Non-targeted, suspect and targeted High Resolution Mass Spectrometry (HRMS) approaches for the profiling of oenological matrices and different food commodities

A Ph.D. dissertation by

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Abbreviations

HRMS: high resolution mass spectrometry

MS/MS: mass/mass

LC: liquid chromatography

QqQ: triple quadrupole

QIT: quadrupole ion trap

SRM: selected reaction monitoring

dd-MS/MS: data-dependent MS/MS

RT: retention time

ESI: electrospray ionization

TIC: total ion current

NMR: nuclear magnetic resonance

MS^n: Tandem or multistage mass spectrometry

Q: quadrupole

IT: ion trap

CID: collision-induced dissociation

HCD: higher-energy collisional dissociation

AIF: all ion fragmentation

AGC: automatic gain control

SIM: single ion monitoring

NL: neutral loss

S/N: signal-to-noise

Asp: aspartic acid

Glu: glutamic acid

S_N2: Nucleophilic substitution

GC: gas chromatography

CoA: coenzyme A

ACN: acetonitrile

MeOH: methanol

SPE: solid-phase extraction

UHPLC: ultra high-performance liquid chromatography

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Aim of work

The increasing number of foodomics studies based on non-targeted methods shows that this approach is considered by scientists to be an efficient way of evaluating food safety and quality. In the last few years, high resolution mass spectrometry (HRMS) has indeed gained wider acceptance thanks to the high selectivity and sensitivity achievable during analysis. In contrast to the classic unit-mass-resolution MS/MS approach, HRMS provides more information on sample composition through collection of full-scan spectra and thanks to the possibility of performing retrospective data analysis. Consequently, even without defining compound-specific tuning, HRMS data can be used for identification of suspect compounds or for structural elucidation of unknowns. HRMS can only compete with classic MS/MS methods using the targeted approach, even if it allows the simultaneous detection of a higher number of compounds. In contrast, HRMS is a more promising approach when suspect and non-targeted screening analysis is performed, not only because full-scan and retrospective analysis is feasible, but also because the accurate mass of both precursor and product ions and their isotope patterns are provided. Furthermore, a non-targeted approach leads to specific profiling of biological systems through a wide selection of chemical descriptors, and provides the fingerprint of the system under investigation, useful for more easily identifying potential adulteration.

The aim of this work was to extend comprehension of the three different HRMS approaches (non-targeted, suspect and targeted screening), examining both their potential and limitations in relation to the analysis of the compounds of interest in different matrices. Initially, the objectives concerned the possibility of developing new methods – one for each HRMS screening approach – for the analysis of glycosides and phenolic compounds, in order to furnish innovative and well-performing analytical tools for food safety and quality control at all stages of food production, processing and distribution. Furthermore, they regarded the possibility of investigating the nature and occurrence of glycosides and phenolic compounds in widely consumed beverage and food commodities, such as grape, wine, spirits, cocoa and honeys.

The thesis, which includes both published and *in litteris* papers, describes newly developed analytical methods and their technological applications in the study of different matrices, focusing on:

- Investigation of Neutral Loss experiments as an instrument for non-targeted screening analysis of glycosides, and performance evaluation of this analytical approach in relation to the glycosidic profiling of international monovarietal wines;

- Investigation of the distribution of free and glycosylated low-molecular-weight phenolic compounds in skin and seeds of color-rich *Vitis vinifera* grapes cultivated in southern Uruguay, combining Neutral loss experiment and suspect screening analysis;
- Investigation of the selectivity and sensitivity of the HRMS approach for targeted analysis of free and glycosylated low-molecular-weight phenolic compounds and suspect screening analysis of the latter, together with evaluation of the best sample clean-up procedure for reducing matrix interference;
- Investigation of the distribution of free and glycosylated low-molecular-weight phenolic compounds in skin, pulp and seeds, focusing on both *Vitis vinifera* and hybrid grapes;
- Investigation of the impact of alcoholic fermentation on the free and glycosylated phenolic profile of wines produced from grapes of hybrid varieties;
- Study of the occurrence of glycosylated low-molecular-weight phenolic compounds in tannins of different botanical origin, in order to evaluate the alteration of the phenolic profile of wines after tannin addition;
- Study of the free phenolic composition of wood barrels, in order to evaluate phenolic enrichment during ageing, and investigation of the possible impact of different barrel sanitation treatments on the phenolic transfer from wood to wine;
- Study of the free and glycosylated low-molecular-weight phenolic profile in Primitivo di Manduria and Negroamaro wines of different vintages and evaluation of the effect of wine ageing;
- Investigation of the possibility of considering free or glycosylated low-molecular-weight phenolic compounds as new markers for beverages and food characterization and their geographical traceability, focusing on wine, spirits, vinegar, food tannins, cocoa beans and honeys;
- Implementation of investigative methods of suspect screening analysis using naturally rich matrices as available sources of compounds of interest. This approach was applied on plant products for alkaloid identification;
- Investigation of the selectivity and sensitivity of the HRMS approach for suspect screening analysis of flavonoids in flowering plant (C. *pareira*) extracts.

1. Introduction

1.1. High-resolution mass spectrometry

In the last twenty years over 5,200 studies have been carried out combining liquid chromatography with high-resolution mass spectrometry (LC-HRMS) and more than 50% of these have been carried out using Orbitrap™ as the detector. The majority of these papers have been published since 2011, dealing principally with clinical and forensic toxicology (Jiwan *et al.*, 2011; Himmelsbach, 2012; Meyer, & Maurer, 2012; Meyer *et al.*, 2014; Maurer, & Meyer, 2016), omic sciences (proteomics, metabolomics and lipidomics; Gallien, & Domon, 2015; Lesur, & Domon, 2015; Ghaste *et al.*, 2016), food safety and control (Botitsi *et al.*, 2011; Kaufmann, 2012; Hernández *et al.*, 2014; Senyuva *et al.*, 2015), and environmental pollution (Hernández *et al.*, 2012; Gosetti *et al.*, 2016).

HRMS has gained wide diffusion due to improvement of detection specificity as compared to the unit-mass-resolution approach, and to the high selectivity and sensitivity of analysis (Kaufmann, 2012). This has been achieved by reducing errors in the assignment of the mass of analytes co-eluted with interference with the same nominal mass and by providing narrower mass-extraction windows (Senyuva *et al.*, 2015).

Furthermore, HRMS provides more information on sample composition through the collection of full-scan spectra and thanks to the possibility of performing retrospective data analysis (Righetti *et al.*, 2016). Consequently, even without defining compound-specific tuning, HRMS data can be used for identification of targeted and suspect compounds or for structural elucidation of unknowns (Kaufmann, 2012). Indeed, HRMS can only compete with classic MS/MS methods in targeted approaches, where specific compounds are quantified and all other matrix components are ignored. In contrast, it is one of the most promising approaches when suspect and non-targeted screening analysis is performed (Senyuva *et al.*, 2015).

1.1.1 Targeted analysis

Targeted analysis allows identification and quantification of the compounds of interest using reference standards. For a large selection of compounds, triple quadrupole (QqQ) or quadrupole ion trap (QIT) have been workhorse instruments in targeted analysis, thanks to the sensitivity and selectivity provided by selected reaction monitoring (SRM) of precursor-product ion transitions. However, monitoring only one transition can lead to false positive identification, while requiring two or more transitions can create several limitations (Krauss *et al.*, 2010):

- some targeted analytes produce only one product ion with a sufficient signal intensity, while others are barely detectable due to low intensity;
- in the event of considering at least two transitions, SRM methods are limited to about 100-150 targeted analytes, depending on chromatographic separation, otherwise insufficient peak

resolution or a short acquisition time for each MS/MS transition can considerably affect the accuracy and sensitivity of analysis;

- some targeted analytes show only non-specific transitions, such as the neutral loss of H_2O or CO_2, also detected for matrix interference.

HRMS offers promising solutions to these limitations of the SRM approach in targeted analysis. Theoretically, all the compounds present in a sample can be detected simultaneously using full-scan mode, without limiting the number of targeted compounds to be identified. Furthermore, through data-dependent MS/MS acquisition (dd-MS/MS), MS/MS analysis is triggered for each compound from the targeted ion list detected in the full-scan. In this way, many more product ion spectra can be recorded within the same run and targeted analytes showing only one transition or a non-specific one can also be identified, without risking false positive identification (Krauss *et al.*, 2010). However, HRMS selectivity requires good preceding LC separation in order to prevent co-elution of isobaric compounds, which could not be distinguished if filtered together for dd-MS/MS analysis.

As reported by Krauss *et al.* (Krauss *et al.*, 2010; Figure 1.1), the targeted workflow usually moves from defining the targeted ion list and filtering exact masses (the *m/z* range of the extraction window depends on the mass accuracy and resolving power of the instrument), to matching of the measured retention time (RT) and MS/MS fragmentation with those of reference standards, and quantification of targets.

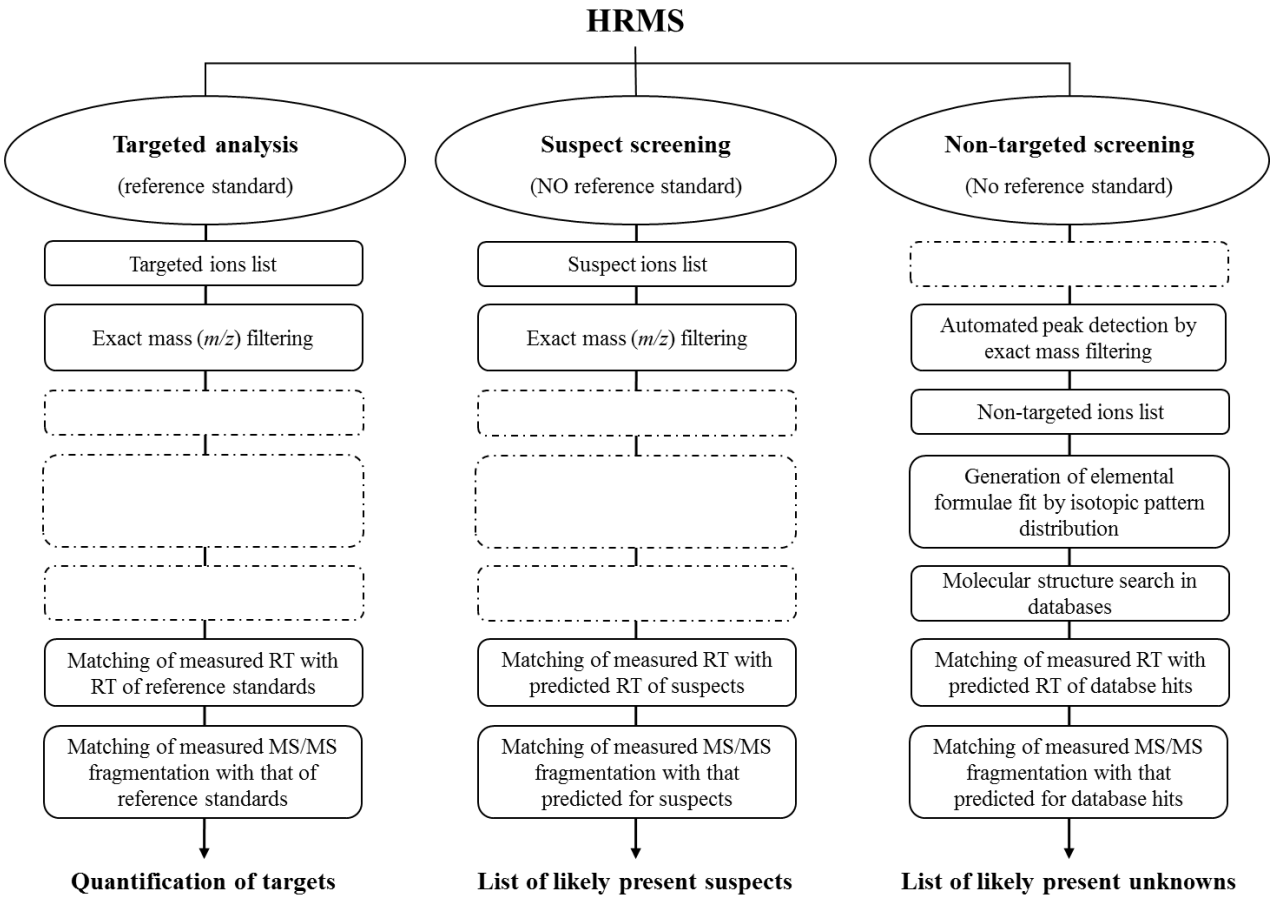


Figure 1.1. Comparison of systematic workflow for targeted analysis, suspect screening and non-targeted screening using LC-HRMS (adapted from Krauss *et al.*, 2010).

1.1.2. Suspect screening

For a large selection of compounds of interest reference standards are currently not available, but specific information such as the molecular formula and structure is accessible. Consequently, identification of these compounds can be based on the suspect screening approach (Krauss *et al.*, 2010). The exact mass of the expected ion can be calculated from the molecular formula and then extracted from the high-resolution full-scan chromatogram, since with electrospray ionization (ESI) predominantly $[M+H]^+$ and $[M-H]^-$ ions are formed. Only when the molecular ion cannot be detected due to in-source fragmentation or in-source adduct formation, the suspect screening approach cannot be applied. In the event of positive findings, compound identification can be confirmed by comparing MS/MS-derived structural information with that available for the suspect, and verifying the match of the observed isotope pattern with the theoretically predicted one from the molecular formula of the expected compound (Krauss *et al.*, 2010).

As regards false negatives, without an analytical standard it is not possible to exclude the possibility of a compound getting lost during sample preparation, not being ionized in the analytical conditions established or ionization being suppressed by a strong matrix background due to insufficient clean-up. Thus careful validation of the whole analytical procedure, using a selection of reference standards with similar physicochemical properties to those estimated for the suspects, is recommended to minimize the occurrence of false negatives (Krauss *et al.*, 2010).

Therefore, thanks to high-resolution, which resolves nominally isobaric ions, and the simultaneous collection of full-scan and dd-MS/MS spectra for a wide selection of suspect compounds, a HRMS suspect screening approach is more suitable than SRM.

The systematic workflow for suspect screening usually moves from defining the suspect ion list and filtering of exact masses (depending on the mass accuracy and the resolving power of the instrument) to matching of the measured isotope pattern, RT and MS/MS fragmentation with those predicted for suspects, and listing of suspects likely to be present (Figure 1.1; Krauss *et al.*, 2010).

1.1.3. Non-targeted screening

Non-targeted screening allows the tentative identification of unknown compounds without any *a priori* information about the analytes to be detected. This approach is more challenging than those previously described (Sections 1.1.1. and 1.1.2.), since structural elucidation of unknowns requires several specific processing steps (Krauss *et al.*, 2010):

- automated peak detection, through exact mass filtering from the total ion current (TIC) chromatogram;
- assignment of an elemental formula to the exact mass of interest;
- database search of plausible structures for the elemental formula determined.

For automated peak detection and prediction of elemental composition from accurate mass measurements, several software packages based on different algorithms are available and usually furnished with the MS instrument (Krauss *et al.*, 2010). The measured mass accuracy should be less than 3 ppm and the relative isotopic ratio accuracy less than 5% (Kind, & Fiehn, 2007).

As regards the third step, the search for plausible structures for a defined elemental formula generally produces an extensive list of compounds, whose tentative identification requires comparison of MS/MS-derived structural information with that reported in MS/MS or in-source fragment ion libraries. However, such an approach is limited by the reduced number of recorded experimental spectra, due to the lack of available reference standards, and by the limited comparability of different source ionization. Furthermore, computational fragmentation spectra predict a large number of product ions, but only a small number of them are actually observed

(Krauss *et al.*, 2010). Comparison of experimental retention time with the theoretical log K_{ow}, calculated based on the predicted structure of database hits, can also be helpful in unknown structural elucidation (Hogenboom *et al.*, 2009).

Non-targeted HRMS screening is a useful approach for providing meaningful structure suggestions for unknowns, but unequivocal identification requires complementary techniques or reference standards. For this reason, HRMS is usually combined with ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy, although these approaches require a higher concentration of the purified unknown compound.

Non-targeted screening workflow moves from automated peak detection through exact mass filtering (depending on the mass accuracy and resolving power of the instrument), production of a non-targeted ion list and the definition of elemental formulae and correlated plausible structures, to matching of measured RT and MS/MS fragmentation with those predicted for database hits, and listing of the unknowns most likely to be present (Figure 1.1; Krauss *et al.*, 2010).

However, in a non-targeted screening approach, ion suppression due to analyte co-elution can affect mass accuracy and consequently the number and accuracy of the molecular formulae generated. Due to the consequent low signal-to-noise ratios, missing of suppressed compounds can occur during automated filtering processing (Senyuva *et al.*, 2015). Therefore, the use of procedures for sample clean-up aimed at removing matrix background and for stable isotope labelling (SIL) may successfully assist a non-targeted screening approach (Righetti *et al.*, 2016).

