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► **To cite this version:**

Isaac Benito-González, Mahrokh Ebrahimi, María del Mar Ortiz-Gimeno, Le Vinh Hoang, Victor Acha, et al.. Ozonation of vine shoots as a source of lignocellulosic phenolic-rich fractions. *Cellulose*, 2023, 30, pp.10795 - 10810. 10.1007/s10570-023-05558-1 . hal-04309864

HAL Id: hal-04309864

<https://hal.science/hal-04309864v1>

Submitted on 16 May 2024

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Ozonation of vine shoots as a source of lignocellulosic phenolic-rich fractions

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Received: 26 July 2023 / Accepted: 10 October 2023 / Published online: 25 October 2023
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Abstract Ozonation is a barely explored methodology proposed in the present work as an alternative and sustainable method for the valorization of vine shoots' from two grape varieties (*Tempranillo* and *Verdejo*). For this aim, several phenolic-rich fractions have been obtained from the vine shoots via ozonation by the modification of two parameters: time (10, 30, and 60 min) and pH (10, 7, and 3). An additional control fraction was obtained for both varieties via a conventional chemical lignin extraction method (acid hydrolysis with NaClO₂) for comparison. All fractions were characterized by means of their yield,

composition (phenolic (2–14%), monosaccharides ($\leq 13\%$) and ash (majoritarian) contents) and antioxidant capacity (30–129 $\mu\text{mol TE/g}$ fraction). In addition, a semi-quantitative analysis of the fractions was carried out derived from their FT-IR and TGA spectra, showing significant differences between times and specially pHs. These results confirm ozonation as a promising and greener alternative for vine shoots' valorization and could be extended to other lignocellulosic waste biomass sources for obtaining novel phenolic-rich fractions.

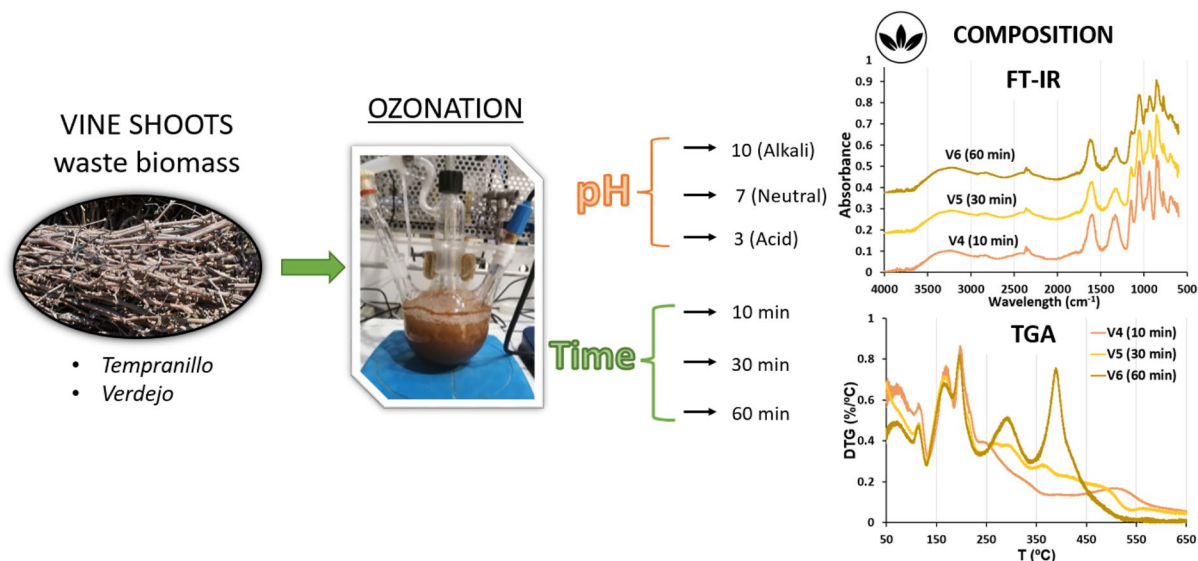
Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10570-023-05558-1>.

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Graphical abstract



Keywords Ozonation · Vine shoots · Lignocellulose · pH · Antioxidant capacity

Introduction

Antioxidants are used to maintain and protect foods from oxidation-induced deterioration and are important for health improvement and disease prevention (Figueiredo-Gonzalez et al. 2016). The search for new sources of natural antioxidants has also led to the exploration of agro industrial wastes (Buranov and Mazza 2008) as they are sources of phenolic compounds, often found in bound form, ester linked to lignocellulosic components of the cell wall (Moung-uengui et al. 2016). In this sense, lignin is the most abundant source of natural phenols with a complex three-dimensional structure that is found in the cell walls of plants (de Melo et al. 2020; Lu et al. 2022). It is made of phenylpropane units named as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) linked by various C–C and C–O interunit linkages, such as β-aryl ether (β-O-4), α-aryl ether (α-O-4), 1,2-diaryl propane (β-1), phenyl coumarin (β-5), biphenyl (5–5, α-1) and diphenyl ether (5-O-4) linkages by β-O-4 bonds being the most abundant in hardwoods (Martínez-Abad et al. 2018; Ralph et al. 2019).

In this sense, lignin can be extracted as a side stream (where cellulose is the main objective) from conventional pulp making processes such as Kraft, sulphite and organosolv treatments due to their cost-efficiency, rapidness and scalability. However, greener and less aggressive alternatives could be used to valorise lignocellulosic biomass for obtaining lignin-rich extracts due to the high amount of downstream chemical residues generated in the aforementioned treatments (Bajpai and Bajpai 1992).

Ozone as a powerful oxidizing agent ($E^\circ = 2.07$ V) can be used to degrade lignin into low molecular weight compounds (Travaini et al. 2016). This degradation is essentially limited to lignin, with hemicelluloses being slightly attacked and cellulose barely affected. As a result, ozonation has the following advantages: (1) it produces limited inhibitory compounds such as furfural or HMF (hydroxymethyl furfural) for downstream processes (Travaini et al. 2015) (2) the reactions are carried out at room temperature and atmospheric pressure; and (3) the generation can be done on site which eliminates chemical supply and storage problems. Ozonation is considered among the greenest methodologies by not leaving any chemical residue (Sun and Cheng 2002). Also, ozone has relatively low productions costs (11–13 kWh/kg), when compared to other well-known alternatives:

Enzymatic can be considered as another green but less efficient alternative due to the required pre-treatments involved (hydrothermal, mechanical or chemical) ranging from 5 to 66 kWh/kg (Licari et al. 2016; Phojaroen et al. 2022). Chemical treatments such as NaOH coupled with vibrational ball milling is able to reduce the production costs (1.42–8.37 kWh/kg) but possess severe residues in the downstream process which require further neutralization (Loustau-Cazalet et al. 2016). Meanwhile, the application of organosolv treatments (i.e. ethanol) has been shown to increase the production costs for lignocellulosic biomass conversion (29.02 kWh/kg) (da Silva et al. 2018).

