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Study of polyphenolic compounds in wines and different parts of the grapevine

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ABSTRACT

This paper describes research conducted on the polyphenolic compounds found in wine and different parts of the grapevine. The research consisted of two experiments. In the first, extracts of polyphenols from the leaves, stems, skins and seeds were measured. In the second, these parts were macerated and left in the must during fermentation. For this experiment, the Souvignier gris wine grape variety was used. In both cases, 33 polyphenolic compounds were measured. These measurements were made using the liquid chromatography–mass spectrometry (LC-MS) method. Based on the results, the individual concentrations of all the polyphenolic compounds in different parts of the plant were measured. Addition of the individual parts of the grapevine to the must during fermentation was shown to increase the concentration of the individual polyphenols in the wine. It is therefore important not to forget the importance of the stems and the maceration of the grapes during the winemaking process.

Keywords: LC-MS, maceration, skins, stems, Souvigner gris

INTRODUCTION

Phenolic substances are important in viticulture, winemaking and wine marketing (De la Cerda-Carrasco et al., 2015). They are responsible for the notable characteristics of the wine, in particular the way its flavour is expressed and its antioxidant properties (El Gharras, 2009). The main reason for an investigation of polyphenols in wine is their impact on human health. Polyphenols have several biological effects; they have antioxidant, antiviral and antibacterial properties. In addition, they protect against oxidative stress, thereby preventing the development of many diseases (Jordao et al., 2001; Liu and White, 2012). The flavonols contained in grape seeds affect the taste and colour of the wine and have attracted attention for their potential in the prevention of cancer (Jordao et al., 1998; Fontana et al., 2013; Chen et al., 2014).

During the fermentation of wine, phenolic compounds are transferred from the solid parts of the grape cluster, such as the skins, stems or seeds, into the wine. The amounts of these compounds that are transferred into the wine depend on the winemaking technique used. In addition to its positive impact on human health, fermentation helps to improve the overall character of the wine. The addition of wood, in the form of oak chips or barrel ageing, is also used in the winemaking process (Ribéreau-Gayon et al., 2017).

According to Sun et al. (1999), one way to increase the polyphenol content of wine is to add different parts of the plant to the must before fermentation. In their research, they stated that the seeds and stems are very good sources of catechins. When making wine that is intended to be archived for a longer period of time, they recommend that the stems be added before fermentation. When these parts are macerated, it is important to know that the process through which the individual phenolics

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begin to leach only begins once the alcohol content has slightly increased. This processing technique removes a small amount of liquid before fermentation, thus significantly increasing the concentration of solids in the must (Meyer and Hernandez, 1970; Kantz and Singleton, 1991; Sun et al., 1999).

The skins are slightly more important in terms of the ease with which polyphenols leach into the wine (Meyer and Hernandez, 1970; Kantz and Singleton, 1990, 1991). However, they contain a substantially lower concentration of polyphenols than, for instance, seeds or stems. These parts are the main sources of the polyphenolic substances in wine. The seeds contain the highest concentration of polyphenolic compounds, and the most common of these compounds is the group of flavan-3-ols, otherwise known as catechins, proanthocyanidins or condensed tannins (Singleton, 1991; Cheynier et al., 1997). There are also a considerable number of phenolic compounds in the stems; of these, about 40%–50% are in the form of polymers. In addition, wines made by macerating the stems have a higher content of both total phenols and polymeric phenols (Kantz and Singleton, 1990, 1991; Zeng, 2017). In some regions of the world, it is traditional to use the entire bunch of grapes. The stalk, if sufficiently mature, is considered a natural additive, bringing complexity, freshness and phenolic structure to the wine and facilitating its chemical stability during ageing (Hashizume et al., 1998).

This article is focused on the study of polyphenolic compounds in wine and different parts of the grapevine. Two experiments were carried out. The first involved the preparation of extracts from the leaf, seed, skin, stems and pulp. In the second experiment, the different parts were added to the wine before fermentation. The individual compounds were measured using liquid chromatography–mass spectrometry (LC-MS).

MATERIALS AND METHODS

Design of experiment

Two experiments were conducted during this research. One was intended to study polyphenolic compounds in parts of the grapevine (Figure 1). The other was focused on the fermentation of wine with the addition of the individual parts of the plant (Figure 2).

Extraction from parts of the grapevine

The Souvignier gris grapes were separated into their individual parts: seeds, skins, flesh and clusters. On the day of harvesting, leaves were also collected. The individual parts (from five bunches) were freeze-dried for 12 hr in a freeze-drier (model BK-FD18P vertical freeze–dryer; Biobase: Hangzhou, China). The seeds were then ground in a coffee grinder, and the leaves, stems and skin were crushed in a mortar.

A sample (1 g) of each of the single homogenised mixtures was weighed into a tube. A total of three tubes were filled for each variant (seeds, leaves, stems and skin).