1.1.4. Ion suppression and data quality

Ion suppression is the reduction in the measured ion abundance of an analyte due to ionization of a highly abundant co-eluting compound (Knolhoff, & Croley, 2016). Depending on ion suppression intensity, the analytes of interest can be detected in traces or not detected at all. In this case, although counterintuitive, sample dilution reduces ion suppression and improves detection of the analytes of interest (Stahnke *et al.*, 2012). Furthermore, efficient sample extraction procedures, good preliminary chromatography and a high detection resolving power are key steps in reducing ion suppression.

The sample extraction method, particularly for suspect and non-targeted screening approaches, should be unselective in order to solubilize and recover a wide range of chemically different compounds, simple and fast to prevent metabolite loss or degradation during procedure, able to extract low abundant analytes and well reproducible (Vuckovic, 2012). Therefore, considering the large number of detectable compounds, ion suppression can be prevented or at least reduced if compounds are sufficiently resolved through efficient and selective chromatography (Knolhoff, &

Croley, 2016). Indeed, by minimizing chemical background and the likelihood that compounds with the same nominal mass can co-elute, ion cloud overlap and distortion of the measured frequency can be prevented (Croley *et al.*, 2012). Furthermore, a higher resolving power, which is the ability to separate neighboring peaks from one another (Knolhoff, & Croley, 2016), results in better mass assignment and is beneficial for detection of less abundant analytes (Berendsen *et al.*, 2015).

1.1.4.1. Sample extraction and purification procedures

Sample extraction is a key step in the analytical process since it reduces matrix interference and increases method accuracy, but also because it can induce analyte loss or degradation when not appropriate (Dai, & Mumper, 2010). Before extraction, solid samples require preliminary steps for milling, grinding and homogenization, which can also be preceded by air- or freeze-drying (Dai, & Mumper, 2010). On the other hand, liquid samples can be directly analyzed after filtration or centrifugation or submitted to purification procedures before analysis (Stalikas, 2007).

Solvent extraction (SE) is the most common approach to isolating phenolic compounds from solid samples with a diffusion-based process (Baiano, & Del Nobile, 2016), but its performance depends on solvent polarity and its affinity with the analytes, the duration and temperature of extraction, and solvent-to-sample ratio (Dai, & Mumper, 2010). MeOH is generally used for low-molecular-weight phenolic compounds, while a mixture of water and acetone is used for high-molecular-weight compounds. The addition of an acid modifier can accelerate cell membrane denaturation, but if excessive can hydrolyze acylated or glycosylated phenols, negatively affecting their extraction. Furthermore, raising the extraction temperature increases analyte solubility and extraction rate, but at the same time enhances the risk of phenolic degradation and oxidation (Robards, 2003).

Ultrasound-assisted extraction (UAE) is an inexpensive method in which analytes extraction is facilitated by the disruption of sample biological membranes induced by implosion of cavitation bubbles (Baiano, & Del Nobile, 2016). It is faster than other extraction methods and affects the integrity of phenolic compounds less (Herrera, & De Castro, 2005).

Pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE) uses organic solvents at high pressure and temperature (above their boiling point; Baiano, & Del Nobile, 2016). When the solvent used is water, PLE is called subcritical water extraction (SWE) and takes advantage of organic solvent-like behavior of water when heated up to 200 °C (Dai, & Mumper, 2010). However, before using PLE it is recommended to assess the risk of oxidation and degradation for the phenolic compounds of interest.

Supercritical fluid extraction (SFE) separates one component from the matrix using supercritical fluids. The most widely used is carbon dioxide, but an organic modifier can be added to increase the

extracting solvent polarity (Baiano, & Del Nobile, 2016). SFE uses high pressure but low temperature, is performed in the absence of air and light, and is less risky due to phenol oxidation (Dai, & Mumper, 2010).

Microwave-assisted extraction (MAE) is based on the rupture of cells due to the pressure generated by cell moisture evaporation during heating (Baiano, & Del Nobile, 2016). It is characterized by a reduced extraction time and solvent volume compared to other extraction methods, but it can affect phenol integrity (Dai, & Mumper, 2010).

As regards liquid samples, matrix interference can be reduced simply by dilution or using liquid-liquid extraction (LLE) and solid-phase extraction (SPE; Motilva *et al.*, 2013).

LLE is a traditional purification process consisting of the distribution of analytes between two immiscible phases (Baiano, & Del Nobile, 2016). It can be performed with different solvents, such as water, methanol, and acetonitrile, pure or mixed, and with or without acid modifiers (mainly formic and acetic acids) but requires a long time to extract the analytes of interest and uses a large volume of solvents (Motilva *et al.*, 2013). For these reasons, it is usually replaced by faster and more environmentally-friendly methods.

SPE is a more diffuse traditional purification method and is based on the distribution of analytes between the solid and liquid phase (Baiano, & Del Nobile, 2016). It can either retain interference by eluting the analytes of interest or retain phenolic compounds by washing out matrix interference. In the latter case, the retained compounds can later be recovered with suitable solvents (aqueous or organic solvents, with or without acid modifiers), yielding quantitative extraction (Motilva *et al.*, 2013). Of the stationary phases, Amberlite, silica-based C8-C18, copolymer-based HLB, PH and ENV+ are the most commonly used (Dai, & Mumper, 2010). The most time-consuming step in SPE is total or partial evaporation of the elution solvent after analyte recovery, but the possibility of performing online SPE allows this step to be skipped by eluting the compounds directly from the SPE cartridge to the analytical column (Motilva *et al.*, 2013).

1.1.5. Tandem mass spectrometry

Tandem or multistage mass spectrometry (MS^n) allows consecutive isolation and fragmentation of the ions of interest, creating a set of hierarchical linked mass spectral data (Figure 1.2*a*; Rojas-Chertó *et al.*, 2011).

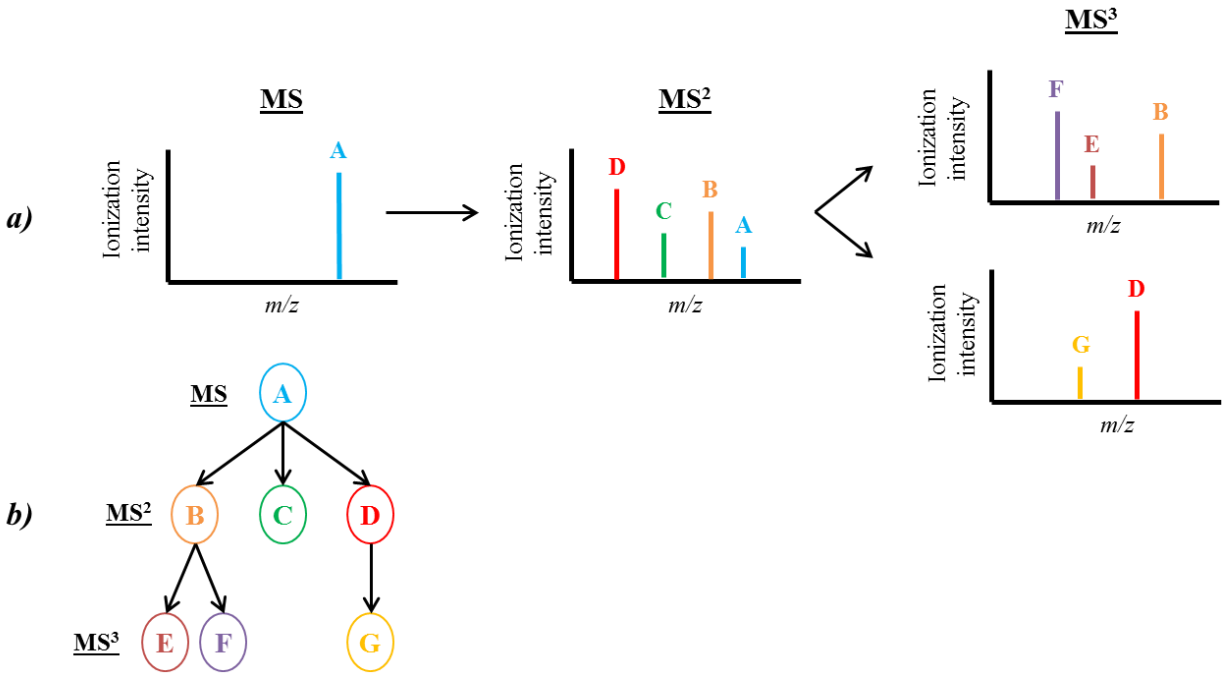


Figure 1.2. Fragmentation scheme of a tandem mass approach (*a*) and an example of a tandem mass spectral tree (*b*).

This approach requires the use of hybrid instruments, which combine both low- and high-resolution detection in the same technique. The first low-resolution detector, such as a quadrupole (Q) or ion trap (IT), isolates the precursor ions that will be fragmented with low-energy collision-induced dissociation (CID). The second high-resolution detector, such as a time-of-flight (TOF), orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) device, provides accurate full-scan data of the fragment ions produced (Gosetti *et al.*, 2016). In appropriate instrument configurations, each fragment ion in turn can be isolated and submitted to new collision-induced dissociation, producing a new mass spectrum every time (Rojas-Chertó *et al.*, 2011). With the last approach, a mass spectral tree is created, which more accurately characterize the analyte of interest, showing the fragmentation pathway of the molecule (Figure 1.2*b*; Vaniya, & Fiehn, 2015). MS^n trees reveal the link between precursor/product ion and product ion/product ion within a single MS/MS experiment and between consecutive ones. This enables recursive reconstruction of fragmentation pathways that associates the specific substructure with complete molecular structures (Vaniya, & Fiehn, 2015).

The MS^n approach can be performed in targeted dissociation, limited to a targeted ion list, in data-dependent dissociation, including all compounds meeting pre-defined criteria (*e.g.* top*N* most abundant ions), or in data-independent dissociation, applied to all precursors without any pre-selection (Knolhoff, & Croley, 2016). Therefore, a high-resolution MS^n approach is a useful tool for targeted confirmation and for the identification of suspects and unknowns (Lin *et al.*, 2015), also

thanks to the possibility of defining the molecular formula of each fragment ion (Knolhoff, & Croley, 2016).

1.2. Orbitrap

The Orbitrap mass analyzer was developed by Alexander Makarov at the end of the 20th century (Makarov, A. 2000) and has become one of the most robust analyzers, routinely used in different fields of analytical chemistry (Perry *et al.*, 2008). Furthermore, in a hybrid configuration, the Orbitrap enables a tandem mass approach for better elucidation of analyte structure (Makarov, & Scigelova, 2010). Its applications range from genomics, proteomics, metabolomics and lipidomics to drug metabolism, doping and food control (Hu *et al.*, 2005; Makarov, & Scigelova, 2010).

1.2.1. Principle of Orbitrap detection

The Orbitrap is a mass analyzer consisting of a central spindle electrode and two surrounding barrel-like electrodes, co-axial with the inner one (Figure 1.3; Hu *et al.*, 2005). It measures mass-to-charge values (*m/z*) from the frequency of harmonic ion oscillations around the central electrode and along the axis of the electric field (Hu *et al.*, 2005).

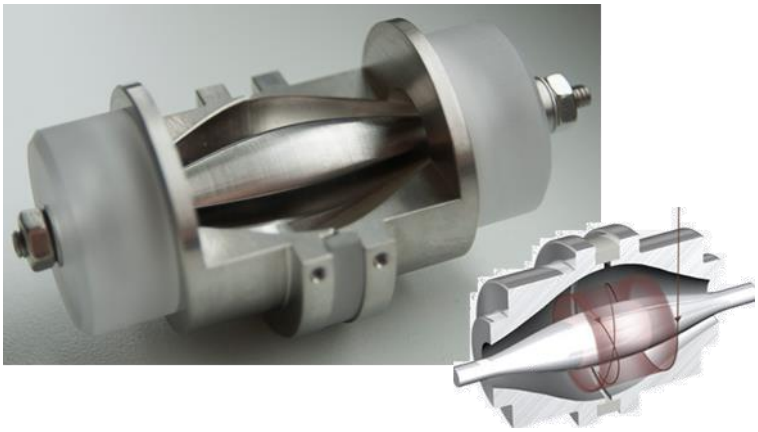


Figure 1.3. Orbitrap mass analyzer.

Ions are formed at atmospheric pressure (electrospray ionization, ESI; atmospheric pressure chemical ionization, APCI; atmospheric pressure photoionization, APPI), moving through a transfer tube to a stacked-ring ion guide (S-lens) and then via an injection multipole into a bent flatapole (Figure 1.4; Michalski *et al.*, 2011). Ion clusters and droplets from the S-lens fly unimpeded out of the flatapole, thanks to its 2-mm-distant rods, to a short octapole that brings ions into a curved RF-only quadrupole, whose central axis follows a C-shaped arc (so called C-trap; Makarov *et al.*, 2006). The C-trap is made up of hyperbolic rods and is enclosed by two flat lenses, through which the ions move. The first lens is between the octapole and C-trap, being the gate electrode for C-trap ion entrance, while the second is between the C-trap and the higher-energy collisional dissociation (HCD) cell, being the gate electrode for C-trap ion exit. When in the C-trap, the ions lose energy in collision with nitrogen gas, without being fragmented thanks to the relatively low pressure of nitrogen (around 1 mTorr).

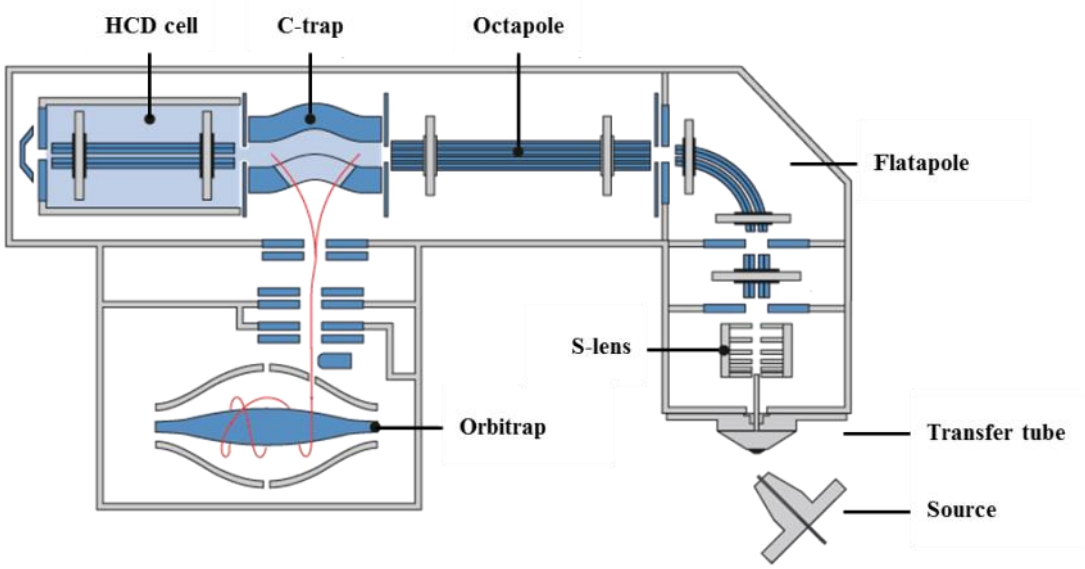


Figure 1.4. Orbitrap instrument (adapted from *www.planetorbitrap.com*).

As a consequence of collisional cooling, the ions form a thin thread along the curved axis of the C-trap, which is compressed axially by applying 200 V to both the gate and the trap electrodes. The RF voltage applied to the C-trap is then rapidly (over 100-200 ns) ramped down and DC pulses are applied to the electrodes as follows: 1200 V to the push-out electrode (i.e. the electrode furthest from the center of C-trap curvature), 1000 V to the pull-out electrode (the electrode closest to the center of curvature), and 1100 V to both the upper and lower electrodes. This voltage distribution forces ions orthogonally to the axis of the C-trap (center of curvature of the C-trap) where they leave via a slot in the pull-out electrode (Makarov *et al.*, 2006). Radial, rather than axial, ion ejection ensures faster and more uniform ion extraction from the C-trap.

After leaving the C-trap, the ions pass through three stages of differential pumping, until they reach the ultrahigh vacuum (circa 2×10^{-10} mbar) compartment of the Orbitrap. In this way, the ions go through curved ion optics, are accelerated to high kinetic energies and converge into a tight cloud. In this form, they enter the Orbitrap tangentially through a small aperture on the outer curved electrode. The short transfer distance between the C-trap and the Orbitrap reduces time-of-flight separation, while the vertical displacement of ions through a dual electrostatic deflector avoids gas carryover to the mass analyzer (Makarov *et al.*, 2006).

As the ions enter the space between Orbitrap electrodes thanks to a rapid increase in the electric field, they gradually spread into rotating thin rings that oscillate axially along the central spindle electrode for a period proportional to $m/z^{1/2}$ (Makarov, & Scigelova, 2010). During injection, narrow spatial (< few mm) and temporal distributions (<100-200 ns) of ions are required, in order to ensure their coherent motion during current signal detection (Perry *et al.*, 2008). After the voltage of central electrode has been stabilized at around 3.5 kV, ion frequencies are measured through

acquisition of the time-domain image current and then converted into a mass spectrum with fast Fourier transformation. Finally, mass spectral data can be stored in full- or reduced-profile format, in the latter case removing all data with the same intensity as the thermal noise of the pre-amplifier (Makarov *et al.*, 2006). The ion pathway described is characteristic of full MS acquisition mode (Figure 1.5).