The use of ozone for pre-treating lignocellulosic biomass such as wheat straw to produce fermentable sugars has already been demonstrated (Bule et al. 2013). However, although biomass sources are still widely available, very little literature has been published on the valorisation of lignocellulosic biomass through ozonation as a novel application (Travaini et al. 2016). In particular, vine shoots represent an abundant agricultural residue derived from the wine industry (with approximately 2.5 million tons of vine shoots generated every year in Spain), and it is in fact extensively grown in Southwest regions of Europe such as France and Spain (Mediavilla et al. 2009). Due to the large costs associated to their management, vine shoots have been traditionally used as fertilizers when directly left at the vineyard or burned

after grape harvest (September–October), which is the time when vine shoots are usually collected from the vineyards. However, burning practices pose severe environmental issues and strict restrictions are applicable in most wine-producing countries (Jenkins et al. 1996). Vine shoots have been studied as a promising source of cellulose (for the production of pulp and paper) due to their high lignocellulosic content (> 90%) (Jiménez et al. 2009; Benito-González et al. 2020). The fractionation of this lignocellulosic structure has been extensively studied by using various methodologies such as organosolv processing, microwave heating or autohydrolysis for further valorisation of their single compounds (i.e. lignin, cellulose and hemicelluloses) (Gullon et al. 2017a; Rivas and Parajó 2021; Rivas et al. 2021). In addition, they have also shown a great potential for the development of bioactive materials, since their high lignocellulosic content is complemented by a significant abundance of bioactive proteins and polyphenols (Rajha et al. 2014).

As a result, vine shoots from two of the most abundant grape varieties (*Tempranillo* and *Verdejo*) are proposed in this work as a lignocellulosic source for an innovative waste biomass valorisation. Their significant lignocellulosic content plus their remarkable bioactivity (due to the high phenolic content) makes them an interesting substrate for exploring ozonation treatments on them. Therefore, the aim of the present

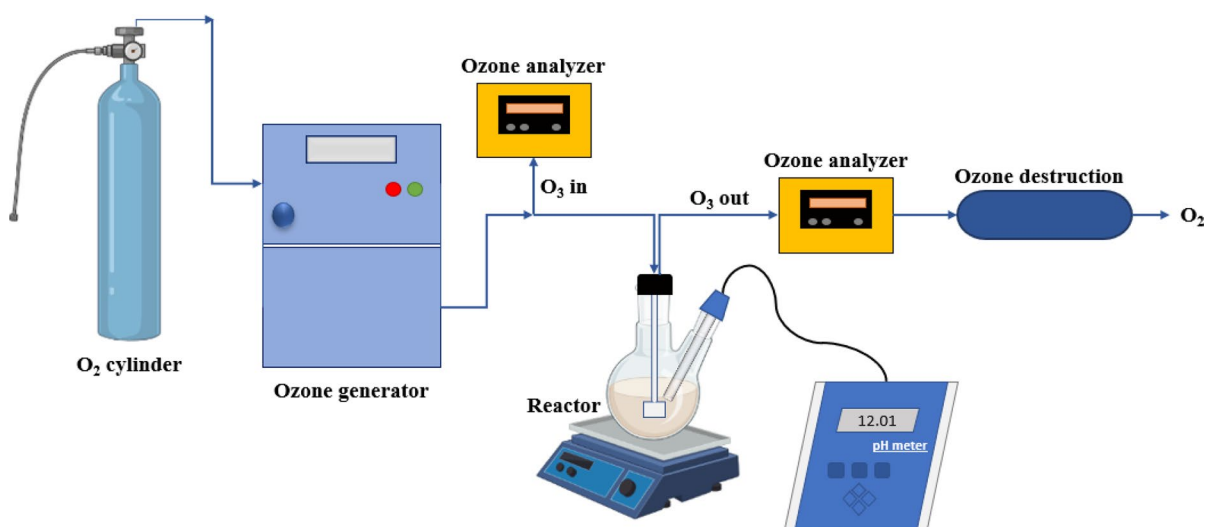


Fig. 1 Schematic representation of the ozonation process of biomass in aqueous solution

study was to investigate the effect of several ozonation conditions (pH and time) on the yields, phenolic content and antioxidant capacity of the extracts obtained from the two vine shoots varieties, in comparison with a traditional chemical extraction process via acid hydrolysis with NaClO_2 .

Experimental

Raw materials

Biomass waste materials consisting of vine shoots from *Tempranillo* (referred to as T) and *Verdejo* (referred to as V) varieties were kindly provided by Bodega Matarromera from its vineyards situated in the D.O. Rueda and Ribera del Duero (Spain, 41.642329, -4.319546) in February 2019. Vine shoots were initially converted into smaller pieces in order to facilitate their further processing by an industrial crusher. The resulting material was milled and manually sieved until obtaining a homogeneous powder (<0.5 mm). The moisture content of vine shoots samples was determined to be 7.1% and 7.7% for T and V, respectively.

Biomass treatments.

Ozonation

The ozonation process was carried out in the Uni-LaSalle ozone platform (UniLaSalle O_3 , Beauvais, France). The ozonation system included two parts: (i) the ozone generator and (ii) the ozone reactor (Fig. 1). Ozone was generated from pure oxygen of 99.5% (Messer, France) by corona electrical discharge (ozone generator: OZAT CFS3-2G, Ozonia, France). Ozone measurements were made and recorded from their absorbance data at 254 nm at the inlet and outlet streams of the ozonation reactor using an ozone analyzer (BMT 964, Germany). Using the same ozone generator, higher ozone concentrations are obtained when using pure oxygen than when feeding the system with dry air.

For the aim of proceeding with the waste biomass ozonation, 3.7 g of dry and milled vine shoots and 200 mL of distilled water were placed into the glass reactor. Ozone gas with a flow rate of 0.1 m^3/h was injected into the reactor using a porous diffuser

producing bubbles transferred through the aqueous solution. Ozone at the outlet was destroyed at 350 °C by a thermal destruction unit (ORV/ODT-020, Ozonia, France). For each vine shoot from the two grape varieties (*Tempranillo* and *Verdejo*), three different pH conditions (3, 7 and 10) and three different ozonation times (10, 30 and 60 min) were studied. The pH of the working solution was initially adjusted by adding 1 M HCl or 1 M NaOH for acidic, and neutral and basic conditions respectively. The required amount of the same buffers was added to keep the pH constant throughout the ozonation experiments. After ozonation, the solution was vacuum filtered using a Whatman filter; the residue was dried in an oven at 45 °C and the extract filtrate was lyophilised for further analysis.