The extraction was carried out using a pre-prepared methanol solution, prepared as follows: 175 mL of methanol (99.9%) and 0.025 mL of acetic acid (99.8%) were measured out into a container, and the total volume was made up to 250 mL with the addition of distilled water. Then, 10 mL of this methanol solution was added

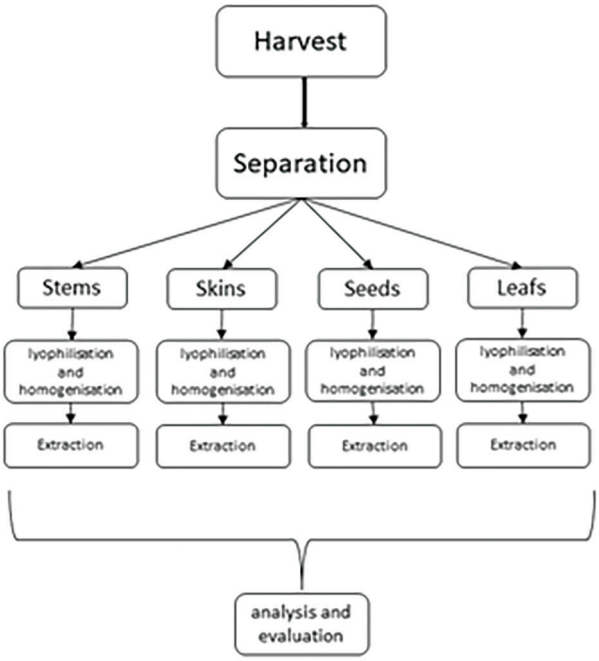

Figure 1. Scheme of the extraction methodology. After harvesting, separation of the leaves, seeds, skins and stems was carried out. Then, extraction with lyophilisation and homogenisation took place. Finally, the individual polyphenolic compounds were measured using LC-MS.
LC-MS, liquid chromatography–mass spectrometry.

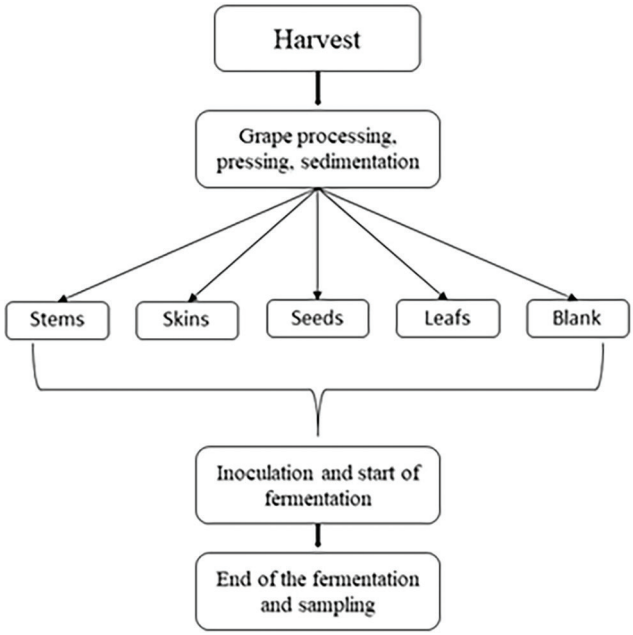

Figure 2. A schematic representation of the experiment with the addition of different plant materials before fermentation. After the processing of grapes, the must was taken away. Before fermentation, parts of the plant were added to each sample. After fermentation, the individual polyphenolic compounds were measured using LC-MS.
LC-MS, liquid chromatography–mass spectrometry.

to the sample tube and left to shake for 30 min (model ES-60+ incubator shaker; Miulab, Hangzhou, China) in an ultrasonic environment at a temperature of 50°C. This was then placed in a centrifuge for 4 min at 3000× *g* (model 5702 R; Eppendorf, Hamburg, Germany). Samples were collected for further experimental measurements.

Fermentation with parts of the grapevine

The Souvignier gris grape variety (Czech Republic; village: Lednice; vineyard: *Na valtické*) was used in the experiment. It was harvested on 21 October 2021. After pressing, the juice was left to settle naturally for 24 hr. Subsequently, the must was divided into fermentation vessels and was twice fermented using active dried wine yeast (*Institut Oenologique de Champagne*, Cedex, France). Each experimental variant was repeated three times. To each fermentation vessel, the following fresh parts of the grapevine were added: the leaves, stems, seeds and skins, with the last variant as the control. The concentration of each part was 72 g · L^{-1} seeds, 250 g · L^{-1} skins, 36 g· L^{-1} stems and 10 g · L^{-1} leaves. The quantities were calculated from the original quantity of grapes that were required to produce 1 L of must. After fermentation, samples were taken, and then the experiment was terminated (Table 1).

Table 1. Parameters of Souvignier gris.

Parameters of Souvignier gris	
Sugar	25°BX
Titratable acids	7.6 g · L^{-1}
pH	3.28
YAN	213 mg · L^{-1}
Village	Lednice

YAN, yeast assimilable nitrogen.