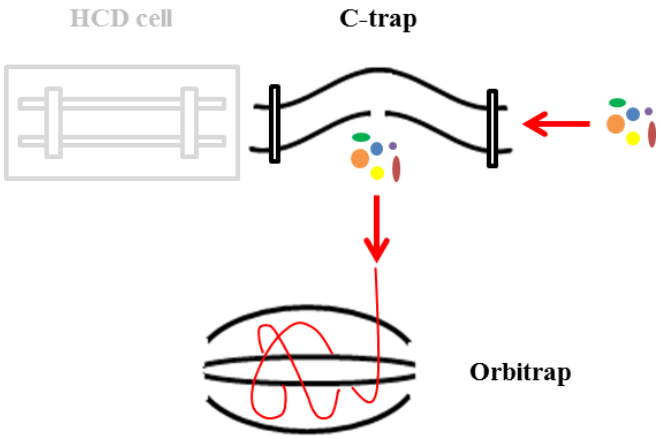


Figure 1.5. Scheme of ion pathway in a full MS scan.

When fragmentation is needed, the C-trap is interfaced directly with the gas-filled HCD cell, where the required collision energy for ion fragmentation is provided by adjusting the inner axial field and the offset of the RF rods. By changing this offset, multiple precursor ions are introduced into the HCD cell and fragmented at their optimum collision energy, without compromising the fragmentation and storage of ions from previous injections. Indeed, as long as the offset is negative compared to the C-trap and HCD exit lenses, all fragment ions produced are trapped inside the HCD cell. At the end of fragmentation, all fragment ions can be transferred back into the C-trap, ejected into the Orbitrap and analyzed in a single detection cycle (Michalski *et al.*, 2011). This ion pathway distinguishes all ion fragmentation (AIF) workflows, in which MS/MS fragment scans are acquired simultaneously in a wide *m/z* range (Figure 1.6). A combined workflow with a preliminary full MS scan (without HCD cell use) followed by an AIF-scan (with fragmentation energy applied in HCD) can be also performed.

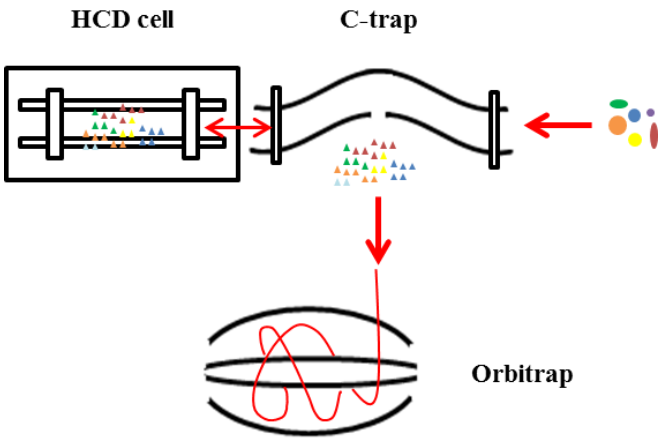


Figure 1.6. Scheme of ion pathway in an all ion fragmentation scan.

This ion storing ability makes it possible to work in "multiplexing mode", which involves combining several ion injections for a single detection cycle, with the limitation that the sum of the individual injection times must be lower than the time required for the Orbitrap scan. This approach is valid both at MS and MS/MS levels. Furthermore, parallel filling, which is the ability to fill the C-trap or the HCD cell while a previous Orbitrap detection cycle is still ongoing, allows improvement the acquisition speed, also in the event of low ion currents, and the quality of spectra (Michalski *et al.*, 2011).

1.2.2. Hybrid Orbitrap configuration

In hybrid instruments, the Orbitrap is combined with a low-resolution mass analyzer (IT or Q) set between the transfer multipole and the C-trap (Figure 1.7). This configuration allows isolation of precursor ions from the matrix background and defines the link precursor/product ions in the event of MS/MS experiments. When compared to the classic QqQ MS approach, hybrid Orbitrap ensures higher sensitivity in full-scan mode and accurate mass detection for both precursor and product ions (Gosetti *et al.*, 2016). Furthermore, MS^n can be achieved with LTQ-Orbitrap, while MS^3 with Q-Orbitrap using in-source fragmentation. An innovative characteristic of the hybrid instrument is the automatic gain control (AGC) procedure, which consists of a short pre-scan of ions in the low-resolution mass analyzer with the purpose of determining the ion current within the mass range of interest and enabling storage of a defined number of ions (AGC target value) in the subsequent analytical scan. Combining the AGC feature with determination of the ion injection time (IT) ensures stability and accuracy for high-resolution mass-to-charge measurements and allows accurate quantitative analysis (Scigelova, & Makarov, 2013).

The LTQ-Orbitrap selects ions 'in time' through mass selective scans in the linear ion trap for both MS and MS^n approaches. In MS mode, the linear ion trap collects the ions of interest before passing

them into the C-trap for the next Orbitrap analysis. In MS^n mode, both collision-induced dissociation (CID) and HCD fragmentation can be performed. In the first mode, the linear ion trap operates as a separate low-resolution mass analyzer, in which fragmentation is activated by a supplemental RF field, producing a mass dependent scan at low resolution. In HCD mode its function is to isolate a particular precursor, which is then fragmented in the HCD cell (Michalski *et al.*, 2011).

The Q-Orbitrap can almost instantaneously select ions for both MS and MS/MS approaches, thanks to fast quadrupole switching times, and enables efficient multiplexing mode due to its ability to separate ions 'in space'. Indeed, only ions with a specified *m/z* value have stable trajectories and pass through the quadrupole towards the storage or fragmentation device before Orbitrap analysis. On their way from the ionization source, ions are transmitted from the bent flatapole into the quadrupole, capable of isolating ions with an isolation width ranging from 0.4 to 2.0 *m/z*, and then into the C-trap via a split lens, used to gate the incoming ion bean, and a short octapole (Figure 1.8; Michalski *et al.*, 2011).

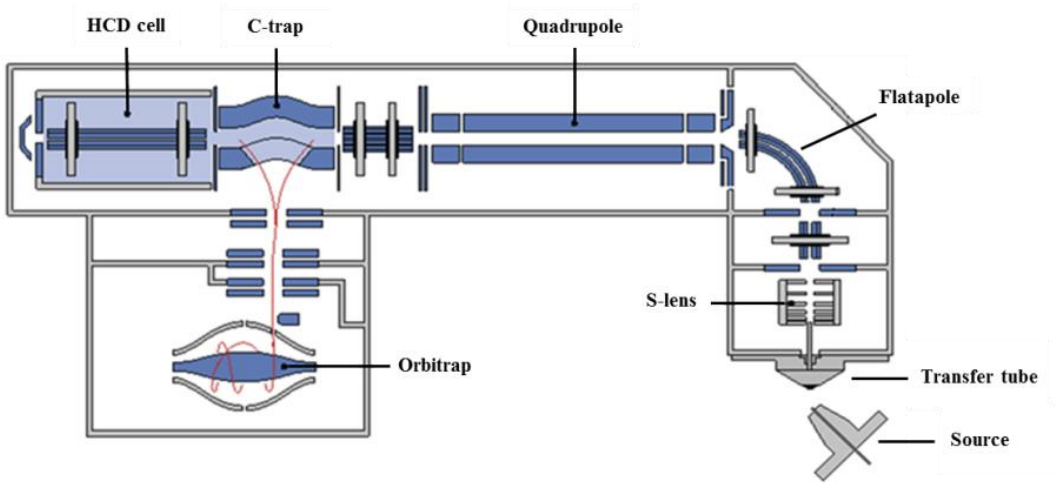


Figure 1.7. Hybrid Q-Orbitrap instrument (adapted from *www.planetorbitrap.com*).

In multiplexed single ion monitoring (SIM) mode, the quadrupole rapidly switches between different mass-to-charge ratios, allowing selected ions to be sequentially accumulated in the C-trap and then jointly analyzed in a single Orbitrap detection cycle. Similarly, in MS/MS experiments, the quadrupole captures different precursor ions in rapid succession, allowing the resulting fragment ions to be retained all together in the HCD cell and then jointly analyzed in the same Orbitrap detection cycle (Scigelova, & Makarov, 2013).

With a hybrid Orbitrap configuration different experiments can be performed, taking advantage of low-resolution mass analyzer isolation capabilities. In full MS / dd-MS^2, a full MS scan is followed by selective fragmentation of ions that satisfy pre-defined criteria (data-dependent MS/MS). In

targeted-SIM and targeted-MS^2, MS or MS/MS scans respectively are acquired only for ions of interest, defined in an 'inclusion list'. In targeted-SIM / dd-MS^2, targeted-SIM scans of precursor ions of interest are followed by their data-dependent triggered MS/MS scans. In full MS / AIF / NL dd-MS^2, a full MS scan is followed by an AIF-scan, in order to recognize user-defined *m/z* neutral losses (NL) between the two scan events and to automatically perform data-dependent MS/MS scans on selected precursor ions.

1.2.2.1. Neutral Loss experiments

Data-dependent analysis is commonly used to carry out both a survey scan (full MS) and product ion scan (MS/MS) in a single analytical run, but success still depends on whether the instrument can automatically and correctly select precursor ions. With hybrid Orbitrap, a variety of criteria for the selection of precursor ions is available, such as ionization intensity (threshold), exact mass, with or without a defined retention time window, or loss of user-defined neutral masses (Yang *et al.*, 2016). In particular, Neutral Loss experiments reveal all precursor ions that have lost a mass equal to the product ion mass + neutral loss mass ± user-defined mass tolerance (ppm), from comparison of full MS and AIF spectra. Data-dependent MS/MS spectra are then automatically acquired for the precursor ions thus identified, effectively providing detailed information on the analytes of interest without loss of time (Figure 1.8a; Yang *et al.*, 2016). Furthermore, a second criterion for the selection of precursor ions can be combined in the neutral loss experiment, defining the intensity threshold required for triggering dd-MS/MS (Figure 1.8b).

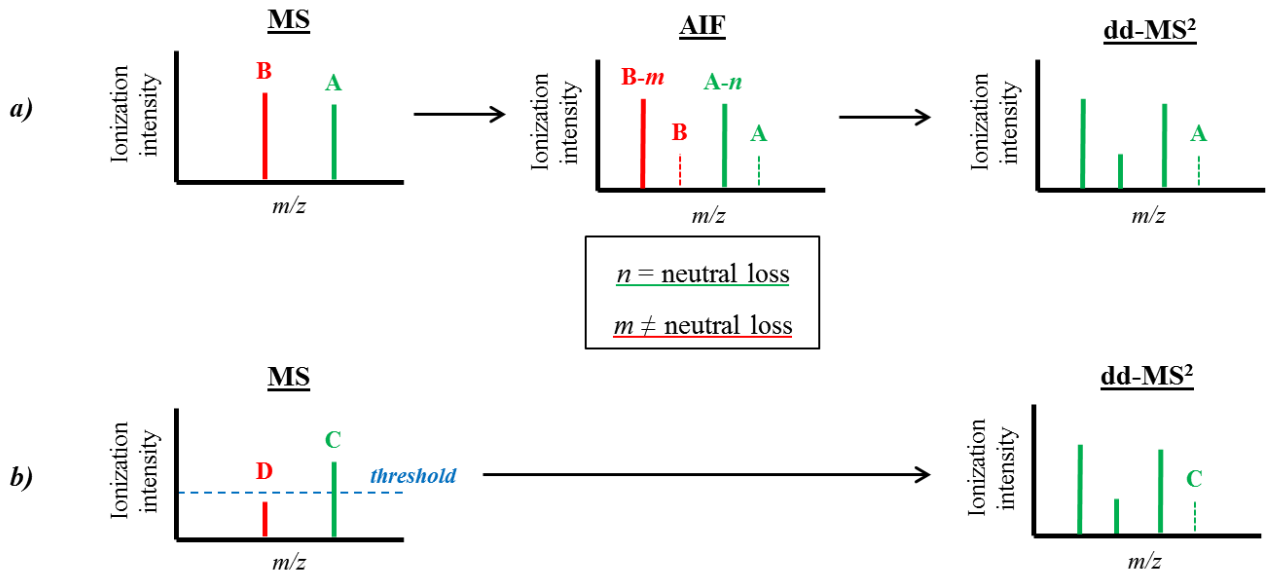


Figure 1.8. Schematic representation of neutral loss experiments and dd-MS/MS-triggering intensity threshold.

Finally, Figure 1.8c summarized the typical workflow of a Neutral Loss experiment, in which a full MS scan and an AIF scan are alternated in order to detect the user-defined neutral loss (for example in the Figure NL=sugar moyeties) and to isolate the precursor of interest to produce its specific dd-MS/MS spectrum.

c)

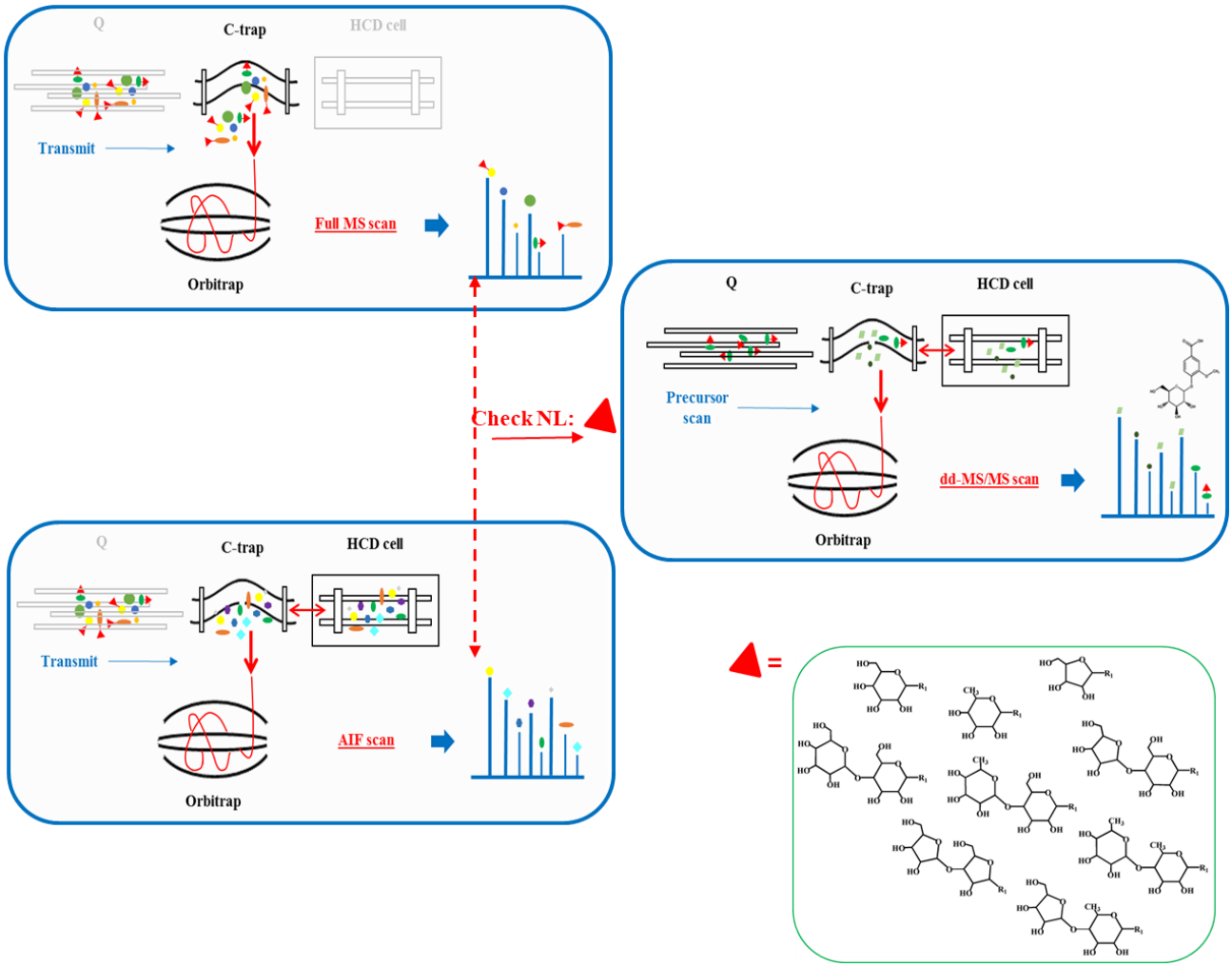


Figure 1.8. Schematic representation of neutral loss experiments and dd-MS/MS-triggering intensity threshold.

1.2.3. Orbitrap performance

The performance parameters that characterize the Orbitrap mass spectrometer are resolving power, resolution, mass accuracy, mass range and ion dynamic range (Hu *et al.*, 2005).