The yield of the water-soluble fractions was calculated as follows:

$$\text{Yield\%} = \frac{\text{Lyophilized watersoluble extract (g)}}{\text{Initial dry vine shoots content (g)}} \times 100 \quad (1)$$

Traditional lignin extraction treatment

A conventional chemical treatment was applied for lignin extraction as a control. Briefly, 7 g of dry and milled biomass powder were subjected to an acid hydrolysis ($t=300$ min) under oxidizing conditions using 1.4% w/v NaClO_2 (700 mL) under stirring at 70 °C and pH=3 adjusted with acetic acid. The resulting supernatant was centrifuged and dialyzed in order to eliminate the excess acid and chlorite and the sample finally freeze-dried. Yield was calculated as follows:

$$\text{Yield\%} = \frac{\text{Dialyzed and lyophilised lignin (g)}}{\text{Initial dry vine shoots content (g)}} \times 100 \quad (2)$$

Compositional analysis.

Total phenolic content (TPC)

The total phenolic content (TPC) of the vine shoot extracts was estimated by the Folin–Ciocalteu colorimetric assay (Singleton et al. 1999). The Folin–Ciocalteu reagent was diluted 1:10 (v/v) with distilled water, and 1 mL of the final dilution was mixed with 0.2 mL of the sample (aqueous

dispersions of the extracts at 20 mg/mL) at room temperature. Finally, 0.8 mL of sodium carbonate at 75 mg/mL were added and the samples were heated up to 50 °C for 30 min. Absorbance values were read at 750 nm wavelength. A calibration curve was built by using gallic acid as the standard and the total phenolic content was expressed as mg of gallic acid (GA)/g extract. The determinations were carried out in triplicate.

Antioxidant capacity of the lignocellulosic fractions (ABTS^{•+} method).

The ABTS^{•+} radical cation scavenging activity was used to determine the antioxidant capacity of the lignocellulosic fraction (Re et al. 1999; Benito-González et al. 2020). 0.192 g of ABTS were dissolved in 50 mL of PBS at pH=7.4 and mixed with 33 mg of potassium persulphate overnight in the dark to yield the ABTS^{•+} radical cation. Prior to use in the assay, ABTS was diluted with PBS to an initial absorbance of approximately 0.70 ± 0.02 (1:50 ratio) at 734 nm, at room temperature. Free radical scavenging activity of samples was assessed by mixing 1.0 mL diluted ABTS^{•+} with 10 μ L of the sample and monitoring the change in absorbance at 0, 1, 3 and 6 min. A calibration curve was built by measuring the scavenging activities of 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox at different concentrations). The antioxidant capacity of the lignocellulosic extracts was expressed as μ mol Trolox equivalents (TE)/g extract. All determinations were carried out in triplicate.

Ash content

Ash content in the different lignocellulosic extracts was determined by the TAPPI T211 om-07 method. The determinations were carried out in duplicate.

Carbohydrate composition

The carbohydrate content and sugar composition of the lignocellulosic extracts were determined after a two-step sulphuric acid hydrolysis, as earlier described (Saeman 1945). The monosaccharides were

analysed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) with a ICS-3000 (Dionex) equipped with a Carbopac PA 1 column (4×250 mm, Dionex). Control samples of known concentrations of mixtures of glucose, fucose, galactose, arabinose, xylose, mannose, galacturonic acid and glucuronic acid were used for calibration. All experiments were carried out in triplicate and the reported values are simple averages of the repetitions.

Attenuated total reflectance (ATR) FT-IR analysis

All lignocellulosic extracts were analysed (after overnight drying at 60 °C) by FT-IR in attenuated total reflectance (ATR) mode using a Thermo Nicolet Nexus (GMI, USA) equipment. The one single reflection ATR crystal is made of ZnSe with diamond cover. ATR spectra were acquired with a resolution of 4 cm^{-1} in the range of 400 to 4000 cm^{-1} with a minimum average of 32 scans.

Thermogravimetric analyses (TGA)

Thermogravimetric curves (TG) were recorded with a Setaram Setsys 16/18 (SETARAM Instrumentation, France). The samples (ca. 10 mg of the lignocellulosic materials) were heated from 30 to 1000 °C with a heating rate of 10 °C/min under nitrogen atmosphere. Derivative TG curves (DTG) express the weight loss rate as a function of temperature.

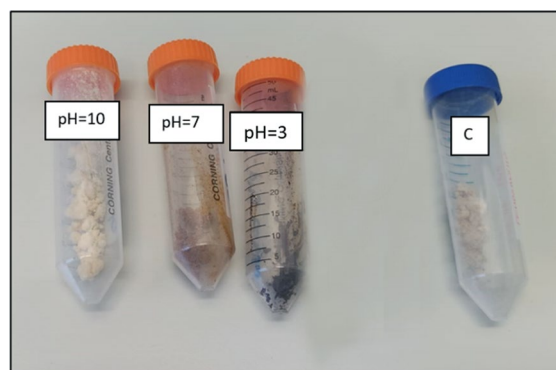


Fig. 2 Visual aspect of the different ozonized and chemically extracted lignin-rich fractions. Basic and neutral pH are shown as soft powders. Acidic are displayed as sticky dark materials. The control fraction C is observed as a fine powder

Table 1 Conditions (time and pH), yield, antioxidant activity, ash and total phenolic content (TPC) of the different ozonised and chemically extracted fractions of both *Tempranillo* (T) and*Verdejo* (V). Standard deviations are shown between brackets while different letters in the same column indicate significance