Methodology

Measurement of the individual phenolic compounds using LC-MS

The following polyphenol compounds were measured: myricetin, quercetin, quercetin-3-glucoside, quercetin-3-galactoside, rutin, kaempferol, kaempferol-3-D-glucoside, isorhamnetin, gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, syringic acid, caftaric acid, grape reaction product, caffeic acid, ethyl caffeate, coutaric acid, *p*-coumaric acid, ethyl coumarate, fertaric acid, ferulic acid, ethyl ferulate, *trans*-resveratrol, *cis*-resveratrol, *trans*-piceid, *cis*-piceid, piceatannol, astringin, catechin, epicatechin, epicatechin-3-gallate, procyanidin B1, procyanidin B2 and procyanidin C.

The concentration of each phenolic compound was determined through a direct injection method. The wines were placed in a centrifuge for 6 min at 3000× *g* and then prepared as follows: white wines: 500 µL of wine, 20 µL of internal standard solution and 480 µL of 10% HCOOH; extracts: 200 µL of extract, 20 µL of internal standard solution and 780 µL of 10% HCOOH. After this treatment, the extracts were again run through the centrifuge and 750 µL of wine was transferred to a vial that was placed in an autosampler for analysis. The internal standard solution used was 2 mM α-cyano-4-hydroxycinnamic acid (HCCA), 2 mM Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Instrumentation: ExionL™ AC binary high-pressure system, which consists of an on-line degasser, two pumps, an autosampler, column thermostat and control unit with a Sciex QTrap 3200 detector, was used. The separation conditions were as follows: separation temperature: 60°C, sample injection volume: 5 µL, mobile phase flow rate: 0.3 µL · min^{-1}, mobile phase A: 1% HCOOH in water, mobile phase B: 1% HCOOH in acetonitrile, column: Arion Polar C18 2.2 µm; 2.1 × 100 mm.

The gradient programme was as follows:

0.00 min: 6% B
6.00 min: 9% B
9.00 min: 12% B
12.00 min: 18% B
15.00 min: 30% B
18.00 min: 60% B
19.00 min: 60% B
19.01 min: 0% B
20.99 min: 0% B
21.00 min: 6% B

The total time between samples was 24 min. The mass detector recorded the analyser output from 0.9 min to 20 min. The determination of the individual components was based on the calibration curves of the standards.

Detector settings

The electrospray ionisation (ESI) source was set in negative mode with an ionisation voltage of -4200 V, the curtain gas was set at 310.26 kPa, the collision-activated dissociation gas (CAD) at medium, the nebuliser gas GS1 at 413.68 kPa, the turbo gas GS2 at 45 psig and a desolvation temperature of 873.15°K. The 3200QTrap™ was operated in the multiple reaction monitoring mode with the settings shown in Table 2.

The parameter settings for the MS/MS transitions are listed in the following table, using the parameters listed in the header.

The determination of basic analytical parameters

The basic parameters (sugar content, titratable acidity and pH) of the resulting wine or must were determined using an Alpha Fourier-transform infrared spectroscopy (FTIR) analyser (Bruker, Karlsruhe, Germany) using the attenuated total reflection sampling technique. Prior to the first measurement, the spectrometer was thoroughly rinsed with deionised water and the background levels were determined using a blank sample (deionised water). For the analyses, 1 mL samples were taken using a syringe; 0.5 mL was used to rinse the system, and the remaining 0.5 mL was analysed three times. The measured values were evaluated automatically using the Opuswin software system (Opuswin Technology and Systems, Lagos, Nigeria).

Statistical methods

Each variant was prepared in triplicate and each sample was measured three times. The arithmetic mean with standard deviation was calculated from the values using STATISTICA 14 statistical software (Tibco, Palo Alto, CA 94304, USA). Further, one-way analysis of variance (ANOVA) was used to evaluate the experimental data, with significant differences between the means set at $p \leq 0.05$. Tukey's honestly significant difference (HSD) post-hoc test was used to determine similar and dissimilar levels of factors (groups). The results are presented as tables in the 'Results and Discussion' section.

RESULTS AND DISCUSSION

In both experiments, the quantities of the 33 polyphenolic compounds were measured. We selected the most important ones found in wine and grapevines. The concentration of each phenolic compound was determined by a previously unpublished direct injection method.

Extracts of parts of the grapevine

The raw material used in the experiment was Souvignier gris. It was divided into the following parts: seeds, skins, pulp and stems. On the day of harvesting, leaves were also added to these samples. The raw material was dried and divided into samples as per the methodology described. After sample preparation, the levels of the individual polyphenols were measured by LC-MS. There were three replicas of each variant, and each variant was subsequently measured three times. The results were averaged and the standard deviation was calculated (see Table 3).