Resolving power is the ability to distinguish between ions differing in the mass-to-charge value by a small increment. It may be characterized by giving the peak width (expressed in mass units, as a function of mass) for at least two points on the peak, specifically at 5% and 50% of the maximum peak height (IUPAC Gold Book, 2014). Resolving power depends closely on the acquisition time (*e.g.* the longer the acquisition time the higher the resolving power) and is unaffected by the AGC target value. Furthermore, for a fixed acquisition time, the resolving power diminishes with the increase in ion mass, because the frequency of axial oscillation is inversely proportional to the square root of *m/z*, since Orbitrap ion trapping is induced by the electrostatic field (Makarov *et al.*, 2006). This decrease has been attributed to the formation of non-coherent packets of ions as a consequence of collision with the background gas. If the center-of-mass collision energy with residual gas remains constant, the collision cross section increases with mass, leading to faster scattering, fragmentation, and transient decay (Makarov *et al.*, 2006). Consequently, the resolving power affects the correct assignment of masses for analytes, and if set lower, the error increases due to co-elution of analytes with nearly-isobaric species (Makarov, & Scigelova, 2010).

Resolution is expressed as $m/\Delta m$, where m is the mass of the ion of interest and Δm is the peak width or the spacing between two equal intensity peaks, with a valley between them of no more than 10% of their height (Murray *et al.*, 2013). When the resolution is higher, mass analysis time increases, since the scan rate (Hz) decreases (Table 1.1).

Resolution	**17,500**	**35,000**	**70,000**	**140,000**
Mass analysis time (ms)	80	150	300	700
Scan rate (Hz)	12	7	3	1.3

Table 1.1. Trend for analysis time and scan rate in relation to resolution.

Mass accuracy depends closely on the instrument's resolving power (Perry *et al.*, 2008) and is typically <5 ppm for externally calibrated mass spectra and <2 ppm for internally calibrated spectra (Marshall, & Hendrickson, 2008). When the signal-to-noise ratio (S/N) is low, mass accuracy is principally affected by noise and there are no differences between mass measurements obtained using external or internal calibration. As the S/N ratio increases, mass accuracy improves considerably (<1 ppm) in the event of measurements obtained with internal calibration (Perry *et al.*, 2008). External calibration is indeed affected by the instability of inner electrode potential, due to

noise, and by thermal sensitivity. Consequently, thermal regulation of the Orbitrap and its high voltage supply makes it possible to keep mass error below 5 ppm for more than 20 h (Perry *et al.*, 2008).

The mass range is the range of mass-to-charge ratios (*m/z*) that the instrument can analyze. When it is modified, the voltage on the central electrode adapts rapidly to reduce ion motional amplitudes during the axial oscillation period and to prevent ion loss due to collision with the outer electrode (Marshall, & Hendrickson, 2008).

The ion dynamic range is the range over which the ion signal is linear with the analyte concentration (Makarov *et al.*, 2006).

1.2.4. Orbitrap applications

The Orbitrap mass analyzer has proven to be a useful tool for targeted and non-targeted screening and for qualitative and quantitative approaches in different fields, such as metabolomics, lipidomics, proteomics, clinical research, drug discovery, forensic toxicology, agricultural science and environmental and food safety (Ghaste *et al.*, 2016). In a targeted approach, the aim is to specifically analyze a limited number of known compound classes, while a non-targeted approach aims to profile as many features as possible in a given complex mixture without any *a priori* information (Scigelova, & Makarov, 2013).

Omic sciences demonstrate the ability of Orbitrap detection to define the composition of samples containing a broad number of analytes distributed over a wide range of mass-to-charge ratios and concentration levels (Perry *et al.*, 2008). Metabolomics aims to provide comprehensive identification and quantification of all metabolites in biological samples (Perry *et al.*, 2008), contributing to defining the architecture of metabolic pathways. It focuses on both primary metabolites, such as organic acids, amino acids, sugars, sugar alcohols, sugar phosphates, amines, fatty acids, polar lipids, hormones and vitamins, as well as specialized metabolites like phenols, flavonoids, monoterpenes, sesquiterpenes, polyketides, alkaloids and others (Ghaste *et al.*, 2016). Lipidomics measures lipid molecular species in cells, tissues, or organisms (*e.g.* many polar lipids, fatty acids, eicosanoids and fat soluble vitamins), but some scientists consider lipidomics to be part of metabolomics, since there is an overlap with metabolites usually covered by these two omic sciences (Ghaste *et al.*, 2016). As regards proteomics, protein identification is the main goal of this field, although complementary studies profile endogenous peptides in human fluids, bioactive peptides arising from proteolytic processing of human precursor proteins and neuropeptides from animal species. Furthermore, quantitative analysis of proteins and the characterization and

monitoring of post-translational modification of intact proteins, so-called top-down proteomics, has become an integral part of more recent proteomic studies (Scigelova, & Makarov, 2013).

In the last few years, food authenticity and safety have aroused concern, requiring the development of new approaches able to understand and control process contamination, food adulteration and food contaminants such as pesticides and mycotoxins in particular (Senyuva *et al.*, 2015). Furthermore, close attention has been paid in agriculture to determining mycotoxins, profiling human health-related metabolites and studying the effect of different diets on animal metabolism (Ghaste *et al.*, 2016).

To conclude, the diffusion of the Orbitrap mass analyzer has been attributed to its high sensitivity, relatively short analysis time, wide dynamic range, high reproducibility and, most importantly, its ability to analyze samples of extreme molecular complexity (Ghaste *et al.*, 2016).

1.3. Glycosides

Carbohydrates represent the principal products of photosynthesis, are the most diffuse organic compounds in plant tissues and constitute the main source of carbon and nutrition for many organisms (Minic, 2008). They can be divided into monosaccharides, such as glucose and fructose, disaccharides, such as sucrose and trehalose, and polysaccharides, such as cellulose and starch. In plants, carbohydrates are part of cell wall polysaccharides, are used to store metabolites or generate glycoconjugates, such as glycolipids and glycoproteins, and have important physiological functions, such as growth, defense against pathogens, signaling and interaction with the environment (Minic, 2008). Due to carbohydrates' great structural diversity and the selectivity of enzymatic reactions in plants, a conspicuous number of enzymes is involved in the carbohydrate metabolism. Glycosyltransferases are responsible for their synthesis, carbohydrate esterases for their modification and glycoside hydrolases and polysaccharide lyases for their breakdown, all being grouped together as carbohydrate-active enzymes (CAZY) and classified on the basis of their amino acid sequence (Minic, 2008).

Glycosides are organic compounds characterized by semi-acetal linkage occurring between the reducing group of sugar and the nucleophilic group of another organic molecule called an aglycone (Swain, 2012). The glycosylation of plant metabolites encompasses chemically different compounds from various biosynthetic pathways (Boeckler *et al.*, 2011) and produces noteworthy modifications in their chemical properties, altering not only their polarity, volatility or chemical stability, but also their chemical and biological activity (Cheynier *et al.*, 2013).

1.3.1. Chemistry of glycosides

Glycosides can be classified by the type of glycosidic bond, the glycone or sugar, and the aglycone (Swain, 2012).

As regards the type of glycosidic bond, glycosides are principally divided into *O*-glycosides, *S*-glycosides, *N*-glycosides and *C*-glycosides, on the basis of the nucleophile involved (Swain, 2012). Division into α- and β-glycosides has also been reported, depending on the relative stereochemistry (R and S) of the sugar anomeric carbon and the stereocenter furthest from C1 (Bertozzi, & Rabuka, 2009). *O*-glycosides, which are most common in plants, involve an alcoholic or phenolic hydroxyl group in the aglycone and can be hydrolyzed to the corresponding constituents (sugar and aglycone) by enzymes or acids. *S*-glycosides or thioglycosides, which are less common in nature, show a sugar moiety linked to a thiolic group, while *N*- and *C*-glycosides involve an amino group and a carbon atom respectively. However, in the last two cases IUPAC discourages use of the term glycosides, suggesting the terms glycosylamines and C-glycosyl compounds respectively (IUPAC

Gold Book, 2014). Finally, depending on whether the anomeric carbon and the stereocenter furthest from C1 share the same stereochemistry or not, an α- or β-glycosidic bond occurs respectively (Bertozzi, & Rabuka, 2009).

As regards glycones, several different sugars are detected in glycosides, but the most common are aldohexoses, especially glucose, deoxyhexoses such as rhamnose, and pentoses. Furthermore, glycosides can show more than one sugar moiety bond to the aglycone, both as monosaccharides linked to different hydroxyl groups and as di- or trisaccharides linked to the same hydroxyl (Swain, 2012). However, glycones are quite homogeneously diffuse in nature and are not specific to any particular group of plants (Swain, 2012).

Glycoside classification by aglycones is based on their chemical nature and is the most useful, in particular for biochemical and pharmacological purposes. It distinguishes between alcoholic and phenolic glycosides, flavonoid glycosides, coumarin glycosides, anthraquinone glycosides, cyanogenic glycosides, cardiac glycosides, saponins and terpene glycosides (Swain, 2012).

1.3.1.1. Glycosides of phenolic compounds

Glycoside formation allows storage of phenols in plants in a form in which they do not interfere with more vital cellular mechanisms. When free phenols naturally occur in higher plants, they are usually accumulated in storage tissues, such as seeds and berries, or in dying or dead tissues, such as heartwood of trees (Harborne, 1964). Generally, only anthraquinones, hydroxycinnamic and hydroxybenzoic acids can occur in the free form and are usually found in lower plants (Hopkinson, 1969). In some cases, phenols can escape glycosylation if conversion to less reactive derivatives occurs, through methylation, esterification or other similar reactions. In the biological literature, the process with which plants convert phenolic compounds to glycosides or other derivatives is referred to as ‘detoxification’. Glycosides, once produced, are not moved from the tissues of synthesis and are stored in the vacuole, which is a site of low metabolic activity in cells, where they remain until the cell dies (Harborne, 1964).

As regards low-molecular-weight phenols, glycosylation can occur both on aryl alcohols and phenols, but when alcoholic and phenolic hydroxyl groups coexist in the same molecule, the former is the only one to be glycosylated (Harborne, 1964). The occurrence of hydroxyalkylphenyl glycosides is attributed to the glycosylation of substituted phenolic acids and aldehydes, which then incur reducing reactions to the corresponding phenolic alcohols (Harborne, 1964). In particular, phenolic acids can be combined with sugars through the glycosidic bond, ester bond or contemporaneously through glycosidic and ester bonds. Glucose esters of phenolic acids are widely distributed in the natural world, since they appear to be important in the normal physiology of

plants and vary depending on the organ or tissue where they are synthesized (Harborne, 1964). Five regioisomers for each pair of α- and β-anomers exist for the glucose esters of hydroxycinnamic acids (Jaiswal *et al.*, 2014), but only one out of ten isomers was unambiguously characterized using NMR spectroscopy (Du *et al.*, 2006). Multiple isomers have indeed been observed in LC-MS analysis of plants, without any assignment of regio- and stereochemistry.

As regards flavonoids, the formation of flavonol and flavone glycosides is strongly influenced by light, and consequently their occurrence is predominant in leaves and fruit skin (Shahidi, & Naczk, 2003). Flavonol glycosides are the most diffuse in plants and can occur as mono-, di- and triglycosides, although the latter is less frequently reported. In monoglycosides, the sugar moiety is generally linked to the 3-position, glycosylation in position 5, 7, 3′ and 4′ rarely being reported, while diglycosides can occur as 3-*O*-diglycosides and 3,7-di-*O*-glycosides (Harborne, 1964). Glucose and rhamnose are the most common sugars, but galactose, apiose and arabinose have also been detected. Furthermore, the flavonol glycosidic pattern includes acylated glycosides in which hydroxycinnamoil residues are attached to the sugar moiety (Shahidi, & Naczk, 2003). In flavones and flavanones the sugar moiety, principally glucose and rutinose, is linked to position 7 and rarely to position 5, in flavononols it often occurs as disaccharides attached to position 7, while for isoflavones glycosylation is relatively irrelevant. Anthocyanidins are described as monoglycosides with the sugar linked to position 3 and as diglycosides with the sugars attached to positions 3 and 5 (Shahidi, & Naczk, 2003). In all these cases, the glycosidic bond occurring between the flavonoidic and sugar moieties involves a hydroxyl group, but analogues with a carbon-carbon bond have also been detected. They are known as C-glycosylflavonoids and always show linkage with sugars in the position adjacent to that of the phenolic hydroxyl group. The latter indeed seems to be necessary for the activation of the adjacent position involved in glycosylation (Shahidi, & Naczk, 2003).

Finally, anthraquinone glycosides are colored compounds with limited distribution in plants. Their aglycone is an aromatic organic compound, where the keto groups are located on the central ring and one or more hydroxyl groups on the external aromatic rings. The most common sugars are glucose and rhamnose and they are generally bound through oxygen in position 8, or more rarely in position 1. Furthermore, *O*-rhamnose at position 6 and *O*-glucose at position 8 can also be contemporaneously present in the same molecule (Brown, 1980). *C*-glycosides, such as aloin, have also been reported as anthraquinone derivatives and are mainly diffuse in plants of the *Aloe* species (Swain, 2012).

1.3.1.2. Non-phenolic glycosides

Cyanogenic glycosides derive from hydroxynitriles, are bound to sugar through oxygen and release hydrogen cyanide after sugar hydrolysis. Three types of cyanogenic glycosides can be distinguished on the basis of the chemical structure of the amino acid of origin. The first type derives from phenylalanine and yields benzaldehyde and hydrogen cyanide after sugar hydrolysis. The most representative are amygdalin (Figure 1.9*a*) and prunasin. The second type is the glucoside of hydroxyisopropyl cyanide and yields acetone and hydrogen cyanide after sugar hydrolysis. The most representative is linamarin. The third type is gynocardoside which yields hydrogen cyanide and a diketone of unknown structure on hydrolysis (Swain, 2012).

Cardiac glycosides are structurally related to steroids, since a lactone ring and a sugar are attached at position 3 of the cyclopentanophenanthrene skeleton (Figure 1.9*b*). They can be divided into two groups on the basis of the size of the lactone ring. Cardenolides are characterized by a five-membered ring, while bufanolides are marked by a six-membered one. Sugars are usually derivatives of deoxymethylpentose and can be further acetylated, while hexose is rarely common (Swain, 2012).

Saponins combine both hydrophilic and lipophilic groups in their structure, showing surfactant properties and giving a stable foam when shaken with water (Osbourn, 1996). The hydrophilic moiety is constituted by one or more sugar residue, usually glucose and galactose, which can only be attached to position 3, as in the case of monodesmosides, or to both positions 3 and 22, as in the case of bisdesmosides (Rao, & Gurfinkel, 2000). The lipophilic moiety can be characterized by a triterpenoid or steroidal structure, depending on the number of carbons, and a subclass incorporating nitrogen can be distinguished in the second group (Figure 1.9*c*). The latter compounds are also called steroidal amines and show the same chemical and pharmacological properties as natural alkaloids (Sparg *et al.*, 2004).

Terpene glycosides are natural compounds made up of two or more 2-methylbutane residues, also referred to as isoprene units, and can be divided mainly into monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes on the basis of the aglycone structure (Breitmaier, 2006). The isopropyl part of 2-methylbutane residue is defined as the head, while the ethyl residue as the tail, and each isoprene unit is linked to the others from head-to-tail (Figure 1.9*d*). One or more sugar moieties can be attached to terpenes, depending on the number of hydroxyl groups born by aglycones. Glucose and galactose are most common among monosaccharides, while rutinose, arabinosylglucose and xylosylglucose are most common among disaccharides (Stahl-Biskup, 1987).