Ozonated samples	Ozonation time (min)	pH	Extraction yield of the fraction (%)	ABTS ($\mu\text{mol TE/g}$)	TPC (mg GAE/g sample)	Ash content (%)
T1	10	10	21.9 (0.7) ^c	72 (6) ^d	24 (3) ^{fg}	51 (0) ^c
T2	30	10	26.5 (0.7) ^b	49 (5) ^e	23 (3) ^{fg}	56 (2) ^{bc}
T3	60	10	31.1 (0.9) ^a	40 (4) ^e	38 (1) ^e	57 (1) ^{bc}
T4	10	7	13.3 (0.4) ^d	83 (4) ^d	33 (1) ^{ef}	54 (1) ^c
T5	30	7	13.9 (0.4) ^d	83 (8) ^d	34 (3) ^{ef}	57 (2) ^{bc}
T6	60	7	14.5 (0.5) ^d	83 (2) ^d	23 (1) ^{de}	66 (1) ^a
T7	10	3	9.8 (0.4) ^e	81 (5) ^d	125 (6) ^{ab}	43 (2) ^{de}
T8	30	3	10.3 (0.3) ^e	129 (15) ^c	126 (10) ^{ab}	48 (4) ^{cd}
T9	60	3	11.1 (0.2) ^e	80 (7) ^d	120 (9) ^{ab}	44 (1) ^d
V1	10	10	22.8 (0.5) ^c	80 (12) ^d	28 (3) ^f	57 (1) ^{bc}
V2	30	10	27.0 (0.4) ^b	43 (4) ^e	27 (3) ^f	60 (2) ^{ab}
V3	60	10	31.1 (0.7) ^a	30 (5) ^e	21 (1) ^g	60 (0) ^b
V4	10	7	13.3 (0.7) ^d	83 (1) ^d	39 (1) ^e	63 (2) ^{ab}
V5	30	7	13.9 (0.3) ^d	83 (5) ^d	55 (5) ^d	62 (1) ^{ab}
V6	60	7	14.5 (0.4) ^d	83 (8) ^d	31 (2) ^{ef}	62 (0) ^{ab}
V7	10	3	9.5 (0.2) ^e	104 (5) ^{cd}	72 (5) ^{cd}	57 (3) ^{bc}
V8	30	3	10.0 (0.3) ^e	80 (10) ^d	95 (10) ^{bc}	50 (3) ^{cd}
V9	60	3	10.5 (0.7) ^e	81 (3) ^d	92 (8) ^{bc}	45 (2) ^d
(Chemical control samples)	NaClO ₂ time (min)	pH	Extraction yield of the fraction (%)	ABTS ($\mu\text{mol TE/g}$)	TPC (mg GAE/g sample)	Ash content (%)
T	300	3	13.7 (1.4) ^d	28 (4) ^e	12 (3) ^h	43 (0) ^d
V	300	3	15.1 (2.1) ^d	31 (4) ^e	27 (5) ^{efg}	40 (2) ^{de}

Statistical analysis

Analysis of variance (ANOVA) followed by a Tukey-b test were used when comparing more than two data sets, after confirming the homogeneity of variances by the Levene test using IBM SPSS Statistics software v.26. All data have been represented as the average \pm standard deviation. Significant differences ($p \leq 0.05$) are denoted by showing the data provided in tables and figures with different letters.

Results and discussion

Images of the different ozonized and chemically extracted phenolic rich fractions are collected in Fig. 2, where the samples treated with ozone at basic and neutral pH conditions were soft powders which facilitated their handling while those ozonized at

acidic conditions were sticky materials with darker colors. The control extract C (obtained following a conventional chemical lignin extraction treatment) also consisted on a fine easy flowing powder (Fig. 2).

All the ozonation conditions as well as the main derived results have been collected in Table 1. The first clear observation was that there was a correlation between pH and final yield. Ozonation at the higher pH condition (10) led to greater yields (~30%) while when applied at acid pH, led to lower extraction yields (~10%). This could be explained by ozone decomposition in water under alkaline conditions, which would generate $-\text{OH}$ radicals which can have indirect and non-selective reaction with lignin and carbohydrates, thus increasing the final extract yield (Coca et al. 2016).

These yields were higher than those reported from antioxidant-rich extracts derived from the autohydrolysis of vine shoots followed by ethyl acetate

extractions (<4%) (Gullon et al. 2017b). Meanwhile, ozonation time did not greatly affect extract yields, with only slight improvements at longer times (60 min). It was also interesting to note that the yields obtained through the traditional chemical lignin extraction process were similar to those obtained through ozonation at pH 7, while the time required for extraction was much lower (300 min for the chemical treatment vs. only maximum 60 min for ozonation).

The antioxidant capacity of the different extracts was measured by the ABTS test as ozone is considered to be a milder treatment in comparison with chemical protocols and, thus, the antioxidant capacity of the extracted compounds is expected to be preserved. As it can be seen in Table 1, all ozonized samples showed higher antioxidant capacities than the chemical control, the latter ones displaying values ca. 30 $\mu\text{mol/g}$ (Table 1). Interestingly, the extracts obtained at lower pH values, displayed higher antioxidant capacities, with a maximum value of 129 $\mu\text{mol/g}$ for the *Tempranillo* sample obtained at pH 3. Again, ozonation time did not seem to have a clear effect on the resulting antioxidant capacity, but there was a negative tendency with longer ozonation times. A possible explanation might involve the generation of more carbonyl groups which can have a detrimental effect on the antioxidant activity (Tang et al. 2012; Xiao et al. 2020). While antioxidant capacity of extracts has been normally ascribed to the presence of phenolic compounds (San Miguel-Chávez 2017), from the values in Table 1 it can be seen that there was not a direct correlation between antioxidant capacity and phenolic content. This could be explained by the experimental method used, as Folin measures the ability of free, available phenol groups to form a coloured complex. Medium molecular weight lignans may have antioxidant activity but little Folin reaction and could be in this way not quantified as polyphenols but still present significant antioxidant capacity via ABTS (Rumpf et al. 2023). A similar behaviour has been previously observed in other lignin-rich samples (Alzameem et al. 2018), and could be explained by the presence of other compounds with antioxidant capacity (Qin et al. 2020), like certain polysaccharides (Xiang et al. 2012) or lignin-cuts (Travaini et al. 2016; Meng et al. 2019). While the antioxidant capacity of the extracts obtained at pH 3 and 7 was rather similar, a clear trend with pH was observed regarding phenolic extraction, being the

highest values obtained at low pH conditions and a decrease in phenolic yields observed when increasing pH values. But still, it should be noted that all the extracts obtained through ozone treatment yielded significant phenolic contents (21–126 mg Gallic acid equivalence (GAE)/g), thus confirming the suitability of these extraction method for producing phenolic-rich samples. Regarding the literature, very little information about biomass ozonation and its resulting antioxidant capacity is available. In particular, there is only a previous study where enzymatic lignin was subjected to ozonation as an effective way of improving its reactivity and antioxidant capacity measured by the DPPH assay. Results showed a promising EC₅₀ of 2.5 obtained after ozonation, being EC₅₀ the concentration of antioxidant required to decrease 50% of the concentration of DPPH (Shi et al. 2019).

Vine shoot extracts have been previously obtained using more conventional techniques, including heating and microwave assisted extractions in a mixture of ethanol/water and their antioxidant capacities ranged between 90–150 $\mu\text{mol TE/g}$ and TPC of 1–2% (w/w) (Jesus et al. 2019), while ethyl acetate extracts obtained at 180–215 °C displayed similar ABTS values too (50–180 $\mu\text{mol TE/g}$) (Gullon et al. 2017b). These results were comparable and eventually higher than several medicinal Indian plants (Surveswaran et al. 2007) highlighting the potential of ozonation as a greener alternative to produce bioactive phenolic-rich fractions from lignocellulosic biomass in a simple and effective way.