Significant differences were measured in the experimental extracts from different parts of the grapevine. Caftaric acid and quercetin-3-glucoside were the most abundant compounds found in the leaves (Table 3). The values of these two compounds were considerably higher than for any of the others. They were followed by kaempferol-3-D-glucoside and coutaric acid. Myricetin, ethyl coumarate, ethyl caffeate and isorhamnetin were found to have the lowest levels of the polyphenols measured. As for the skins, the main polyphenol found was quercetin-3-glucoside (Table 2). In the case of the skins, a substantially greater difference was seen between the concentrations of the most abundant polyphenols. Caftaric acid was found at the second highest concentration. Similar results were found in the experiment by Ostroukhova et al. (2016). In their experiment, quercetin, kaempferol and their glycosides dominated in the leaves (Ostroukhova et al., 2016). No value was measurable for the polyphenols myricetin, vanillic acid, syringic acid, ethyl caffeate, ethyl coumarate and piceatannol. In the seeds, two substances predominated, namely catechin and epicatechin; they only displayed minimal differences in concentration. It is worth mentioning that procyanidin B1 had the third highest concentration. However, this is not the case with the stems, wherein procyanidin B1 was the most dominant. Once again, for the stems, we can see a considerable difference between the polyphenols.

Table 2. Detector settings

Q1	Q3	DT (ms)	Compound name	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
169.0	125.0	10	Gallic acid	-42.0	-9.2	-5.1	-21.9	-0.3
153.0	109.0	10	Protocatechuic acid	-40.3	-9.2	-6.8	-21.4	-0.1
137.0	93.0	10	4-Hydroxybenzoic acid	-35.4	-8.7	-6.8	-22.6	-0.3
167.0	152.0	10	Vanillic acid	-33.0	-9.9	-4.9	-20.0	-0.6
197.0	123.0	10	Syringic acid	-32.1	-10.2	-6.1	-33.6	-0.3
311.0	179.0	10	Caftaric acid	-34.3	-4.2	-9.1	-22.2	-0.3
616.1	149.0	10	Grape reaction product	-63.0	-6.0	-18.0	-48.0	-0.9
179.0	135.0	10	Caffeic acid	-38.9	-10.7	-9.8	-24.2	-0.5
207.0	135.0	10	Ethyl caffeate	-55.0	-4.8	-11.0	-32.0	-0.6
295.0	163.0	10	Coutaric acid	-41.0	-4.1	-16.5	-22.0	-0.9
163.0	119.0	10	*p*-Coumaric acid	-37.5	-8.7	-8.8	-22.5	-0.3
191.0	117.0	10	Ethyl coumarate	-50.0	-4.2	-9.0	-42.0	-1.0
325.0	193.0	10	Fertaric acid	-44.0	-3.3	-9.0	-23.0	-0.3
193.0	134.0	10	Ferulic acid	-40.0	-10.2	-5.9	-24.4	-0.5
221.0	206.0	10	Ethyl ferulate	-55.0	-7.9	-15.2	-25.0	-0.5
227.0	143.0	10	Resveratrol	-70.0	-10.6	-11.7	-38.2	-0.7
389.0	227.0	10	Piceid	-70.0	-10.0	-17.3	-28.1	-0.3
243.0	159.0	10	Piceatannol	-70.0	-10.0	-7.2	-39.1	-0.6
405.0	243.0	10	Astringin	-63.0	-5.6	-20.0	-31.0	-0.3
289.0	123.0	10	Catechin	-60.0	-9.9	-9.5	-44.0	-0.3
317.0	151.0	10	Myricetin	-74.0	-4.0	-9.5	-36.3	-0.6
301.0	179.0	10	Quercetin	-68.0	-7.0	-8.2	-29.0	-0.3
463.0	300.0	10	Quercetin-3-glycosides	-81.0	-4.0	-13.0	-41.0	-0.9
609.0	300.0	10	Rutin	-93.0	-4.3	-17.0	-54.0	-0.9
285.0	285.0	10	Kaempferol	-120.0	-8.8	-16.0	-17.0	-0.5
315.0	300.0	10	Isorhamnetin	-74.0	-5.0	-19.0	-36.0	-0.3
447.0	284.0	10	Kaempferol-3-D-glucoside	-78.0	-4.2	-12.0	-40.0	-0.6
441.1	169.0	10	Epicatechin-3-gallate	-64.0	-3.8	-12.0	-31.0	-0.3
577.1	289.0	10	Procyanidin B	-60.0	-4.0	-15.0	-37.0	-0.7
865.2	289.0	10	Procyanidin C	-80.0	-7.5	-25.0	-55.0	-0.6
188.0	93.0	10	α-Cyano-4-hydroxycinnamic acid	-36.0	-9.4	-10.7	-32.6	-0.1
249.0	205.0	10	Trolox	-58.0	-10.0	-7.2	-26.6	-0.3

Q1 and Q3 are the parent and daughter ions.
DT, dwell time, DP, declustering potential, EP, entrance potential, CEP, collision cell entry potential, CE, collision energy, CXP, collision cell exit potential.

Catechin was the second most abundant and the third was *trans*-resveratrol. Similar results were presented by Souquet et al. (2000), who conducted similar experimental work and measured the extracts. They confirmed that the seed was the main source of catechins and epicatechins and reported that stems came in second place. For comparison with the merlot variety, the value of catechins was 60 mg · kg^{-1} (Souquet et al., 2000). From the results, it can be seen that the main source of *trans*-resveratrol is the stem (Table 1). Ethyl coumarate was not measured in any of the parts of the grapevine tested. A study by Rothwell et al. (2005) claimed that the largest source of quercetin and its compounds is the stem. This claim was not confirmed by our results (Rothwell et al., 2005).