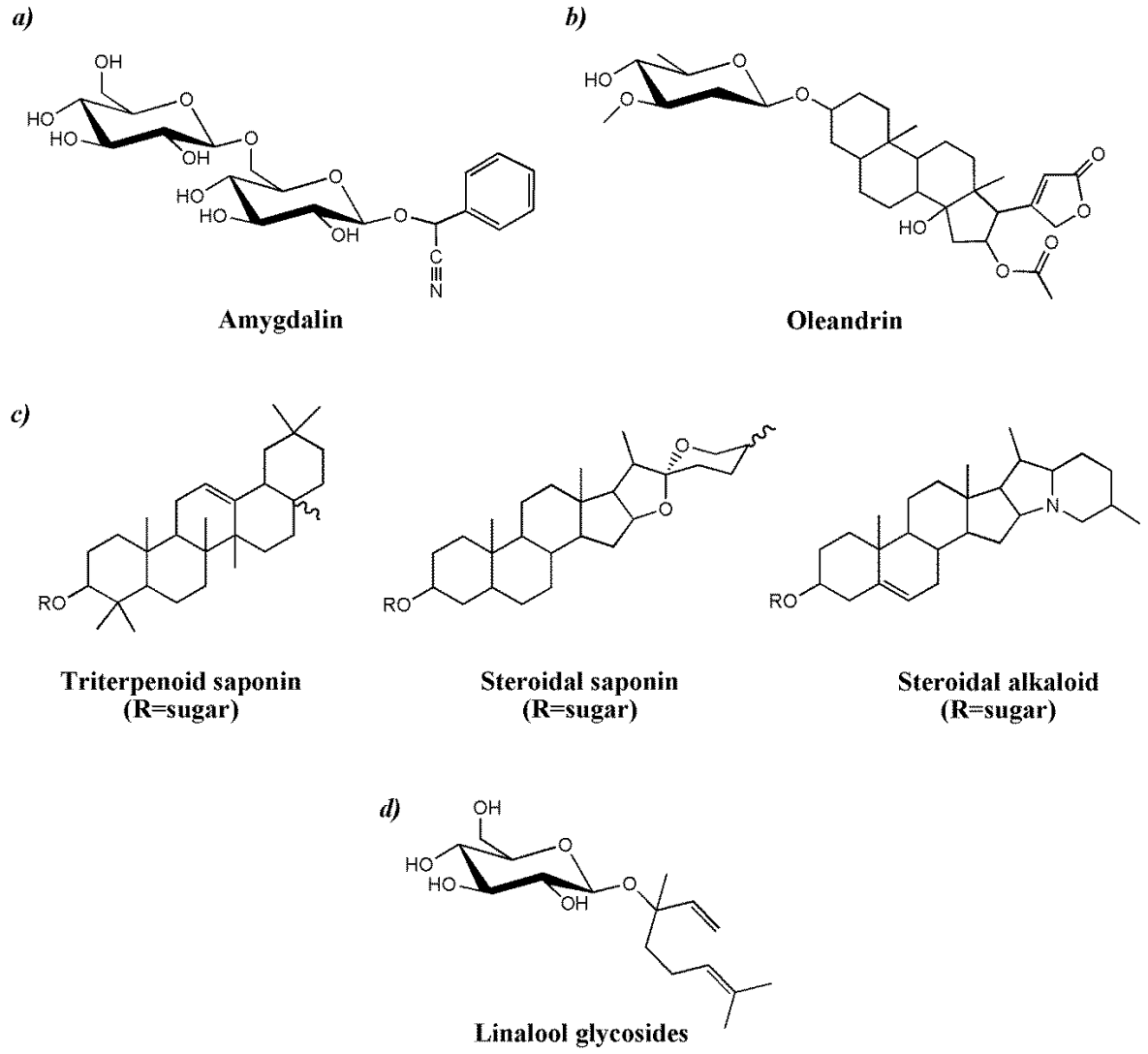


Figure 1.9. Examples of non-phenolic glycosides.

1.3.2. Biosynthesis of glycosides

Plants can form glycosides through highly specific enzymes such as glycosyltransferases, which catalyze the transfer of sugar moieties from donor molecules to a specific aglycone through highly stereo- and regiospecific reactions (Cheynier *et al.*, 2013).

Glycosyltransferases can be classified in 103 families (carbohydrate-active enzymes database; CAZy) on the basis of similarities between their amino acid sequences and are defined as Leloir and non-Leloir enzymes, depending on the type of glycosyl donor used (Xu *et al.*, 2016).

Leloir glycosyltransferases are the most common in nature, require activated glycosyl donors, such as nucleotides, and can retain (α→α) or invert (α→β) sugar anomeric configuration depending on the reaction mechanism (Xu *et al.*, 2016).

For retaining glycosyltransferases, a double displacement mechanism has recently been suggested (Figure 1.10; Weijers *et al.*, 2008). In the first step, the enzyme carboxylate residue, generally provided by aspartic acid and glutamic acid (Asp; Glu), performs a direct S_N2 attack on the anomeric position of the nucleotide sugar moiety, forming a β-covalently-linked enzyme-glycosyl intermediate. Then, the glycosyl acceptor, activated through deprotonation by another enzyme carboxylate residue, performs a direct S_N2 attack on the anomeric position of the intermediate sugar moiety, producing glycosides with the same anomeric configuration as the glycosyl donor.

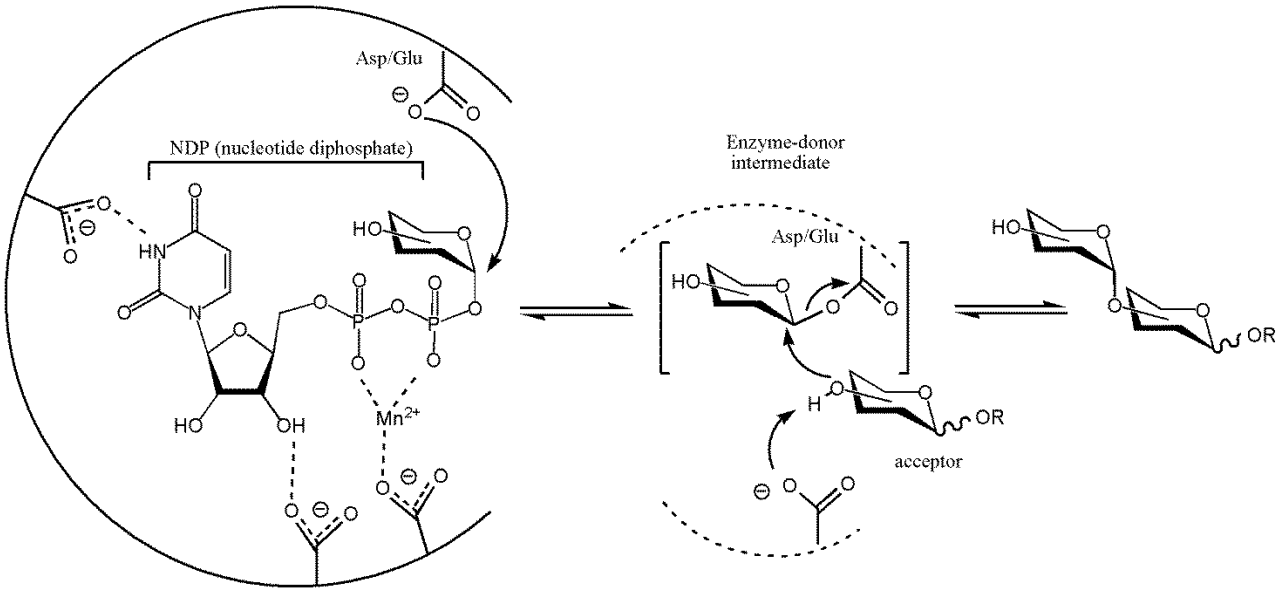


Figure 1.10. Reaction mechanism for retaining glycosyltransferases (adapted from Weijers *et al.*, 2008).

For inverting glycosyltransferases, a single-step mechanism is reported (Figure 1.11; Weijers *et al.*, 2008). The enzyme carboxylate residue (Asp; Glu) deprotonates the glycosyl acceptor, inducing its S_N2 attack on the anomeric position of the nucleotide sugar moiety and the formation of an oxocarbenium-like transition state. Subsequent formation of the glycosidic bond between the acceptor and the sugar yields glycosides with the opposite anomeric configuration to the glycosyl donor.

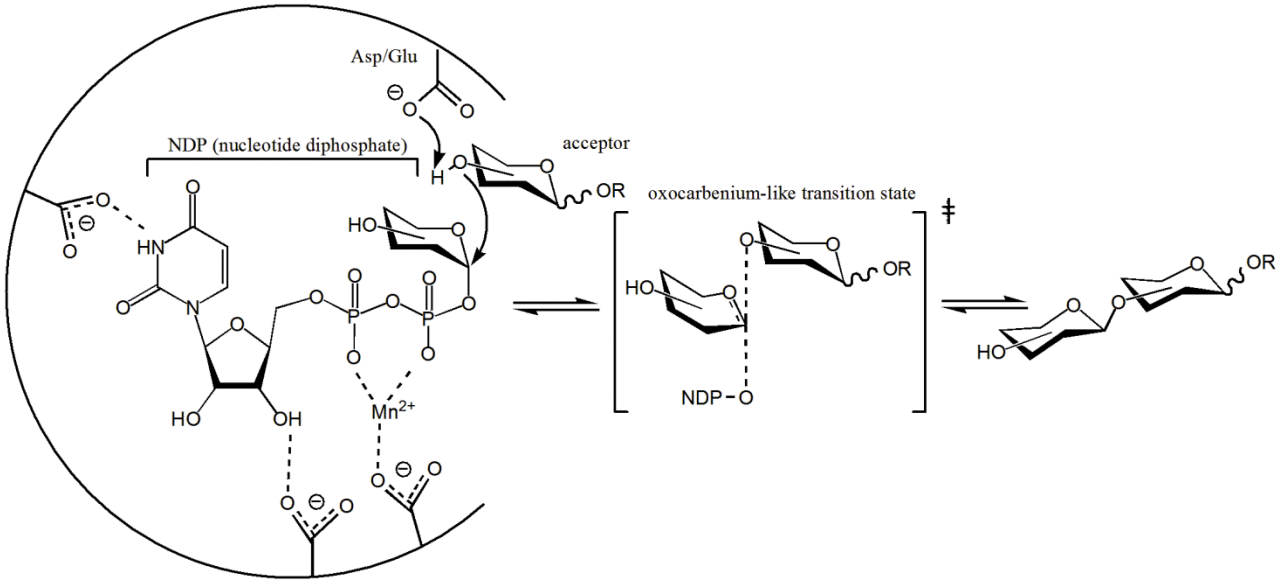


Figure 1.11. Reaction mechanism for inverting glycosyltransferases (adapted from Weijers *et al.*, 2008).

For both retaining and inverting glycosyltransferases, the Leloir pathway starts with the activation of sugar, first to phosphate-sugar and then to nucleoside 5′-triphosphate, and with its conversion to nucleotide-activated sugar, which is used in stoichiometric amounts (Weijers *et al.*, 2008).

1.3.3. Functions of glycosides in plants

No single and specific function can be assigned to glycosides, since their biological activity depends closely on aglycones and glycones occurring in the structure (Hopkinson, 1969). Generally speaking, glycosylation increases the water solubility of many compounds, can protect hydroxyl or phenolic groups from chemical and enzymatic oxidation when occurring and can suppress or decrease the toxicity of phytotoxic aglycones. Furthermore, it can increase the bioavailability of drugs that need to pass through the blood-brain barrier and improve pharmacokinetics, facilitating membrane transport through sugar transporters and mediating cell-specific delivery by targeting carbohydrate receptors (Xu *et al.*, 2016). Finally, glycosylation can be a required step in metabolites biosynthesis, as in the case of coumarins generated from cinnamic acid glycosides (Harborne, 1964) or lignins presumably produced from glucosides of cinnamyl alcohols (Pridham, 1965).

1.3.3.1. Role of the glycosides of phenolic compounds in plants

Glycosides of low-molecular-weight phenolic compounds are widespread in plants and are mainly involved in plant defense, since they can be converted into active substances in damaged plant tissues (Reichardt *et al.*, 1988). They can be readily translocated from the site of synthesis or storage to that of attack and enzymatically converted to defensive substances. Glycosylation

increases the water solubility of metabolites, facilitating their translocation, and attenuates their toxic properties when not required. Glycosides of phenolic compounds can also defend plants against herbivores, rendering the edible parts of plant undesirable as foodstuffs thanks to their bitter taste (Boeckler *et al.*, 2011). Furthermore, they can regulate plant growth, induce enzymatic inhibition and act as H_2O_2 scavengers during water stress (Le Roy *et al.*, 2016).

Flavonoid glycosides constitute the stored form of free flavonoids and can be easily moved through the plant, particularly under UV stress conditions. They play a significant role in determining flower, leaf, seed and fruit color or in attracting animals for flower pollination and seed dispersion, can contribute to defining fruit flavor and aroma, accumulating as bitter or non-bitter species, and are involved in plant defense against pathogens (Le Roy *et al.*, 2016). Finally, flavonoid glycosides can also control indole-3-acetic acid oxidase activity (Pridham, 1965).

1.3.3.2. Role of non-phenolic glycosides in plants

Cyanogenic glycosides participate in defending plants from the action of phytopatogens, act as feeding deterrents or phagostimulants, depending on the insect species, and are considered to be effective in deterring herbivores (Vetter, 2000). Furthermore, they improve plant robustness and viability in response to environmental challenges (Gleadow, & Møller, 2014). Cyanogenic glycosides are stored in the vacuole as inactive forms, but are activated in the cytoplasm by enzymatic hydrolysis when the plant is attacked, releasing toxic hydrogen cyanide (Gleadow, & Møller, 2014).

Cardiac glycosides are involved in plant defense, although their synthesis is significantly correlated with latitude and higher content develops in the tropics. Abiotic and biotic stress also impacts the production of cardiac glycosides (Agrawal *et al., 2012)*. In particular, production increases with the exposure of plants to CO_2, ozone fumigation, herbivore damage and bacterial infection, while decreasing with water stress and nitrogen fertilization. Therefore, although the effects are highly variable in different species, cardiac glycosides increase plant adaptation to environmental change.

Saponins constitute another class of glycosides involved in plant defense against bacteria and fungi. Monodesmosidic saponins are more active than bisdesmosidic saponins, but the latter can be converted into biologically active forms through the hydrolysis of sugar residues in position 22. Consequently, when mechanical damage or pathogen attacks occur, enzymes come into contact with saponins and proceed with hydrolysis. The mechanism of action of saponins towards pathogens involves the formation of complexes with membrane sterols, which results in the formation of pores and the loss of membrane integrity. Finally, in the case of steroidal glycoalkaloids, defense activity is pH-dependent (Osbourn, 1996).

Terpene glycosides are surmised to be involved in the biosynthesis of free monoterpenes in plants, since the highest glycoside content is usually detected before the maximum levels of the corresponding free forms. Furthermore, terpene glycosides are considered to be the preferred form for accumulation of free terpenes in undifferentiated cultures and their transport from the site of accumulation to that of metabolism. Indeed, due to their lipophilicity, free terpenes are unable to penetrate cells without destroying membranes (Stahl-Biskup, 1987).

1.3.4. Glycosides in food sources

The occurrence of glycosides in foods and beverages depends on the aglycone considered and the matrix examined. Generally speaking, flavonoids mainly occur as glycosides and phenolic acids as glucose esters. Stilbenes occur almost equally as aglycones and glycosides, while lignans never occur in the glycosidic form (Pérez-Jiménez *et al.*, 2010).

Of phenolic acid glycosides and glucose esters, derivatives of hydroxycinnamic acids are the most abundant (Neveu *et al.*, 2010). In particular, verbascoside, an ester of caffeic acid bound to hydroxytyrosol through the sugar unit, is characteristic of verbena (>1350 mg/100 g) and the black olive (68 mg/100 g). Hydroxybenzoic acid glycosides are present at lower levels, mainly in berries (1-5 mg/100 g) and fruit juices (3-10 mg/100 mL).

Flavonol glycosides are relatively widespread in food and beverages, with content ranging from a few mg/100 g (or mL) to several tens of mg. The main sources are spices (100-250 mg/100 g), black chokeberries (46 mg/100 g), olives (45 mg/100 g), onions (3-77 mg/100 g, increasing from the white to yellow and red varieties), beans (16-40 mg/100 g) and cereals (8-36 mg/100 g), followed by lingonberries, black raspberries, leafy vegetables, bottled tea and fruit juices, with concentrations ranging from 10 to 20 mg/100 g (or mL). In nuts, tea (infusion), beer and wine, flavonol glycoside content does not exceed 5 mg/100 g (or mL; Neveu *et al.*, 2010).

Flavone and flavanone glycosides are less common in foods and beverages, although the former are relatively abundant. Flavone glycosides are mainly present in herbs (300-1100 mg/100 g) and spices (100-600 mg/100 g), followed by cereals (7-45 mg/100 g), vegetables and fruit juices (1-5 mg/100 g and mg/100 mL). Flavanone glycosides are mainly present in herbs (80 mg/100 g), while in nuts, fruit, vegetables and fruit juices they are found at lower levels (1-5 mg/100 g and mg/100 mL).

Flavanol glycosides are characteristic of cocoa powder and chocolate (>5 mg/100 g), while dihydroflavonol glycosides characterize wines (1-5 mg/100 mL) and dihydrochalcone glycosides fruit and fruit juices (1-5 mg/100 g and mg/100 mL; Neveu *et al.*, 2010).

Anthocyanidin glycosides, also known as anthocyanins, are mainly present in dark-colored fruit and vegetables. Specifically, the main sources are black elderberries (1316 mg/100 g), black

chokeberries (878 mg/100 g) and black-currants (595 mg/100 g), followed by red wine, colored beans, blood oranges, red lettuce and red onions (Neveu *et al.*, 2010).

Stilbene glycosides are relatively widespread in food and beverages, but are present at trace levels (<1 mg/100 g and mg/100 mL). The main sources are grapes (0.06-0.2 mg/100 g), wine (0.2-0.9 mg/100 mL), chocolate (0.1 mg/100 g), nut oil (0.01 mg/100 g) and lentils (0.09 mg/100 g; Neveu *et al.*, 2010). Anthraquinone glycosides are rarely diffuse in food sources, but are mainly present in Aloeaceae and Rubiaceae plants (Swain, 2012), where they are found in fresh leaves, with content ranging from 0.5% to 1% of their weight.

As regards glycosides of non-phenolic compounds, cyanogenic glycosides are the most diffuse in foods and mainly occur in almonds, sorghum, barley, flax, cassava, lima beans, fruit (*e.g.* apples, peaches, apricots, plums, nectarines and cherries) and bamboo shoots. Their levels vary widely with the *cultivar*, climatic conditions, plant part and degree of processing. The most widespread compounds are amygdalin, prunasin and linamarin and content ranges from 70 to 200 mg/100 g (Haque, & Bradbury, 2002). Amygdalin is the cyanogenic glycoside responsible for the toxicity of the seeds of many species of Rosaceae, such as bitter almonds, peaches and apricots.