Ashes were also quantified in the different fractions obtained from ozonation and, interestingly, they constituted a very significant fraction of the dry extract weight (between ~40 and 60%), part of them being the salts required for pH buffering stabilization. In this sense, a further dialysis step could be carried out in order to improve the antioxidant capacity of the extracts by removing the salts present.

Monosaccharide analysis was also conducted for all the ozonated and chemically-treated fractions (Table 2). It could be seen that low contents were reported from all ozonated fractions (<15%) except from C samples (~30%). Apparently, total monosaccharide content decreased while increasing reaction times. It should also be highlighted that glucose was the major component of all fractions probably due to either hemicellulose (e.g. xyloglucan) or cellulose degradation. Low carbohydrate contents were

Table 2 Monosaccharide content (mg/g) of the ozonized and chemically extracted lignin-rich fractions from *Tempranillo* (T) and *Verdejo* (V) vine shoots. Values in the same column followed by different letters are significantly different ($p \leq 0.05$)

Ozonated samples	Arabinose (mg/g)	Galactose (mg/g)	Glucose (mg/g)	Xylose (mg/g)	GalA (mg/g)	Total (mg/g)
T1	—	9.3 (3.2) ^c	67.6 (6.2) ^{ef}	—	18.9 (1.3) ^{cd}	101.6
T2	—	—	24.5 (3.3) ^h	—	26.0 (4.5) ^{bc}	60.0
T3	—	—	25.9 (1.4) ^h	—	34.5 (6.1) ^b	77.2
T4	—	9.8 (2.7) ^c	72.6 (5.1) ^e	—	10.0 (0.4) ^d	98.9
T5	—	—	27.2 (2.0) ^h	—	—	42.3
T6	—	—	11.3 (3.5) ⁱ	—	—	22.1
T7	—	9.8 (1.1) ^c	106.7 (12.4) ^{cd}	—	—	126.1
T8	—	11.6 (4.2) ^{bc}	105.9 (10.3) ^{cd}	—	—	133.4
T9	—	—	40.2 (2.2) ^g	—	12.0 (3.7) ^d	63.2
V1	—	—	30.6 (7.7) ^{gh}	—	10.3 (0.1) ^d	48.4
V2	—	—	—	—	—	17.0
V3	—	—	—	—	—	10.4
V4	—	—	22.5 (6.9) ^{hi}	—	—	29.5
V5	—	—	—	—	—	16.1
V6	—	—	—	—	—	14.2
V7	—	9.9 (1.4) ^c	72.3 (8.8) ^e	—	—	94.5
V8	—	—	47.1 (4.7) ^{fg}	—	—	67.9
V9	—	—	15.5 (1.1) ⁱ	—	10.1 (2.1) ^d	31.9
Chemical control samples T	44.4 (4.2) ^a	28.6 (0.6) ^a	167.0 (7.9) ^{ab}	13.4 (1.1) ^b	19.5 (0.9) ^c	272.9
V	47.6 (3.9) ^a	26.6 (3.1) ^a	136.9 (18.8) ^{bc}	30.2 (6.5) ^a	48.0 (2.6) ^a	289.4

* — indicates < 10 mg/g

expected as ozone preferentially attacks lignin aromatic bonds with minor selectivity for cellulose and hemicelluloses (Coca et al. 2016). Indeed, it has been reported a loss of cellulose after ozone pre-treatment to be less than 5% in wheat straw biomass (Ben-Ghedalia and Miron 1981). However, pH is a system parameter that significantly affects the release and yield of radicals as well as their reaction rate during the ozonation process (Ragnar et al. 1999). Under acidic conditions, ozone reacts directly with saturated bonds while in neutral or alkaline conditions, it generates hydroxyl radicals which can have non-selective reaction with both lignin and carbohydrates, thus increasing their degradation. On the contrary, acidic conditions lead to more efficient hydrolysis of organic substrates, thus expecting higher extraction selectivity for lignin derivatives at lower pH compared to neutral or basic pH.

It should also be mentioned that minor differences were observed between both vine shoots varieties (T

and V) in terms of yield, antioxidant capacity and composition. This results were somehow expected due to high compositional similarity between varieties as previously reported in the literature (Benito-González et al. 2020).

The analysis of the different fractions obtained through ozonation was carried out through FTIR in attenuated total reflectance (ATR) mode. Fractions obtained at basic pH showed a broad peak at 3100–3600 cm^{-1} characteristic from O–H stretching vibrations. The more intense and defined peaks were located at 1582 cm^{-1} (characteristic from aromatic skeletal vibrations), 1328 cm^{-1} (C–H vibrations of cellulose and C–O vibrations of S ring) and a broad shoulder at around 1400 cm^{-1} which is expected to come from the lignin, cellulose and hemicelluloses present in the extracts, as they are expected to be extracted in greater yields at basic pH conditions (Bule et al. 2013). Differences in monosaccharide analysis can be related to ozone excessive

aggressiveness at basic conditions being hemicelluloses degraded rather than depolymerized (Table 2). Other spectral bands also indicating the presence of cellulose and hemicelluloses are the ones at 1146, 1044, 930 and 850 cm^{-1} corresponding to C–O–C vibrations, C–O stretching and C–H deformation (Bule et al. 2013). In this sense, slight differences were observed between T and V samples with additional peaks in T at 960 and 775 cm^{-1} suggesting a different monosaccharide extraction profile between varieties at pH=10.

Neutral pH conditions led to less intense spectra although showing similar peaks, probably due to the lower presence of non-selective –OH radicals with regards to the alkaline conditions, thus having a lower incidence in cellulose and hemicelluloses (1582 and 1328 cm^{-1} peaks). However, it should be noted that highest soluble lignin contents have been reported for ozonized biomass at neutral pH suggesting a great efficiency of these conditions, although minor differences were seen with regards to pH=3 and pH=10 in the present work (Barrera-Martínez et al. 2016). It is worth highlighting that their antioxidant capacity was in line with the rest of the conditions tested thus providing neutral pH as optimum as the others for the extraction of green and highly bioactive fractions (Table 1). At longer ozonation times, the band corresponding to the stretching of hydroxyl groups at 3600–3100 cm^{-1} became less defined, while both 1582 and 1400–1328 cm^{-1} peaks were still representative. Also, 1100–700 cm^{-1} region showed significant differences with previous fractions obtained at basic pH suggesting differences in the monosaccharide profile, in line with differences previously shown in Table 2. In this sense, no significant differences were observed between T and V varieties.