Boso et al. (2019) performed an experiment in which they focused on flavanols in different parts in Albarino and Mencía varieties of grapes. The Albarino variety had the highest level of flavanols in the stems, followed by the seeds. With regard to polyphenols in the stems, this variety had the highest level of catechins, followed by procyanidin B1; in our results, they came in the other way around. Their findings for the seeds were remarkably similar to ours, with catechin and epicatechin being the most abundant. A match was also found for procyanidin B1 in the skins;

Table 3. The measured results for each polyphenolic substance from different parts of the grapevine.

	Leaves (mg · L^{-1})	Skins (mg · L^{-1})	Seeds (mg · L^{-1})	Stems (mg · L^{-1})	*p*-value
Myricetin	LOD	LOD	0.24 ± 0.033 b	LOD	0.001832
Quercetin	12.02 ± 3.50 d	9.18 ± 0.48 c	0.21 ± 0.053 a	3.07 ± 0.178 b	0.000000
Quercetin-3-glucoside	1586.05 ± 195.78 d	1093.44 ± 46.97 c	10.92 ± 0.920 a	240.15 ± 10.32 b	0.000000
Quercetin-3-galactoside	170.04 ± 33.81 c	37.24 ± 1.91 b	0.18 ± 0.022 a	13.88 ± 0.33 a	0.000039
Rutin	62.75 ± 13.36 d	20.76 ± 1.01 b	0.21 ± 0.032 a	47.67 ± 2.31 c	0.000000
Kaempferol	5.11 ± 1.04 c	1.09 ± 0.16 b	0.13 ± 0.088 a	0.66 ± 0.098 ab	0.000014
Kaempferol-3-D-glucoside	698.54 ± 95.79 b	57.82 ± 4.32 a	0.25 ± 0.018 a	25.87 ± 1.02 a	0.000000
Isorhamnetin	0.113 ± 0.08 a	0.91 ± 0.08 c	0.10 ± 0.087 a	0.224 ± 0.082 b	0.000001
Gallic acid	5.50 ± 0.77 a	2.14 ± 0.17 a	223.13 ± 21.73 c	32.91 ± 2.27 b	0.000160
Protocatechuic acid	7.03 ± 1.60 a	8.46 ± 1.03 ab	11.13 ± 0.745 c	9.76 ± 1.23 bc	0.000000
4-Hydroxybenzoic acid	31.43 ± 7.35 b	1.74 ± 0.48 a	1.13 ± 0.216 a	51.82 ± 4.92 c	0.000001
Vanillic acid	0.176 ± 0.50 a	LOD	LOD	0.000 ± 0.000 a	0.324174
Caftaric acid	1975.65 ± 287.12 d	403.73 ± 30.41 b	9.35 ± 0.934 a	684.42 ± 58.49 c	0.000001
Grape reaction product	21.26 ± 5.43 c	10.25 ± 1.050 a	0.016 ± 0.044 b	13.98 ± 0.93 a	0.000000
Caffeic acid	12.20 ± 2.57 c	1.28 ± 0.08 a	0.85 ± 0.103 a	3.75 ± 0.29 b	0.000002
Ethyl caffeate	0.102 ± 0.08 b	0.002 ± 0.007 a	0.002 ± 0.005 a	0.059 ± 0.039 ab	0.000251
Coutaric acid	479.01 ± 93.55 d	223.19 ± 15.22 b	25.01 ± 2.070 a	341.44 ± 29.60 c	0.000000
p-Coumaric acid	4.27 ± 0.74 d	0.50 ± 0.07 a	3.48 ± 0.172 c	2.44 ± 0.24 b	0.000000
Ethyl coumarate	LOD	LOD	0.004 ± 0.010 a	LOD	0.324174
Fertaric acid	76.06 ± 20.50 d	19.64 ± 1.21 b	1.00 ± 0.114 a	53.42 ± 3.48 c	0.000000
Ferulic acid	1.85 ± 0.65 b	1.03 ± 0.14 a	0.87 ± 0.157 a	2.65 ± 0.27 c	0.000000
Ethyl ferulate	9.42 ± 5.23 b	3.60 ± 2.33 a	3.61 ± 1.114 a	4.59 ± 2.19 a	0.000000
Trans-resveratrol	19.83 ± 2.57 a	4.28 ± 0.47 a	0.77 ± 0.158 a	446.94 ± 59.15 b	0.000903
Cis-resveratrol	12.33 ± 4.56 c	1.16 ± 0.23 a	LOD	6.07 ± 0.830 b	0.000005
Trans-piceid	59.49 ± 12.95 b	4.17 ± 0.44 a	3.26 ± 0.245 a	65.31 ± 6.58 b	0.000000
Cis-piceid	186.94 ± 39.45 c	6.28 ± 0.55 a	0.09 ± 0.022 a	33.07 ± 2.24 b	0.000151
Piceatannol	1.35 ± 0.55 a	0.04 ± 0.08 a	LOD	69.08 ± 6.76 b	0.001344
Astringin	7.38 ± 2.36 b	0.10 ± 0.12 a	0.296 ± 0.088 a	10.50 ± 1.08 c	0.000001
Catechin	187.73 ± 34.56 b	13.50 ± 0.67 a	1423.37 ± 91.967 d	1175.31 ± 109.86 c	0.000000
Epicatechin	15.98 ± 5.20 a	8.56 ± 0.56 a	1407.11 ± 83.606 c	135.47 ± 24.64 b	0.000384
Epicatechin-3-gallate	2.77 ± 0.75 a	0.16 ± 0.07 a	57.41 ± 4.970 b	118.90 ± 23.12 c	0.000006
Procyanidin B1	95.91 ± 22.18 a	34.51 ± 3.64 a	365.06 ± 42.64 b	3091.25 ± 174.01 c	0.000198
Procyanidin B2	19.48 ± 5.14 a	8.03 ± 1.05 a	611.43 ± 39.36 c	157.25 ± 21.89 b	0.000032
Procyanidin C	9.28 ± 2.03 a	4.76 ± 0.51 a	383.95 ± 39.36 c	287.76 ± 36.08 b	0.000001

