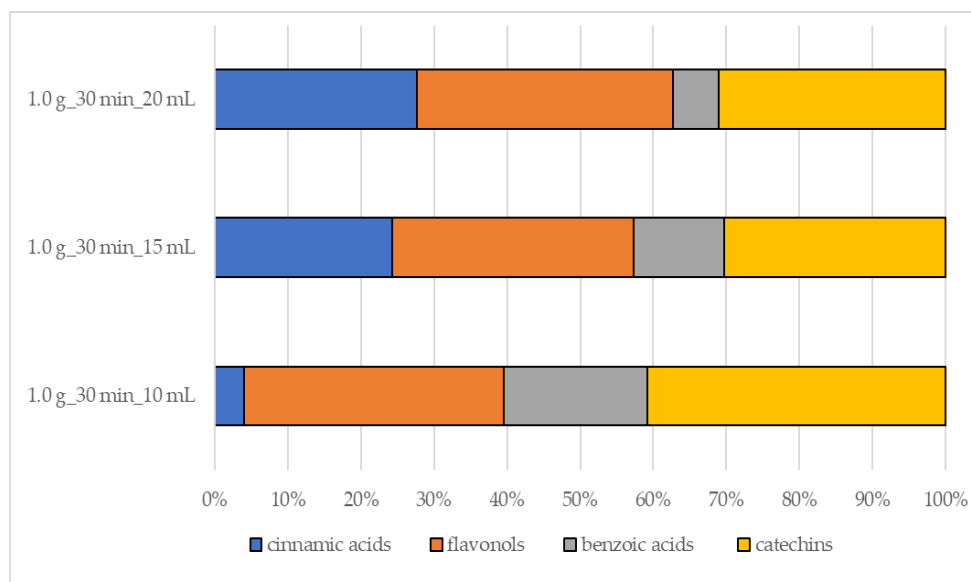

Figure 6. Phytochemical fingerprint according to the volume of solvent tested for the extraction of phenol compounds.

The use of 20 mL of extraction solvent provided the most effective and comprehensive results in terms of characterizing cinnamic acids, flavonols, and catechins. Nevertheless, there were no statistically significant differences with the results of using 15 mL. Due to the lack of works on table grape that focused on the expression of phenol compounds related to graft incompatibility, the main phenol classes involved in graft incompatibility according to earlier works on other fruit crops have been taken into account [7,20,37]. Considering the focus on multiple markers rather than on single/few specific compounds, 20 mL was proposed as the proper volume of organic solvent, since it gave the broader bands for the four phenolic classes analyzed.

The low yield of benzoic acids, and particularly the absence of gallic acid, could further support the compatibility between the genotypes analyzed in this study, given the well-recognized role of graft incompatibility marker on multiple fruit species of this compound [9,22].

Investigations on the level of extraction solvent should be deepened, since the results of this study suggest that it could be slightly reduced between 10 and 15 mL for certain phenol compounds without compromising the extraction potential. Lessening the quantity of organic solvent would be a great enhancement in terms of extraction process sustainability.

The identification of potential markers for graft compatibility, such as benzoic acids, catechins, and cinnamic acids, could help to identify new genotypes with high compatibility rates and resilience to environmental stressors such as climate change. The development of new, resilient rootstocks could lead to increased yields, improved quality, and reduced environmental impact, making viticulture more sustainable and profitable.

4. Conclusions

The present study is the first to address the matter of graft incompatibility in table grape from a biochemical perspective. In particular, the work focused on the development of a preliminary extraction method for phenol compounds in woody tissues. 1 g of plant material, 30 minutes of sonification, and a range between 15 and 20 mL of organic solvent resulted in the most efficient fingerprint, both quantitatively and qualitatively, with the four phenol classes significantly represented. The results provided a promising and sustainable methodology for the study of phenol compounds involved in the grafting process, which can be useful in exploring the interactions between new rootstock genotypes and table grape cultivars. Overall, the presence of the chlorogenic acid, whose biosynthesis seems to be stimulated in the case of compatible unions, coupled with the absence of the gallic acid, one of the most well-known markers of incompatibility, suggest the compatibility of the grafting combination tested. This work focused on three key parameters influencing the extraction of phenol compounds. Therefore, further studies are planned to validate and verify these findings, testing additional extraction variables and new grafting combinations with the aim of developing an optimized extraction protocol of polyphenols from table grape woody tissues.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

Authors contributions

Conceptualization, Giovanni Gamba, Dario Donno and Gabriele Beccaro; Data curation, Giovanni Gamba and Dario Donno; Formal analysis, Giovanni Gamba and Dario Donno; Investigation, Giovanni Gamba and Dario Donno; Methodology, Giovanni Gamba and Dario Donno; Resources, Giovanni Gamba; Supervision, Gabriele Beccaro; Validation, Paolo Sabbatini and Gabriele Beccaro; Writing—original draft, Giovanni Gamba; Writing—review & editing, Dario Donno, Zoarilala Razafindrakoto, Paolo Sabbatini and Gabriele Beccaro. All authors have read and agreed to the published version of the manuscript.

Supplementary

Table S1. Chromatographic conditions of the used methods.

Method	Compounds of Interest	Stationary Phase	Mobile Phase	Flow (mL min^{-1})	Wavelength (nm)
A	cinnamic acids, flavonols	KINETEX—C18 column (4.6 × 150 mm, 5 μm)	A: 10 mM KH_2PO_4/H_3PO_4, pH = 2.8 B: CH_3CN	1.5	330
B	benzoic acids, catechins	KINETEX—C18 column (4.6 × 150 mm, 5 μm)	A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1 v/v/v), pH = 2.5 B: $CH_3OH/HCOOH$ (100:0.1 v/v)	0.6	280

Method A—gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min (2 min conditioning time).

Method B—gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min (2 min conditioning time).

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