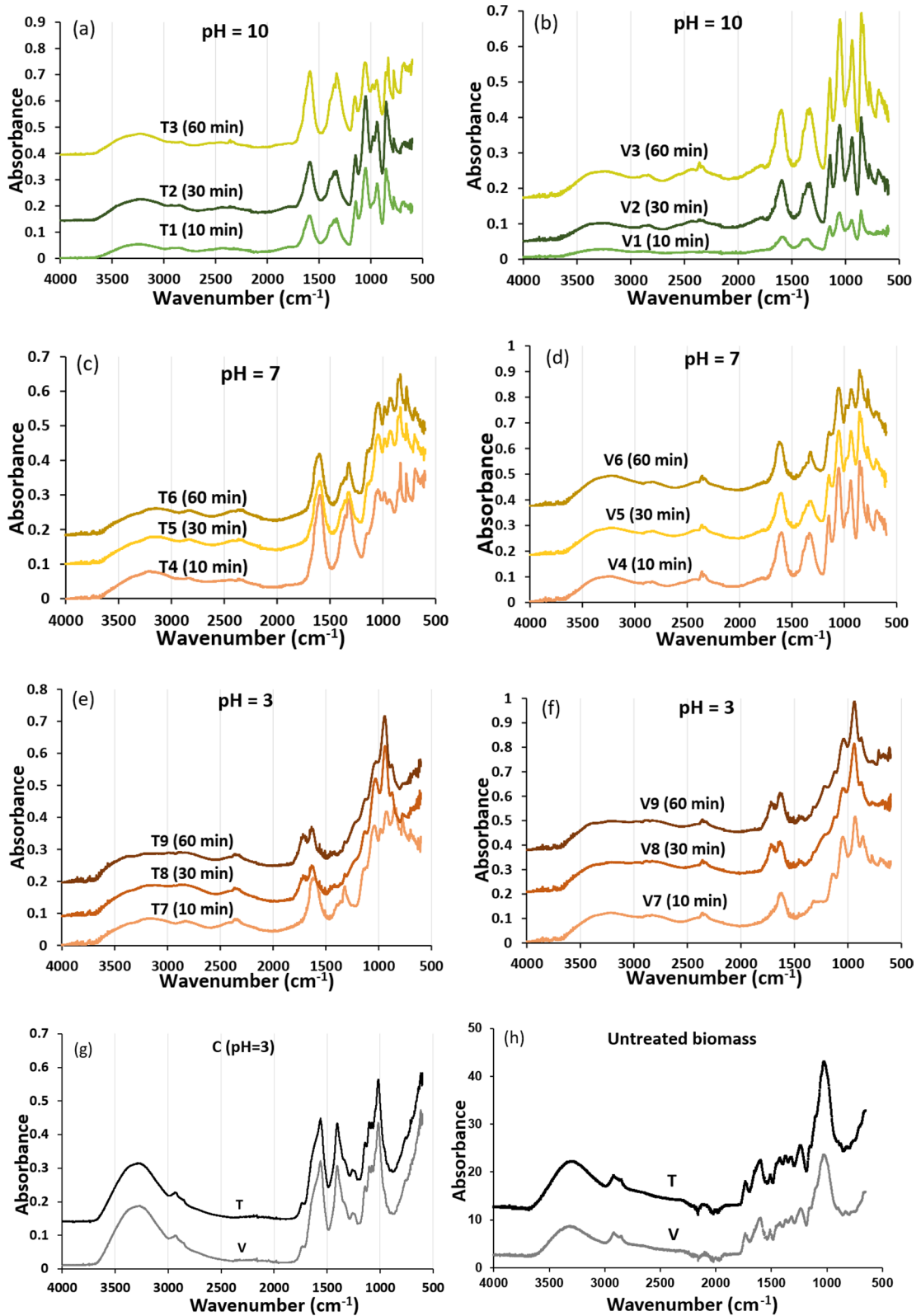
Acidic pH led to some significant differences with regards to their neutral and basic counterparts. Interestingly, there was a clear decrease on the 1400–1328 cm^{-1} peak intensity, as this peak was only present in the fraction extracted at the shortest time (10 min.). This could be the result of limited ozone reaction with hemicellulose and cellulose in acidic pH, with significantly decrease 1400–132 cm^{-1} peak intensity. On the other hand, an additional peak at 1700 cm^{-1} was shown at longer reaction times as opposed to the disappearance of the previously mentioned peak. This has been previously ascribed to

C=O stretching vibrations of ester groups, unconjugated ketones and carboxylic/carbonyl groups from lignin and hemicelluloses (Bule et al. 2013; Benito-González et al. 2020), as a result of ozone attacking aromatic saturated bonds from phenolic rings thus leading the formation of C=O bonds.

Finally, control C samples showed sharper peaks at 3600–2800 cm^{-1} too, as well as intense peaks at 1599 and 1395 cm^{-1} , similarly to those previously reported at basic pH. A minor peak could also be seen around 1730 cm^{-1} , all suggesting significant compositional differences with regards to their pH=3 ozonized counterparts. Again, minor differences were observed between vine shoots varieties at same conditions while monosaccharide profile also differed between acidic and control pH, as previously seen in Table 2 (Fig. 3).

TGA analyses were also carried out to determine the thermal stability of different ozonized and chemically extracted lignin-rich fractions (Fig. 4). Again, significant differences were observed at different pH values, but there were also clear trends when applying longer ozonation times (10, 30 and 60 min).

At pH=10, longer ozonation times appeared to have a decreasing effect on the broad 340–500 °C peak previously attributed to lignin degradation (Ma et al. 2016), as well as the 200–250 °C peak which has been linked to the degradation of hemicelluloses (Benito-González et al. 2020), while the peak around 150 °C (usually related to the presence of low molecular weight (LMW) compounds) became sharper and more intense due to the greater effectiveness of longer ozone treatment. On the contrary, at pH 7, when ozone treatment time increased (from 10 to 60 min), an increase in the peak related to lignin degradation (350–500 °C) was observed (especially in *Verdejo* vine shoots), in agreement with previous works reporting that this pH value leads to the highest soluble lignin contents (Barrera-Martínez et al. 2016). In contrast, ozonation time at pH 7 led to a decrease in the degradation peak ascribed to LMW compounds (~150–200 °C), while additional peaks were observed at higher degradation temperatures (peaks T5 and T6, pH 7, Fig. 4), probably related to the presence of hemicelluloses in the extracts. The TGA curves from the extracts obtained at acidic conditions (pH=3) showed less clearly defined peaks, probably as a result of higher compound degradation. In fact, a clear increase in the band related to thermal



◀**Fig. 3** FT-IR spectra of all ozonized, control fractions and untreated raw biomass from both *Tempranillo* (T) and *Verdejo* (V) vine shoots, being **a** T at basic pH, **b** V at basic pH, **c** T at neutral pH, **d** V at neutral pH, **e** T at acid pH, **f** V at acid pH, **g** control samples and **h** untreated raw biomass

degradation of low molecular weight compounds (LMW) was seen, which could be related to lignin degradation giving raise to lower molecular weight phenolic compounds (explaining the greater phenolic content and antioxidant activities of these extracts) (Travaini et al. 2016). This might be due to the fact that at acidic conditions ozone is less aggressive but longer lasting, resulting in lignin depolymerization but not completely degraded (Fig. 2).