Results are expressed as the mean value of nine measurements ± standard deviation; values are the result of ANOVA. The division into homogeneous groups (a, b, c, d) was based on Tukey's test, and the significance level was $\alpha = 0.05$.
ANOVA, analysis of variance; LOD, limit of detection.

it was at the highest level in both experiments (Boso et al., 2019).

Jara-Palacios et al. (2014) did similar research, and we have confirmed their results. They performed their work on the Zalema grape variety. They measured 31 polyphenolic compounds in different parts of the fruit (seeds, flesh, stems and skins) through rapid resolution LC (RRLC)/MS. They reported that flavanols were the most abundant phenolics found, with concentrations in the range of 121–613 mg · 100 g^{-1} dry weight, followed by flavanols (8–146 mg · 100 g^{-1}) and phenolic acids (9–27 mg · 100 g^{-1}). The highest levels of flavanols were found in the seeds (613 mg · 100 g^{-1}), followed by the stems (348 mg · 100 g^{-1}) and the pomace (282 mg · 100 g^{-1}), while the skins had the lowest concentration (122 mg · 100 g^{-1}). In contrast, the flavanols were most abundant in the skins and pomace (146 and 144 mg · 100 g^{-1}, respectively) with no significant differences between them, whereas the seeds were low in these compounds (8 mg · 100 g^{-1}). The derivatives of

Table 4. Results of the measurement of polyphenols in wines, fermented with the individual parts of the grapevine.