Cardiac glycosides are very rare in foods but are quite diffuse in ornamental shrubs, where they are present in the stem, sap, leaves, fruits and seeds. The main sources are oleander, wintersweet, bushman's poison, sea-mango, frangipani, balloon cotton, redheaded cotton-bush, king's crown, rubber-vine and cane toad. Furthermore, cardiac glycosides can also occur in mono- and dicotyledons, and their geographical distribution is irregular (Radford *et al.*, 1986).

Saponins are relatively widespread in food and dietary intake has been estimated at 15-240 mg daily, depending in particular on the type and the amount of legumes consumed (Rao, & Gurfinkel, 2000). The main sources are soya and its derivatives, beans, legumes, edible seeds, tomatoes, asparagus, tea, peanuts, spinach, blackberries, liquorice, herbs and ginseng (Price *et al.*, 1987). Saponins are also present in many medicinal plants and are found in different parts of the plant, such as the roots, stems, bulbs, leaves and fruits (Rao, & Gurfinkel, 2000).

Terpene glycosides are broadly distributed in the natural world, both in essential oil and non-essential oil-bearing plants, and can often exceed the amount of the corresponding free forms in a ratio ranging from 2:1 to 5:1 (Winterhalter, & Skouroumounis, 1997). In food and beverages, grapes and wine are the main sources of terpene glycosides, where they are mostly present in the form of *O*-β-D-glucopyranosides, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides, 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides and 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides (Schwab *et al.*, 2015). However, other sources are plants belonging to the Actinidiaceae, Apiaceae,

Berberidaceae, Cupressaceae, Lamiaceae, Pinaceae, Rosaceae and Solanaceae families, where they are found in the roots, stems, leaves, fruits and petals (Winterhalter, & Skouroumounis, 1997).

1.3.5. Nutritional and physiological effects of glycosides

Many bioactive compounds are glycosides, where the glycosidic residue can be essential for their activity or only to improve the pharmacokinetic parameters. However, it is difficult to broadly define the biological properties of glycosides compared to the respective aglycones, because their activity depends closely on timing and placing of glycosidic cleavage. Many glycosides are hydrolyzed in the stomach by the acidic environment or in the intestine by the action of glycosidases, while others cannot be easily hydrolyzed in this way and should be metabolized later in the colon through the action of intestinal microflora (Kren, & Martínková, 2001).

Only after the hydrolysis of the sugar moiety the aglycone becomes small enough to be transported through the gut wall into the bloodstream and then around the body (Aldred, 2009).

1.3.5.1. Bioavailability and health effects of the glycosides of phenolic compounds

Glycosylation of phenolic compounds plays a significant role in the prevention of phenolic groups from air or enzymatic oxidation, ensuring antioxidant and nutraceutical properties for molecules taken in through the diet. However, it usually decreases the antioxidant activity of glycosylated phenolic compounds compared to that of the corresponding aglycones. In particular, glycosylation of flavonols mainly affects their antioxidant activity when the substitution occurs in the B ring and when the number of sugar moieties at the same position increases. The same trend also characterizes sugar esterification of hydroxycinnamates (Williamson *et al.*, 1999).

The glycosides of phenolic compounds are generally metabolized in the large intestine by local microflora, although aglycones sometimes appear in the plasma within 30 minutes of ingestion, indicating the rapid absorption of several compounds (*e.g.* flavonols and isoflavones) in the small intestine (Day *et al.*, 2000). Anthocyanins are an exception, since intact glycosides are the predominant form in blood probably due to direct absorption at gastric level (Gutiérrez-Grijalva *et al.*, 2016).

As regards anthraquinone glycosides, they remain unaltered until they enter the gut and are cleaved off by microorganisms of the caecum, where they induce water and electrolyte secretion producing cathartic and direct irritant effects (Aldred, 2009). They can also act as antifungal agents, inhibitors of excessive renal tubular cell proliferation and modulators of inflammation by partially inhibiting cyclooxygenase. Finally, antimicrobial activity has been reported against *Bacillus subtilis*, *Bacillus*

cereus, *Saccharomyces cerevisiae* and *Candida albicans* but not against *Escherichia coli* (Brown, 1980).

1.3.5.2. Bioavailability and health effects of non-phenolic glycosides

Cyanogenic glycosides are potentially toxic compounds because they can produce hydrogen cyanide after enzymatic degradation, resulting in acute cyanide poisoning. In the intact plant these cyanogenic compounds are stored separately from hydrolytic enzymes, but crushing of plant materials during technical processes or following chewing obliterates this separation and induces enzymatic hydrolysis of cyanogenic compounds (Bolarinwa *et al.*, 2016). Acute cyanide poisoning causes rapid respiration, a drop in blood pressure, rapid pulse, headache, dizziness, vomiting, diarrhea, mental confusion, stupor, cyanosis, twitching and convulsions, while chronic cyanide intoxication yields to ataxic neuropathy that comprises lesions of skin, mucous membranes, optic and auditory nerves, spinal cord and peripheral nerves (Vetter, 2000). Cyanide detoxification in human body can be achieved though the conversion of hydrogen cyanide into thiocyanate, occurring in the presence of sulphur-containing amino acids through the action of rhodanase, but at doses between 0.5 and 3.5 mg/kg body weight cyanide poisoning can occur. However, due to the lack of quantitative toxicological tests and epidemiological information, it is difficult to establish a safe level for cyanogenic glycoside intake in many foods (Bolarinwa *et al.*, 2016).

Cardiac glycosides have both positive inotropic and negative chronotropic effects on the heart during cardiac failure. They increase the pumping capacity of the heart muscle and reduce its rate by inhibiting the Na^+/K^+-ATPase (Aldred, 2009). The role of these compounds as drugs for treatment of cystic fibrosis, ischemic stroke, heart ischemia, neurodegenerative diseases, spinobulbar muscular atrophy and other polyglutamine related diseases has also been reported (Prassas, & Diamandis, 2008).

Saponins are amphipathic molecules able to enhance the penetration of macromolecules such as proteins through cell membranes, due to their surfactant properties. For this reason, saponins have also been used as adjuvants in vaccines, even though they can induce red blood cell hemolysis by cell sterol complexation as a side effect (Sparg *et al.*, 2004). Furthermore, saponins have been shown to have hypocholesterolemic, anticoagulant, anticarcinogenic, hepatoprotective, hypoglycemic, immunomodulatory, neuroprotective, anti-inflammatory and anti-oxidant activity (Rao, & Gurfinkel, 2000).

Terpene glycosides are appreciated not so much for their biological activity as for their role as aroma precursors. Indeed, terpenes are odor-active compounds widespread in the natural world, while their glycosides are water soluble, storage-stable and odorless molecules that can release the

desired volatile compound through acid or enzymatic hydrolysis. The possibility of performing controlled release of odorous compounds has made terpene glycosides interesting aroma precursors employable in the food and cosmetic industries in order to prolong the perception of aroma and flavor (Schwab *et al.*, 2015).

1.3.6. LC-HRMS in the analysis of glycosides

Glycosides are generally analyzed in biological matrices, such as plant extracts or biological fluids, and so adequate sample clean-up approaches and separation methods become necessary for their profiling and structural characterization. Gas chromatography (GC), appropriate only for analysis of free aglycones after glycoside hydrolysis, liquid chromatography (LC) and capillary electrophoresis (CE) are suitable techniques for glycoside analysis (Kachlicki *et al.*, 2016). However, the LC-HRMS approach is the most useful in glycoside structural characterization, since measurement of four-decimal figure accurate mass with an error lower than 5 ppm makes it possible to define the elemental composition of both protonated/deprotonated molecules and their fragments, and to distinguish between isobaric compounds with different substitution patterns. Furthermore, the availability of hybrid HRMS spectrometers allows sequential fragmentation of precursor ions and structural identification of both sugar residues and aglycones (Forcisi *et al.*, 2013).

1.3.6.1 Cleavage of the glycosidic bond

The most characteristic fragmentation of glycosides is cleavage of the glycosidic bond, which occurs through different pathways, depending on whether they are *O*- or *C*-glycosides.

In the case of *O*-glycosides, cleavage generally occurs with the retention of glycosidic oxygen by the species formed from the reducing end, producing ions (B_i or Y_j; Figure 1.12) that can be detected in both positive and negative ion mode (Domon, & Costello, 1988). Depending on the number of sugar moieties present in the glycosides, cleavage of the glycosidic bond can directly release the aglycone (Y_0) or its still glycosylated derivatives Y_j, with j = n-1, where *n* is the number of sugar moieties remaining after each break of the terminal residue (Figure 1.12).

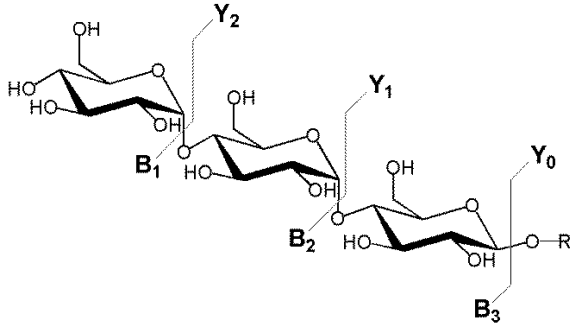


Figure 1.12. Example of glycosidic bond cleavage in a trisaccharide-glycoside (R=aglycone).

In positive ion mode, protonation of glycosidic oxygen can yield the B_i oxonium ion and the Y_j neutral fragment or *vice versa* the Y_j ion and the B_i neutral fragment, if cleavage is followed by a proton transfer from B to Y (Figure 1.13; Domon, & Costello, 1988).

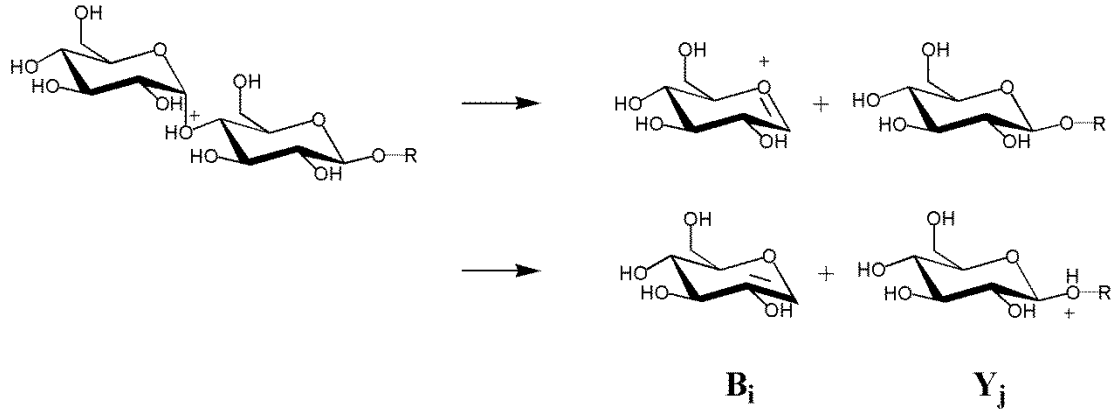


Figure 1.13. Cleavage mechanism of *O*-glycosides in positive ion mode (adapted from Domon, & Costello, 1988).

In negative ion mode, deprotonation generally affects a sugar hydroxyl group in position 4 or 6 and is accompanied by epoxide formation, which induces opening of the sugar ring and consequent cleavage of the glycosidic bond (Figure 1.14*a*; Domon, & Costello, 1988). This fragmentation pathway yields the Y_j ion and the B_i neutral fragment, but the B_i ion and Y_j neutral fragment are produced if proton transfer occurs between B and Y.

An alternative cleavage of the glycosidic bond, which can only occur in negative ion mode, consists of the retention of glycosidic oxygen by the terminal sugar residue, producing fragments C_i and Z_j (Domon, & Costello, 1988). In this case, deprotonation affects a hydroxyl group adjacent to the terminal sugar moiety and is accompanied by epoxide formation, which induces sugar release in the form of a C_i ion (Figure 1.14*b*). Here again, in the event of proton transfer from Z to C, a Z_j ion can also be produced.

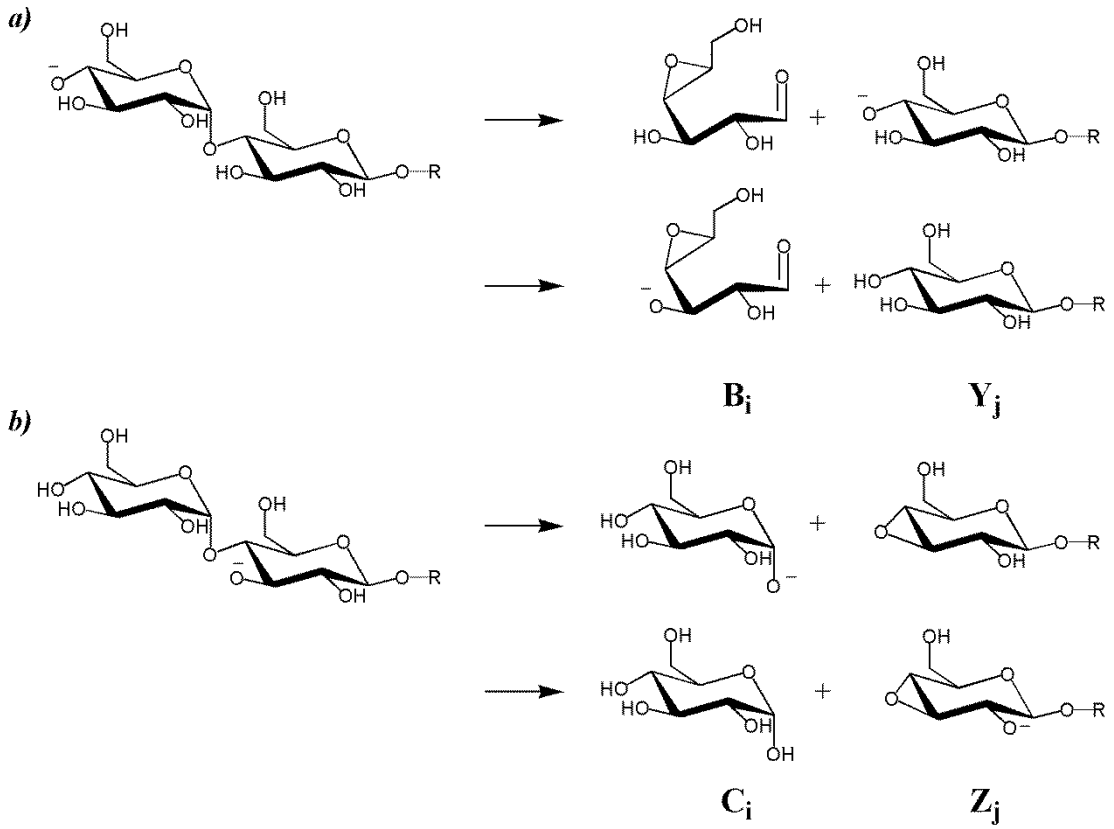


Figure 1.14. Cleavage mechanisms of *O*-glycosides in negative ion mode (adapted from Domon, & Costello, 1988).

The nature of the sugar moieties (*e.g.* hexose, deoxyhexose, pentose, hexose-hexose, etc.) that constitute glycosides can be established on the basis of the *m/z* value of ions B_i or C_i, or based on the difference between the *m/z* value of the precursor ion and fragments Y_j or Z_j. However, sugar isomers cannot be distinguished (Forcisi *et al.*, 2013).

In the case of the *C*-glycosidic bond, the carbon-carbon bond is more stable than the corresponding oxygen-carbon bond and thus cleavage of *C*-glycosides consists in the breaking of carbon-carbon bonds within the sugar ring (Forcisi *et al.*, 2013). The ions produced are labelled $^{k,l}X_j$ and $^{k,l}A_i$, depending on whether or not they include the aglycone, and the superscript *k* and *l* indicate the sugar ring bonds that have been broken (Figure 1.15; Domon, & Costello, 1988).

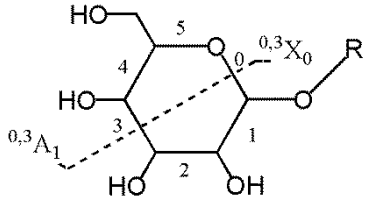


Figure 1.15. Sugar ring fragmentation and relative bond numbering.

1.4. Phenolic compounds

Phenolic compounds are among the most abundant and widespread secondary metabolites in the plant kingdom, with more than 8000 phenols currently known (Bravo, 1998), and they are accumulated in plants at the end of the pentose phosphate, shikimate, and phenylpropanoid pathways (Balasundram *et al.*, 2006). Structurally, they are characterized by an aromatic ring bearing one or more hydroxyl groups and are very heterogeneous, ranging from low molecular-weight compounds, such as simple phenols, to highly polymerized phenols, such as tannins (Bravo, 1998).