On the other hand, chemically extracted fractions (C, acidic pH, also shown in Fig. 2) showed a completely different TG profile, probably due to a significant degradation induced by both longer reaction times (300 min) and a different degradation mechanism (being ozone more reactive and specific than conventional bleaching treatments), thus only showing a very broad peak between 170–350 °C (Fig. 4). These results highlight the potential of ozone as an alternative treatment for obtaining less degraded and highly bioactive extracts from waste biomass sources.

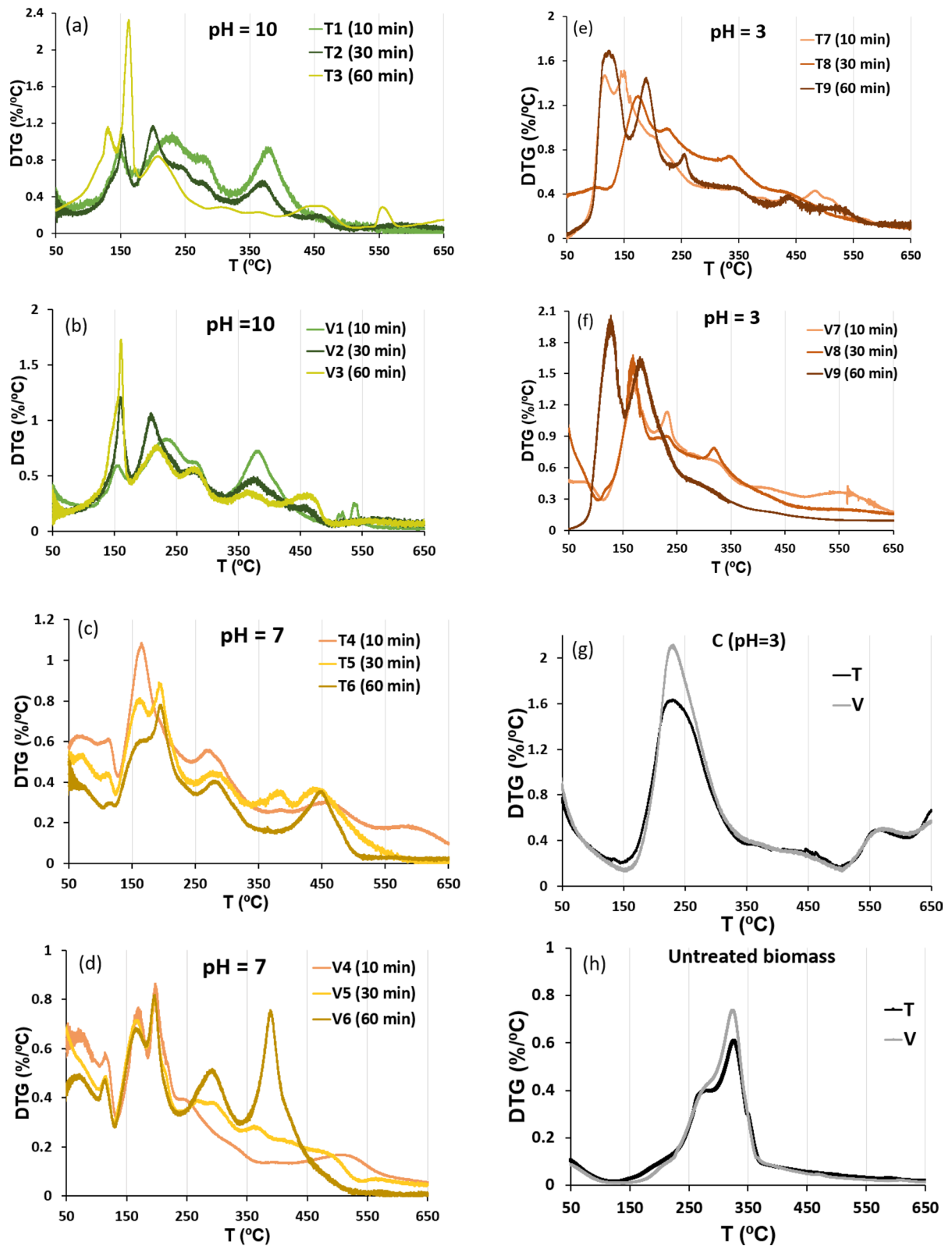
Finally, although minor differences in the results of the DTG curves were observed for the *Tempranillo* and *Verdejo* vine shoots, trends remained unaltered so that no distinction will be made between the two varieties in these analyses.

In addition, the peaks of the DTG curves (Fig. 4) were analysed in terms of weight loss for a semi-quantitative analysis of the composition of the fraction. The results are summarized in Table 3. This analysis was conducted as a result of the clear and differentiated peaks that could be seen in the different DTG curves, and based on previously reported temperature ranges of degradation of different compounds (Fig. S1) (Benito-González et al. 2018). Thus, 6 different regions were characterized: Water (< 100 °C), LMW compounds (lignin cuts and/or small monosaccharides, 100–180 °C), hemicelluloses (180–300 °C), cellulose (300–350 °C), lignin (350–500 °C) and ashes (> 500 °C). These LMW compounds are especially relevant due to their expected abundance in smaller lignin precursors as a result of ozone activity (among other partially-degraded compounds such as short-chain carbohydrates). These lignin-cuts can

possess remarkable antioxidant capacity due to the scission of their phenolic groups, being more accessible to react and so increasing their antioxidant capacity as it was previously shown in Table 1, which is one of the main objectives of the present research. Therefore, their presence in terms of sharper peaks might be indicative of higher antioxidant activities. It is also worth highlighting that ashes (mainly derived from the required salts to equilibrate pH) have been removed and corrected for estimating the relative quantity of the most relevant compounds in order to simplify the resulting data as they are not expected to possess any antioxidant capacity (Table 3).