	Skins (mg · L^{-1})	Stems (mg · L^{-1})	Leaves (mg · L^{-1})	Seeds (mg · L^{-1})	Control (mg · L^{-1})	*p*-value
Myricetin	0.002 ± 0.004 a	LOD	LOD	LOD	LOD	0.325582
Quercetin	0.035 ± 0.008 b	0.037 ± 0.008 a	0.021 ± 0.009 c	0.168 ± 0.023 d	0.002 ± 0.004 a	0.000081
Quercetin-3-glucoside	0.346 ± 0.038 b	0.099 ± 0.007 a	0.094 ± 0.013 a	0.059 ± 0.005 c	0.016 ± 0.004 d	0.000004
Quercetin-3-galactoside	0.037 ± 0.002 ab	0.027 ± 0.001 ab	0.036 ± 0.012 a	0.020 ± 0.002 b	0.034 ± 0.011 a	0.000000
Kaempferol-3-D-glucoside	0.014 ± 0.001 a	0.004 ± 0.001 a	0.005 ± 0.001 a	0.003 ± 0.001 a	0.003 ± 0.003 b	0.000000
Isorhamnetin	LOD	LOD	LOD	0.031 ± 0.006 b	0.003 ± 0.007 a	0.007477
Rutin	0.001 ± 0.002 b	0.002 ± 0.002 ab	0.003 ± 0.002 ab	0.003 ± 0.002 ab	0.004 ± 0.001 a	0.000000
Gallic acid	10.656 ± 1.483 a	5.259 ± 0.623 b	0.079 ± 0.025 a	31.48 ± 0.65 d	0.165 ± 0.065 c	0.000139
Protocatechuic acid	3.358 ± 0.091 a	2.090 ± 0.178 b	2.926 ± 0.299 c	1.679 ± 0.025 a	1.638 ± 0.100 d	0.000000
4-Hydroxybenzoic acid	0.709 ± 0.015 a	0.611 ± 0.022 b	0.869 ± 0.036 d	0.405 ± 0.008 a	0.432 ± 0.012 c	0.000000
Vanillic acid	0.424 ± 0.030 ab	0.360 ± 0.021 b	0.352 ± 0.027 ab	0.310 ± 0.017 a	0.335 ± 0.024 c	0.000000
Syringic acid	0.101 ± 0.009 a	0.140 ± 0.015 d	LOD	0.088 ± 0.013 ab	0.072 ± 0.014 b	0.000000
Caftaric acid	35.14 ± 2.90 ab	44.56 ± 3.38 c	4.339 ± 0.474 d	42.14 ± 0.77 bc	38.43 ± 1.41 a	0.000000
Grape reaction product	0.527 ± 0.090 b	5.376 ± 0.987 d	0.189 ± 0.021 a	3.996 ± 0.249 c	2.950 ± 0.206 a	0.000000
Caffeic acid	2.498 ± 0.215 b	1.172 ± 0.117 d	0.120 ± 0.017 a	0.904 ± 0.044 c	0.410 ± 0.034 e	0.000000
Ethyl caffeate	1.861 ± 0.048 c	0.572 ± 0.063 a	0.065 ± 0.007 b	0.509 ± 0.029 a	0.162 ± 0.006 d	0.000010
Coutaric acid	12.82 ± 1.23 a	9.37 ± 0.18 b	5.501 ± 0.038 a	9.89 ± 0.13 b	5.05 ± 0.06 c	0.000000
p-Coumaric acid	1.188 ± 0.052 a	0.954 ± 0.013 d	0.524 ± 0.067 c	0.325 ± 0.010 b	0.211 ± 0.018 e	0.000000
Ethyl coumarate	1.135 ± 0.035 a	0.416 ± 0.022 d	0.247 ± 0.020 c	0.167 ± 0.010 b	0.089 ± 0.007 e	0.000002
Fertaric acid	3.046 ± 0.223 a	3.88 ± 0.33 c	0.865 ± 0.054 b	5.67 ± 0.10 d	2.86 ± 0.05 a	0.000000
Ferulic acid	0.635 ± 0.027 b	0.492 ± 0.029 d	0.040 ± 0.002 a	0.330 ± 0.013 c	0.260 ± 0.036 e	0.000000
Ethyl ferulate	0.022 ± 0.022 ab	0.003 ± 0.007 ab	LOD	0.002 ± 0.006 a	0.003 ± 0.006 b	0.034311
Trans-resveratrol	0.271 ± 0.055 a	1.999 ± 0.110 c	0.014 ± 0.015 a	0.248 ± 0.037 b	0.088 ± 0.019 b	0.000707
Cis-resveratrol	2.621 ± 0.336 c	1.038 ± 0.049 a	0.049 ± 0.021 b	0.833 ± 0.027 a	0.403 ± 0.102 d	0.000002
Trans-piceid	0.171 ± 0.011 a	0.491 ± 0.019 a	0.088 ± 0.012 b	0.402 ± 0.008 d	0.541 ± 0.095 c	0.000000
Cis-piceid	0.772 ± 0.063 b	0.634 ± 0.026 a	0.046 ± 0.021 c	1.114 ± 0.031 b	0.965 ± 0.205 a	0.000000
Astringin	0.004 ± 0.005 a	0.001 ± 0.001 a	0.001 ± 0.002 a	0.002 ± 0.003 a	LOD	0.009305
Catechin	29.30 ± 3.31 a	12.37 ± 1.54 b	0.119 ± 0.092 a	107.08 ± 1.70 d	0.342 ± 0.029 c	0.000379
Epicatechin	19.24 ± 2.30 a	1.778 ± 0.689 a	0.053 ± 0.036 a	64.64 ± 0.77 c	0.111 ± 0.017 b	0.000836
Epicatechin-3-gallate	0.041 ± 0.007 a	0.024 ± 0.005 a	LOD	1.084 ± 0.057 b	LOD	0.007249
Procyanidin B1	16.56 ± 2.690 a	17.13 ± 1.13 b	0.031 ± 0.026 a	95.37 ± 2.12 c	0.012 ± 0.017 b	0.000526
Procyanidin B2	11.80 ± 2.00 a	2.174 ± 0.454 b	0.020 ± 0.020 a	79.40 ± 0.94 d	0.025 ± 0.019 c	0.002721
Procyanidin C	6.46 ± 0.989 a	1.336 ± 0.302 a	LOD	66.23 ± 2.84 c	LOD	0.004521

Results (measured using LC-MS) are expressed as the mean value of nine measurements ± standard deviation, values are the result of ANOVA. The division into homogeneous groups (a, b, c, d) was based on Tukey's test, the significance level is $\alpha = 0.05$.
ANOVA, analysis of variance; LC-MS, liquid chromatography–mass spectrometry; LOD, limit of detection.

phenolic acid were minority compounds in the four by-products. The main flavanols in the by-products were the quercetin glycosides, with quercetin 3-*O*-glucoside and quercetin 3-*O*-glucuronide accounting for 32%–42% and 35%–37% of the total flavanol content, respectively. Both compounds were more abundant in the pomace and skins (51–58 mg · 100 g^{-1}) than in the stem (25–27 mg · 100 g^{-1}) or seed extracts (around 3 mg · 100 g^{-1}) (Jara-Palacios et al., 2014).