Although phenol occurrence in plants is attributed to resistance to pathogens and predators (Bravo, 1998), they constitute a fundamental part of the Mediterranean diet (Aguilera *et al.*, 2016). Indeed, the importance of consuming fruit, vegetables and plant-derived beverages (*e.g.* tea, wine and coffee), rich sources of phenolic compounds, has increased as phenols' preventive role in chronic diseases has been documented (Aguilera *et al.*, 2016). The most abundant phenolic compounds usually obtained through the diet are phenolic acids and flavonoids (30% and 60% of the total respectively; Baiano, & Del Nobile, 2016). The former generally occur as aglycones (free compounds), esters and glycosides, while flavonoids, the largest group of phenols including more than 5000 compounds, can be found as aglycones and glycosides (Baiano, & Del Nobile, 2016). Glucose, galactose, arabinose, rhamnose, xylose and mannose are the monosaccharides most frequently detected in glycosides, together with glucuronic and galacturonic acids (Escarpa, & Gonzalez, 2001). Associations with other compounds, such as carboxylic and organic acids, amines, lipids and other phenols have also been reported (Bravo, 1998).

In addition, phenolic compounds have been useful for taxonomic studies or for several industrial applications, such as the production of paint, paper and cosmetics, and they have been used as adulteration markers, tanning agents or food colorants and preservatives (Bravo, 1998).

1.4.1. Chemistry of phenolic compounds

Phenolic compounds are characterized by great structural heterogeneity and thus the general classification of their structure is based on the number of carbons that constitute the basic skeleton (Table 1.2.; Harborne, 1989).

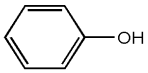
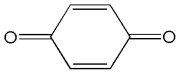
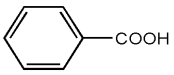
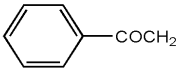
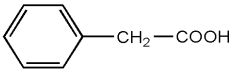
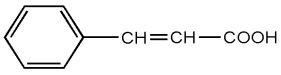
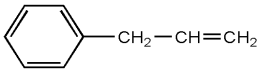
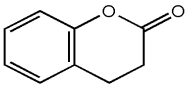
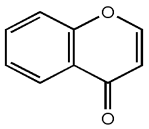
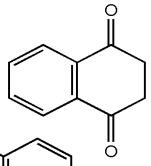
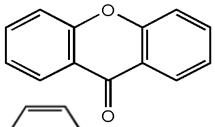
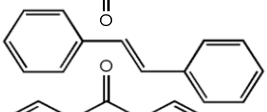
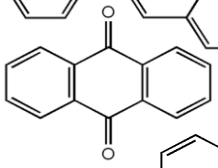
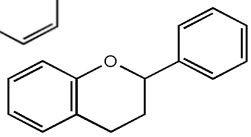
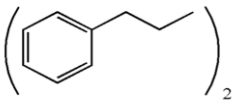
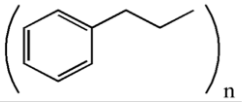
Chemical class	Carbon skeleton	Chemical Structure
Simple phenols	C6	
Benzoquinones	C6	
Penolic acids	C6-C1	
Acetophenones	C6-C2	
Phenylacetic acids	C6-C2	
Hydroxycinnamic acids	C6-C3	
Phenylpropenes	C6-C3	
Coumarins	C6-C3	
Chromones	C6-C3	
Naftoquinones	C6-C4	
Xanthones	C6-C1-C6	
Stilbenes	C6-C2-C6	
Anthraquinones	C6-C2-C6	
Flavonoids	C6-C3-C6	
Lignans	$(C6\text{-}C3)_2$	
Lignins	$(C6\text{-}C3)_n$	

Table 1.2. Phenolic compound classification.

The structure of phenolic compounds plays a decisive role in their activity as radical scavengers and metal chelators (Balasundram *et al.*, 2006). For example, the antioxidant activity of phenolic acids increases with the number of hydroxyl groups and decreases in the event of their substitution with

methoxyl groups. In addition, hydroxyl groups in *ortho*- or *para*- position of the carboxyl function reset antioxidant activity, while the same is not true for the *meta*- position. Furthermore, hydroxycinnamic acids show higher antioxidant activity than hydroxybenzoic acids, thanks to the presence of an α,β-unsaturated carboxyl group, which ensures greater H-donating ability and radical stabilization than the simple carboxyl function (Rice-Evans *et al.*, 1996).

Defining the structure-activity relationships of flavonoids is more complicated due to their greater structural complexity, however the main structural features pertain to rings B and C. For example, flavonoids' antioxidant activity increases with the number of hydroxyl groups in the B ring or with the combined presence of a double bond and a hydroxyl group in the C ring (*e.g.* flavononols). In the first case, hydroxyl groups, particularly if they are *orto*-dihydroxyls, confer higher electron delocalization and act as the preferred metal binding site, while in the second case the C ring structure described ensures co-planarity. Radical scavenging capacity is enhanced in the presence of a double bond conjugated with an oxo group in the C ring (*e.g.* flavones) and is affected by the substitution of hydroxyl groups with methoxyl ones in the B ring, due to the altered redox potential (Balasundram *et al.*, 2006).

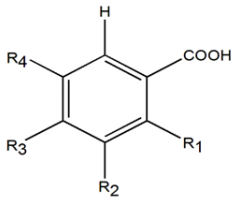
1.4.1.1. Low-molecular-weight phenols

Of low-molecular-weight compounds, the most common and important are simple phenols, phenolic acids and their aldehydic derivatives, phenylacetic acids, acetophenones, phenylpropanoids and their derivatives, chromones and coumarins, and cinnamyl alcohols (Alu'datt, *et al.*, 2017).

Simple phenols and their derivatives (*e.g.*, phenol, cresol, thymol, resorcinol, orcinol and hydroquinone) are widespread among different plant species and are characterized by an aromatic ring bearing one or more hydroxyl and methyl groups (Bravo, 1998).

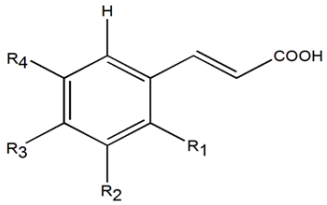
Phenolic acids consist of hydroxybenzoic and hydroxycinnamic acids and can occur in different forms of oxidation (*e.g.*, alcohol, aldehyde and propene). The first group includes gallic, gentisic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which have in common the C6–C1 structure. The latter constitutes the most widely distributed group of phenolic acids, also known as phenylpropanoids, is characterized by a three-carbon side chain (C6–C3) and is principally represented by coumaric, caffeic, ferulic and sinapic acids (Escarpa, & Gonzalez, 2001). They can occur as esters of tartaric and quinic acids, leading to hydroxycinnamoyltartaric and chlorogenic acids respectively, or linked through ester, ether or acetal bonds to other metabolites, such as flavonoids, anthocyanins, saponins, proteins, cellulose and glucose. Furthermore, the phenylpropanoid moiety can cyclize to form coumarins, dimerize to produce lignans, polymerize to

form lignins, or undergo side chain elongation leading to stilbenes and flavonoids (Stalikas, 2007). The structures of the most common hydroxybenzoic and hydroxycinnamic acids are shown in Figure 1.16.



Hydroxybenzoic acids

Compound	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
p-hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Protocatechuic acid	H	OH	OH	H
Syringic acid	H	OCH_3	OH	OCH_3
Gentisic acid	OH	H	H	OH
Salicylic acid	OH	H	H	H



Hydroxycinnamic acids

Compound	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
o-coumaric acid	OH	H	H	H
m-coumaric acid	H	OH	H	H
p-coumaric acid	H	H	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 1.16. Chemical structure of the most common hydroxybenzoic and hydroxycinnamic acids (adapted from Alu'datt *et al.*, 2017).

Finally, acetophenones and phenylacetic acids, which have in common the C6-C2 structure, are less frequently described in the literature, while phenylpropanoid derivatives (C6-C3) constitute an important group of low-molecular-weight phenols (Bravo, 1998).

1.4.1.2. Flavonoids

Flavonoids are characterized by a C6-C3-C6 skeleton and constitute the largest group of phenols naturally occurring in the plant kingdom (Harborne, 1989). Structurally, they consist of two aromatic rings, A and B, linked by a heterocyclic ring, C (Figure 1.17).

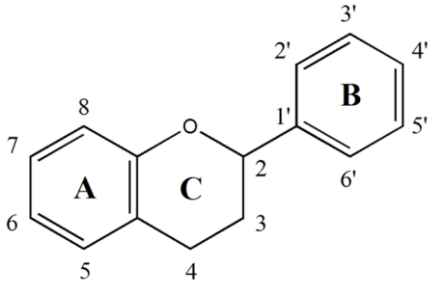


Figure 1.17. Basic chemical structure and numbering system for flavonoids.

Based on the substitution profile of the C ring, it is possible to distinguish between flavones, isoflavones, flavonols, flavanones, flavanonols, flavanols and anthocyanidins (Figure 1.18), while variations in the substitution in rings A and B through glycosylation, malonylation, methylation,

hydroxylation or acylation discriminate between compounds within each class (Balasundram *et al.*, 2006). Furthermore, prenylation and polymerization have an important impact on flavonoid function, solubility and degradation (Weston, & Mathesius, 2013).

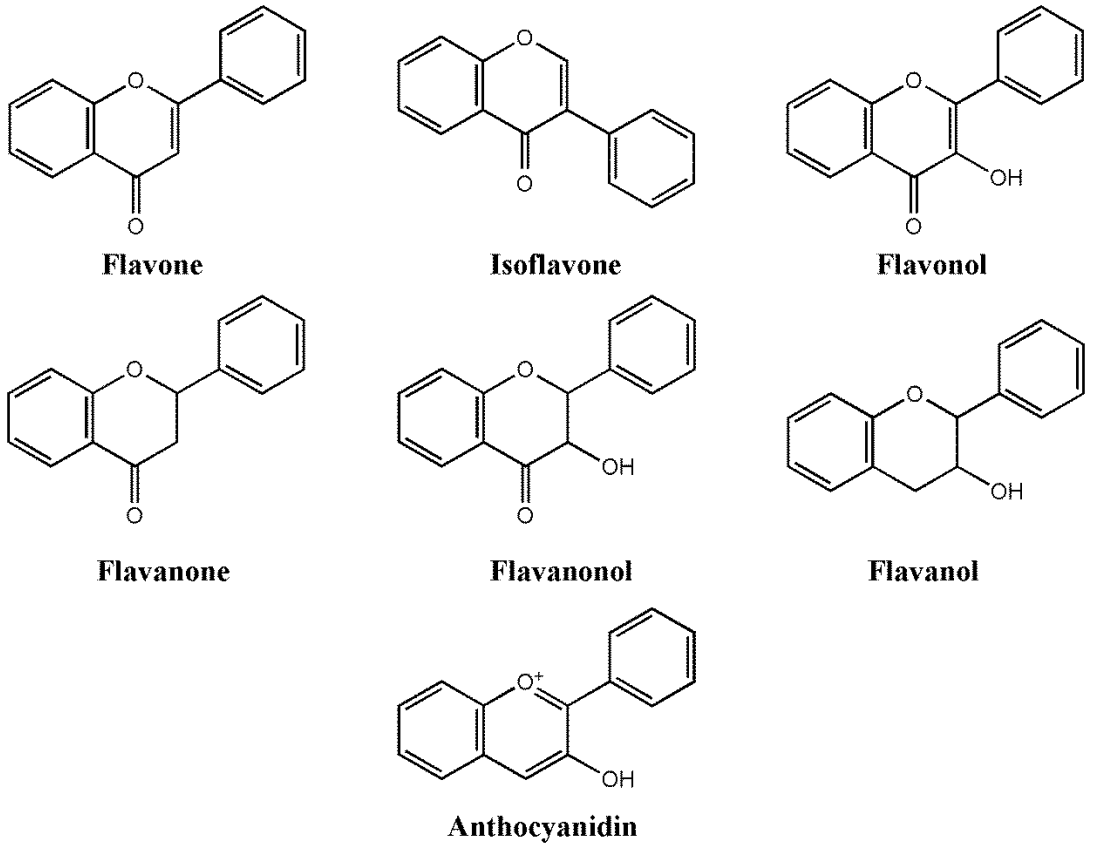


Figure 1.18. Basic chemical structure of the main classes of flavonoids.

Flavonoids can occur naturally both as aglycones and glycosides, and in the latter case they can be found in the form of *O*-glycosides and *C*-glycosides (Escarpa, & Gonzalez, 2001).

Flavones and isoflavones are not widely distributed in food, occurring principally as glycosides, with the most common flavones being apigenin and luteolin, and the most common isoflavones being genistein and daidzein.

In contrast, flavonols are very widely distributed in the natural world and constitute a fundamental part of the human daily diet. The most common are quercetin, kaempferol, myricetin and isorhamnetin. The structural composition of flavonol can vary greatly based on environmental factors, although in the case of glycosylated species only an *O*-glycosidic bond at position 3 is allowed (Escarpa, & Gonzalez, 2001). As regards sugar residues, the most common are glucose, galactose, rhamnose and glucuronic acid, in descending order, and their configuration is D- or L- depending on whether the bond involved is β or α respectively (Escarpa, & Gonzalez, 2001).

Flavanones are not extensively distributed in the plant kingdom, with the exception of citric fruit, where they constitute the main class of flavonoids. Here, they are present as aglycones and the most common are hesperetin, naringenin and narirutin, while in other plants they are diffuse as glycosides. As a rule, flavanone glycosylation occurs at position 7 in the A ring and involves glucose and rhamnose, both as monosaccharides and disaccharides (Escarpa, & Gonzalez, 2001).

Flavanonols differ from flavonones by having a hydroxyl group at position 3, as a result of which they are also known as 3-hydroxyflavonones or dihydroflavonols. They have two centers of asymmetry at positions 2 and 3 and are little present in plants. The most common are taxifolin, also known as dihydroquercetin, and aromadedrin, also called dihydrokaempferol.

Flavanols, or flavan-3-ols, are one of the most widespread flavonoid classes in nature, being principally present as aglycones. They can occur as monomers, such as (+)-catechin and (-)-epicatechin, as oligomers, also called procyanidins and constituted by dimeric associations of catechin and epicatechin, and as esterified forms, such as gallocatechin (Escarpa, & Gonzalez, 2001).

Finally, anthocyanidins are widespread in the plant kingdom, where they are responsible for fruit and flower color. The most common are cianydin, delphinidin, peonidin, pelargonidin, petunidin and malvidin. They can occur as aglycones, glycosides and acetylated forms, in the latter case being conjugated to hydroxycinnamic and organic acids. Conjugation, mainly performed with glucose, arabinose and galactose, can involve positions 3, 5, 7, 3' and 5', but the first is the most common. Anthocyanidin stability depends on pH, light exposure and susceptibility to oxidation (Escarpa, & Gonzalez, 2001).

1.4.1.3. Tannins and lignans

Tannins are phenolic compounds characterized by a high molecular-weight and noteworthy reactivity against proteins and carbohydrates (Escarpa, & Gonzalez, 2001). They can be divided into hydrolysable and condensed tannins, although there is also a third group, phlorotannins, which is only found in marine algae not included in the human diet (Bravo, 1998).

Hydrolyzable tannins are esters of gallic or hexahydroxydiphenic acids, so called gallo- and ellagitannins respectively, and are easily hydrolyzed with acids, bases, hot water and enzymes, yielding polyhydric alcohol and phenylcarboxylic acid. The most common hydrolyzable tannin is tannic acid, which is a gallotannin consisting of a pentagalloyl glucose (Bravo, 1998).

Condensed tannins, also called proanthocyanidins, are high-molecular-weight polymers produced through oxidative condensation of flavan-3-ols and flavan-3,4-diols, which occurs between carbon C-4 in the C ring and carbons C-6 or C-8 in adjacent units (Bravo, 1998). Considering the difficulty

in analyzing highly polymerized molecules, the dimers, trimers and tetramers of proanthocyandins are currently mainly reported in the literature. Furthermore, since interflavanoid linkage can be hydrolyzed by acids yielding anthocyanidins that are easily detected, this reaction is used to investigate the structure of proanthocyanidin oligomers (Bravo, 1998).

Lignans are high-molecular-weight phenolic compounds, constituted by two units of phenylpropanoids (C6-C3) joined together by the central carbons of their side chain. They can be divided into lignans, lignolides, monoepoxylignans and biepoxylignans (Shahidi, & Naczk, 2003).

1.4.2. Biosynthesis of phenolic compounds

Phenolic compounds are produced by two different biosynthetic pathways, namely the shikimate/chorizmate or succinylbenzoate pathway, which yields phenylpropanoid derivatives (C6-C3), and the acetate/malonate or polyketide pathway, which produces high-molecular-weight phenylpropanoids (C6-C3-C6).

The shikimate pathway, used by bacteria and plants but not by animals, is a seven-step metabolic route yielding aromatic amino acids such as phenylalanine, tyrosine and tryptophan (Bentley, & Haslam, 1990). Specifically, their synthesis starts with erythrose-4-phosphate, deriving from the pentose phosphate pathway, and phosphoenolpyruvate, produced during glycolysis, which combine to form a seven carbon sugar, 3-deoxy-*O*-arabino-heptulosonate phosphate (DAHP), which is then cyclized to form 3-dehydroquinate (DHS) and reduced to form shikimate (Figure 1.17; Shuab *et al.*, 2016). From here, metabolites can yield low-molecular-weight phenolic compounds (*e.g.*, phenol, benzoic acids and chlorogenic acids) or proceed to generate either L-tryptophan or L-arogenate. The latter is the starting point for production of L-phenylalanine and L-tyrosine, which are the main building blocks for both protein and phenylpropanoid synthesis (Cohen, & Kennedy, 2010).