Initially, it could be seen that water content increased at lower pH. This result was not unexpected as these fractions showed a stickier appearance (see Fig. 2) where water could not be completely eliminated by freeze-drying due to the higher hydrolysis degree into compounds with free -OH groups (e.g. lignols or monosaccharides as seen in Tables 1 and 2). LMW compounds (i.e. lignols and/or short chain monosaccharides) showed different tendencies depending on the pH followed. While both basic and acid pH increased their content while increasing the reaction time, the opposite occurred at neutral pH. This is due to less aggressive pH, which is only able to solubilize lignin at longer times without substantially degrading it to LMW compounds. Interestingly, it is worth highlighting that in all cases the increase or decrease of LMW content provoked the contrary effect in the peak corresponding to lignin. This correlation suggests a clear implication of small lignin-cuts in the incidence of LMW peak over other partially-degraded compounds such as short-chain carbohydrates. As a result, it could be inferred that longer times were beneficial for the appearance of LMW compounds at both basic and acid pH, while shorter times (10 min) were more optimum at neutral pH, which can be beneficial as shorter reaction times improves the cost efficiency of the whole process. On the other hand, the presence of lignin oligomers or higher molecular weight lignin structures (which remained unattacked and are also worth extracting due to their phenolic nature) followed the opposite tendency.

Regarding hemicelluloses and cellulose content, it was difficult to achieve clear correlations between contents and reaction conditions. This could be due to the difficulty of identifying separate cellulose peaks



◀**Fig. 4** Derivative thermogravimetric (DTG) curves of the ozonized, chemically extracted and raw untreated fractions from *Tempranillo* (T) and *Verdejo* (V) vine shoots being **a** T at basic pH, **b** V at basic pH, **c** T at neutral pH, **d** V at neutral pH, **e** T at acid pH, **f** V at acid pH, **g** control samples and **h** untreated raw biomass

at 300–350 °C region (Miranda et al. 2013), as it usually appears as a broad shoulder next to the hemicelluloses peak. This might also be related to higher ozone aggressiveness at higher pH, which results in degrading hemicelluloses rather than depolymerizing them.

Conclusions

Phenolic-rich fractions have been successfully extracted from *Tempranillo* (T) and *Verdejo* (V) vine shoot varieties through a simple and rapid method that involves the use of ozone. Ozonation time (10, 30 and 60 min) and pH (10, 7, 3) were the studied parameters. Control samples (C) of the two varieties of vine shoots were treated by a conventional chemical extraction method for comparison purposes. Semi-quantitative analyses by FT-IR and TGA showed significant differences when varying the pH, with minor incidence on ozonation time and vine shoot variety. Acidic (pH 3) extracts showed the highest phenolic and antioxidant activities that were directly related to the increase in LMW compounds, although their

Table 3 Semi-quantitative analysis of the different ozonized and chemically extracted fractions from both *Tempranillo* (T) and *Verdejo* (V) vine shoots by means of weight loss in rep-

resentative T(°C) ranges. The corrected compositional analysis eliminates the amount of ashes

Ozonated samples	Corrected compositional analysis (%)						
	Time (min)	pH	Water < 100°C	LMW 100-180°C	Hemi 180-300°C	Cell 300-350°C	Lig 350-500°C
T1	10	10	6	18	33	15	28
T2	30	10	9	19	36	16	20
T3	60	10	26	26	26	9	13
T4	10	7	24	24	24	7	21
T5	30	7	19	16	19	19	27
T6	60	7	18	15	18	15	34
T7	10	3	14	14	35	14	23
T8	30	3	21	25	21	10	23
T9	60	3	29	27	14	11	20
V1	10	10	14	14	26	14	32
V2	30	10	8	22	30	15	25
V3	60	10	10	28	22	18	22
V4	10	7	32	16	24	8	20
V5	30	7	29	16	23	11	21
V6	60	7	21	16	16	21	26
V7	10	3	16	28	26	12	18
V8	30	3	30	24	20	10	16
V9	60	3	29	35	15	9	12
(Chemical control samples)							
T			19	0*	54	11	16
V			18	0*	57	10	15

*Probably not zero but impossible to determine regarding the graphs due to lack of clear peaks

yield and processability were lower than their basic counterparts.

All pH conditions were able to achieve remarkable antioxidant values ($> 70 \mu\text{mol TE/g}$ sample) even at the lowest reaction times, with some final yields superior to 30%. Ozone treatment at basic pH led to highest yields at the expense of a higher degree of degradation and lower antioxidant activities while acid conditions led to highest antioxidant capacity as a result of lignin transformation into LMW compounds (e.g. lignols or soluble lignin) with preserved antioxidant activity. Prolonged ozonation was detrimental for both acidic and especially basic conditions. On the other hand, neutral pH showed promising results without significant differences in antioxidant capacity and phenolic content at shortest (10 min) and longer ozonation times. On the contrary, a chemical oxidation treatment reported yields around 14% with lower antioxidant capacity ($\sim 30 \mu\text{mol TE/g}$ sample). As a result, ozonation has proven to be a fast and effective oxidative pre-treatment to obtain highly antioxidant fractions with minor processing and by-product waste compared to conventional chemical treatments such as sodium chlorite extraction and preserving the cellulose fraction.

This is a promising step for an alternative valorisation of vine shoot residues as well as for alternative lignocellulosic waste biomass sources, following circular economy principles. The remarkable antioxidant capacity of all ozonized extracts makes them promising candidates for various applications, i.e. as additives in food packaging or for biomedical purposes. In this sense, future studies can be directed to increase the antioxidant capacity of the fractions (i.e. by optimizing different parameters such as ozone flow, biomass rate or dialyzing step).

Acknowledgments Authors want to acknowledge the financial support from Hauts-de-France region, France. Mr Benoit Lerzy is thanked for his valuable contribution to the article.

Author contributions I.BG wrote the initial draft and revised the experimental part and conceptualization of the manuscript. M.E, M.M.OG and L.H carried out the experimental part. V.A, T.A, A.MA and A.LR did the conceptualization, funding acquisition and revised experimental work. I.BG and M.E. prepared the figures of the manuscript. I.BG, M.E, V.A, T.A, A.MA and A.LR reviewed the manuscript.

Funding Bodega Matarromera S.L. (IDI-20221019), Pago de Carraovejas S.L.U. (IDI-20221020) and ENCE

Energía y Celulosas S.A. (IDI-20221021) for financial support within the VINEBOX CDTI project (EXP 00149387 / ABS – 20220137). The project CEX2021-001189-S was funded by MCIN/AEI/**10.13039501100011033 and by “ERDF A way of making Europe”. I. Benito-González is recipient of a Torres-Quevedo contract (PTQ2021-011739) from the Spanish Ministry of Science and Innovation (MCIN/AEI/**10.13039501100011033) while M. M. Ortiz-Gimeno was recipient of a JAE-Intro grant from CSIC (JAEINT20_EX_0182).

Declarations

Ethics approval and consent to participate No ethical approval and consent were required for the presented work.

Consent for publication All the authors have provided their consent for the submission of the presented work.

Availability of data and materials N/A.

Competing interests 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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