The influence of maceration of the plant parts on the polyphenol content of the resulting wine

The Souvigner gris variety was used for this experiment. The grapes were handpicked and processed. After pressing, the must was allowed to settle naturally, and then the individual parts of the plant were added. These were the skins, seeds, leaves and stems. The must had not fermented and fermentation started spontaneously. Fermentation lasted for 14 days. After fermentation, samples were taken, and the individual polyphenolic compounds were subsequently measured by LC-MS. Three replicates of each variant were produced, and each variant was measured three times. The results were averaged, and the standard deviation was calculated. In addition to the above variants, measurements were also taken for the wine as a control (Table 4).

According to Table 4, for the variant with the addition of skins, caftaric acid was found to be the most abundant, followed by coutaric acid. The levels of kaempferol, myricetin, ethyl coumarate, piceatannol, astringin and procyanidin C were insignificant. Very similar results were obtained for the variant that had leaves added to the must. Again, here, the most dominant polyphenolic compound was caftaric acid. No detectable levels of concentration were measured for myricetin, kaempferol, isorhamnetin, syringic acid, piceatannol and procyanidin C. It is interesting to note that for the variant that contained the skins, quercetin-3-glucoside and caftaric acid were dominant in the extracts, but in the wine, this dominance was not apparent. Caftaric acid is still the most abundant substance, but it is not at as high a level as it was in the extracts. In terms of the results, we can say that there are only three compounds that were not present: kaempferol, isorhamnetin and piceatannol. For the version with added seeds, on the other hand, the situation is different. According to Tables 3 and 4, it is clear that the main compounds in both the experimental extract and the experimental wine are similar. They were mainly catechin, epicatechin, procyanidin B1 and procyanidin B2. The level of catechin found was several times greater than was found in the other variants. Kaempferol and piceatannol are two polyphenolic substances that were not measured in this variant. In the stems, the substance with the highest concentration was caftaric acid. There is a considerable difference between the concentrations of the most abundant substance versus the second most abundant. As was the case with the extracts, catechin and procyanidin B1 were the predominant substances found. For some substances, such as myricetin, kaempferol, isorhamnetin and piceatannol, no detectable levels were found. These findings confirm a study by Puskás et al. (2010), which reported that the addition of seeds increases the total content of polyphenolic substances. According to that study, the addition of 50% of the picked stems during processing increases the number of phenolic compounds by 60% (Puskás et al., 2010).

In 1999, Sun et al. (1999) conducted an experiment where they attempted to establish the percentage of polyphenolic compounds that could be released into the wine from the solid parts of the plant. They found that between 45% and 50% of the total catechins entered the wine from the seeds. We agree with their results regarding the release of polyphenolic compounds from the skins. The skins contain much lower levels of these substances, but they enter the wine to a much greater extent than from the stems. Our study confirms these results (Sun et al., 1999).

Another interesting study was done by Revilla et al. (1997). They found that the extraction rate of catechins and procyanidins can be considered to be linear during the first 7 days after the start of maceration. In the case of merlot, the catechin and procyanidin contents of the Me-2 wine was approximately 25% higher than in the Me-1 wine. The maceration took the same length of time (92 hr), but the quantity of seeds in contact with the must was 25% higher for the Me-2 wine in comparison to the Me-1 wine. The addition of more pomace led to a higher total phenolic content in the free run wines from the Me-B, Me-C and Fr-B trials than in the control merlot and frankinja wines. As expected, the total content of phenolics in the pressed wines (Me-4 and Fr-3) was higher than in the corresponding free run wines (Me-2 and Me-3 for Merlot and Fr-2 for Frankinja). The total levels of phenolics in the coupage wines (Me-5 and Fr-5) were fairly similar to what was expected given the total phenolic content of the wines (Revilla et al., 1997).

CONCLUSION

In these two experiments, 33 polyphenolic compounds were measured using LC-MS. Higher levels of polyphenolic compounds were found to be naturally present in the extracts. It can be argued that for each part of the plant, a different substance dominated. As far as the experiment with the extracts was concerned, the most abundant substance found was procyanidin B1, specifically in the stems. In addition, it can be argued that it was also the most dominant substance overall in the plant. Other significant substances were quercetin-3-glucoside, caftaric acid, catechin and epicatechin. A polyphenol we tested for, namely syringic acid, was not found in any measurable quantity in any of the plant parts. The highest polyphenol content was found in the seed variant, with catechin as the most abundant. The lowest values of polyphenols were found in the control and the leaf variant. It can also be argued that leaching from the stems is substantially weaker than from the seeds. This phenomenon can mostly be seen for catechin and epicatechin. Considering the results, it is clear that there are many ways to increase the polyphenol content of wine. If necessary, the stems can be used to increase certain polyphenols and to change the sensory

properties. From past experiences, we know that this can also be done for certain styles of wine. This experiment also demonstrates the importance of maceration in varieties of white wine.

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AUTHOR CONTRIBUTIONS

J.H. prepared the paper and compiled the results from the experiments performed. K.K. prepared experimental samples and evaluated the results. J.S. conducted the experiments and was responsible for the entire article. M.K. was responsible for all the measurements and methods. M.B. supervised the entire project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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