The genesis of more complex phenols starts with amino acid deamination, which aims to introduce a double bond in the non-aromatic side chain and is performed by specific enzymes. Phenylalanine ammonia lyase (PAL) generates cinnamic acid, which is then converted into coumarin or *p*-hydroxycinnamic acid, while tyrosine ammonia lyase (TAL) produces *p*-hydroxycinnamic acid directly (Figure 1.17; Shuab *et al.*, 2016). The latter phenolic acid can subsequently be transformed into different cinnamate derivatives, such as ferulic, caffeic and sinapic acids through hydroxylation and methylation (Shuab *et al.*, 2016), converted in the corresponding alcohols in order to produce lignins and used as precursors of flavonoids (Figure 1.17; Le Roy *et al.*, 2016).

Flavonoids derive from the condensation of hydroxycinnamoyl CoA, provided from the shikimate pathway, with malonyl CoA, produced in the acetate/malonate pathway from the carboxylation of acetyl CoA and used for C2 chain elongation (Saito *et al.*, 2013). Specifically, the product of

condensation is a triketide that spontaneously cyclizes, forming naringenin chalcone (Saito *et al.*, 2013), and can then be converted into flavanones and dihydroflavonols (Figure 1.17; Vickery, & Vickery, 1981). Flavanones deriving from chalcone isomerization can yield flavones through 2,3-dehydrogenation and isoflavones through ring migration, as a consequence of flavanone oxidation (Vickery, & Vickery, 1981). Dihydroflavonols can be converted into flavonols through the formation of α-hydroxychalcone, into flavan-3,4-diols and then flavan-3-ols by reduction of the keto group, and into anthocyanidins through the formation of several intermediates (Figure 1.19; Vickery, & Vickery, 1981). Therefore, the flavonoid A ring derives from the acetate/malonate pathway and the B and C rings from cinnamic acids (Vickery, & Vickery, 1981), while further modification of the flavonoid scaffold can be attributed to tailoring reactions carried out by glycosyltransferases, methyltransferases and acyltransferases (Saito *et al.*, 2013).

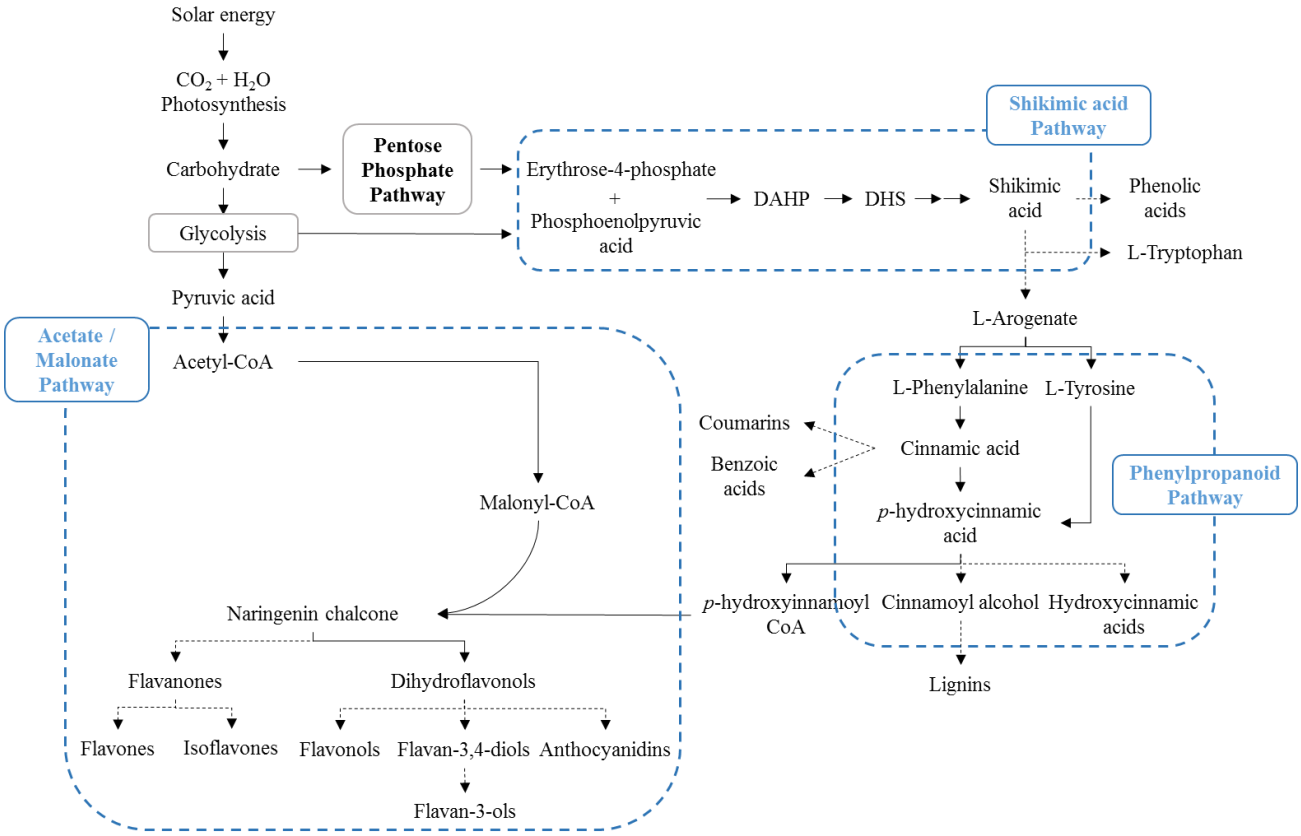


Figure 1.19. Biosynthetic pathway of phenolic compounds.

The biosynthetic pathway described for flavonoids can also generate stilbenes, splitting off one carbon atom of the phenylpropane after the introduction of the second phenyl moiety (Shahidi, & Naczk, 2003).

Cinnamic acids are also the precursors of benzoic acids and coumarins. The former are produced by a β-oxidation of the propenyl side chain of cinnamic acids, which occurs after ester formation with CoA (Vickery, & Vickery, 1981), and can be converted into different hydroxybenzoic acids by

hydroxylation and methylation (El-Basyouni *et al.*, 1964; Shahidi, & Naczk, 2003). Furthermore, the decarboxylation of benzoic and cinnamic acids yields simple and vinyl-substituted phenols, respectively, while their reduction can produce the corresponding aldehydes (Shahidi, & Naczk, 2003). As regards coumarins, biosynthesis starts with the introduction of an *o*-hydroxyl group into cinnamic acid derivatives and its subsequent glycosylation, which can induce isomerization of the propenyl chain from the *trans* to the *cis* form. Then, after sugar hydrolysis, spontaneous cyclization occurs (Vickery, & Vickery, 1981).

1.4.3. The role of phenolic compounds in plants

Phenolic compounds are widespread in the plant kingdom, but their presence and distribution depends closely on environmental conditions and natural selection (Lattanzio *et al.*, 2006). Generally, they are involved in plant defence, pigmentation and growth, or act as antioxidants, metal chelators, signalling agents and UV light screens. Phenolic compounds can be pre-synthesized during plant growth and stored in specific sites (*e.g.* the vacuole of guard, epidermal or sub-epidermal cells), or expressly produced after intense stress such as infection, a high concentration of heavy-metal salts, UV-irradiation and temperature (Lattanzio *et al.*, 2006).

When stored within the epidermal cell layer, phenolic compounds protect plants from the harmful ultraviolet radiation of the sun (280-320 nm), which can damage DNA, proteins and membranes or generate reactive oxygen species (ROS). Consequently, depending on sun exposure and excess light, the occurrence of phenolic compounds can also vary with the latitude and altitude of the growing area. All phenolic compounds absorb UV radiation due to their aromatic ring, but each class can be distinguished on the basis of distinctive spectral maxima. For example simple phenols and hydroxybenzoic acids exhibit intense absorption in the range of 250-290 nm, hydroxycinnamic acids in the range of 290-330 nm and flavones and flavonols at about 250 and 350 nm (Lattanzio *et al.*, 2006). Other phenolic compounds are colored and consequently show strong absorption in the visible region, such as anthocyanins, which show a spectral maximum in the range of 475-560 nm. These compounds play a significant role in plant pigmentation, attracting pollinators to flowers and inducing animals to eat fruit and disperse seeds (Lattanzio *et al.*, 2006). Phenolic compound co-pigmentation with other molecules (*e.g.* purines, alkaloids and metallic cations), together with temperature and pH inside the vacuole, intensify and modify the original color of pigments. The most widely occurring colored phenolic compounds are anthocyanins, flavones, flavonols and quinone derivatives.

Phenolic compounds can influence rates of decomposition and nutrient cycling, directly stimulating or inhibiting germination and the growth of microorganisms, or contribute to plant growth, acting as a reservoir of phenylpropanoid units for lignin biosynthesis (Lattanzio *et al.*, 2006).

Many phenolic compounds have a key role in plant defence against fungi, bacteria and nematodes (Bennett, & Wallsgrove, 1994). Their defence capability mainly depends on the speed of phenol biosynthesis at the moment of attack, rather than the concentration. However, although some of them are stored in plant cells as inactive compounds and readily activated by enzymatic hydrolysis in response to pathogen attack, their level increases through specific synthesis when needed (Lattanzio *et al.*, 2006). Phenolic compounds can act directly as toxins, as in the case of phytoalexins or free radicals produced by lignin degradation, or can form barriers, as in the case of lignin (Bennett, & Wallsgrove, 1994). Lignification is indeed considered to be a resistance mechanism, since the occurrence of lignin increases in the event of plant disease and its presence prevents enzymatic hydrolysis and mechanical penetration of plant tissue by pathogens.

Finally, the poor palatability of many phenolic compounds, such as tannins, is responsible for their use as feeding deterrents for herbivores. These are indeed less attracted by plants rich in high-molecular-weight phenols, due to their astringency and poor digestibility (Bennett, & Wallsgrove, 1994).

1.4.4. Phenolic compounds in food

Phenolic compounds are present in almost all foods (vegetables, cereals, legumes, fruit, cocoa, nuts, etc.) and beverages (wine, cider, beer, tea, etc.) of plant origin, and therefore represent an important part of animal diet. Their levels depend on genetic factors such as the *cultivar*, the site of accumulation in the plant, crop treatments and environmental conditions (Bravo, 1998). However, qualitative and quantitative information about phenolic compound occurrence in plants reported in the literature is sometimes contradictory and difficult to compare, since the content is also affected by preparation and extraction procedures, technological treatments, storage and food processing (Escarpa, & Gonzalez, 2001).

Of the different phenolic compounds, flavanols, flavonols, hydroxycinnamic and hydroxybenzoic acids are the most widespread in food and beverages, while anthocyanins, isoflavonoids, flavanones and stilbenes are less common (Pérez-Jiménez *et al.*, 2010). However, on comparing the total content of each class of these compounds in food and beverages (Table 1.3), anthocyanins and flavanols are shown to be the most abundant, while flavonols, flavones and stilbenes are present in lower amounts. Particular attention has been paid to lignans, which are present in a small number of foods, such as sesame seed oil, flaxseed and sesame seed meal, but always at very high levels.

Phenolic compounds	Content* in foods (mean ± SD)	Richest foods (content*)
Anthocyanins	115±259	black elderberry (1316), black chokeberry (878), black currant (595)
Flavanols	180±560	cocoa powder (3410), dark chocolate (1589), hazelnut (495)
Flavanones	23±25	pure blood orange juice (51), pure grapefruit (46), pure blond orange juice (38)
Flavones	4±12	whole-grain common wheat flour (73), globe artichoke heads (58), black olive (27)
Flavonols	11±21	red onion (158), spinach (119), shallot (112)
Isoflavonoids	66±106	soy flour (466), roasted soy bean (246), soy tempe (148)
Hydroxybenzoic acids	29±121	chestnut (1215), walnut (449), red raspberry (121)
Hydroxycinnamic acids	35±78	coffee (278), red chicory (203), globe artichoke heads (202)
Stilbenes	0.7±1.1	red wine (3.4), lingonberry (3.0), red currant (1.6)
Lignans	550±625	sesame seed oil (1294), flaxseed meal (867), sesame seed meal (776)

*expressed as mg/100 g or mg/100 mL; SD=standard deviation.

Table 1.3. Main phenolic compound content in food and beverages (adapted from Pérez-Jiménez *et al.*, 2010).

1.4.4.1. Occurrence of low-molecular-weight phenols in food

Of low-molecular-weight phenolic compounds, phenolic acids are the most widespread in food and beverages (Pérez-Jiménez *et al.*, 2010).

The most common hydroxybenzoic acids are gallic acid (principally found in nuts, chicory, some berries, tea and wine), ellagic acid (in nuts and berries), 4-hydroxybenzoic acid (in olives, loquats, some berries and carrots), vanillic acid (in dates, some berries, olives, rye flour, Swiss chard and cocoa), and syringic acid (in nuts, olives, dates, pumpkin and cauliflower). The food and beverages with the highest hydroxybenzoic acid content are chestnuts (1215 mg/100 g), with ellagic acid and gallic acid predominating (735 mg/100g and 480 mg/100 g respectively), raspberries (121 mg/100 g), pomegranate juice (55 mg/100 mL) and blackberries (50 mg/100 g). Content ranging from 5 to 50 mg/100 g or mg/100 mL is typical of olives, tea, wine, different berries and dates. Furthermore, hydroxybenzoic acids are also present as esters with quinic acid, such as 5-*O*-galloylquinic acid in tea, or esterified in ellagitannins such as punicalagin in pomegranate juice or sanguiin H-6 and lambertianin C in raspberries (Pérez-Jiménez *et al.*, 2010).

Hydroxycinnamic acids are mostly present as quinic acid esters, with the exception of *p*-coumaric acid, present in free form in olives, and sinapic acid, found as an aglycone in olives and cauliflower (Pérez-Jiménez *et al.*, 2010). The most common esters are chlorogenic acid (an ester of caffeic and quinic acids mainly present in coffee, berries, pome and drupe fruits such as plums, sweet cherries, apples, peaches, pears, nectarines, quinces and apricots, vegetables such as artichokes, chicory, carrots, broccoli, lettuce and tomatoes, olives and potatoes), caftaric acid (an ester of caffeic and tartaric acids, mainly present in grapes and wine), chicoric acid (an ester of tartaric acid with two molecules of caffeic acid, mainly present in chicory), *O*-feruloylquinate (an ester of ferulic and quinic acids, mainly present in coffee) and *O*-coumaroylquinate (an ester of coumaric and quinic

acids, mainly present in sweet cherries and apples). The foods with the highest concentrations of hydroxycinnamic acids are coffee (212 mg/100 mL), artichokes (202 mg/100 g), prunes (192 mg/100 g), red chicory (183 mg/100 g), blueberries (135 mg/100 g), green chicory (108 mg/100 g), green olives (104 mg/100 g), black olives (96 mg/100 g), plums (89 mg/100 g) and sweet cherries (88 mg/100 g).

Of low-molecular-weight phenolic compounds present in a restricted number of foods and beverages, the most abundant are tyrosol derivatives and alkylresorcinols. The first are mainly present in black olives (266 mg/100 g) and olive oil (60 mg/100 g), while alkylresorcinols are present in the highest amounts in cereal bran (286 mg/100 g), rye whole-grain flour (69 mg/100 g) and wheat whole-grain flour (64 mg/100 g;). Finally, stilbenes are mainly present in red wine, lingonberries and red-currants, but always at a lower level (1-5 mg/100 mL and mg/100 g; Pérez-Jiménez *et al.*, 2010).

1.4.4.2. Occurrence of flavonoids in food

Of the most diffuse flavonoids in food and beverages, flavanols are also the most abundant (Table 1.3). The richest sources are cocoa (3411 mg/100 g) and dark chocolate (1590 mg/100 g), followed by black chokeberries (659 mg/100 g), nuts (181-496 mg/100 g), blueberries (330 mg/100 g), strawberries (148 mg/100 g), black-currants (139 mg/100 g) and apples (111 mg/100 g). Other important sources of flavanols are black tea, green tea and red wine (Pérez-Jiménez *et al.*, 2010).

Flavonols, another widespread class of flavonoids, are generally present at lower levels. Higher concentrations are found in onions and shallots (73-158 mg/100 g), spinach (119 mg/100 g) and black chokeberries (88 mg/100 g), while lower amounts characterize green tea, black tea, dark chocolate, and various fruits, vegetables and nuts.

Of the flavonoids detected in a restricted number of food sources, isoflavonoids are those found in higher amounts (Table 1.3). The principal sources are soy foods, with the highest content (>100 mg/100 g) in soy flour, soy nuts and soy tempeh.

Flavones are mainly present in whole grain wheat flour (80-100 mg/100 g), black olives and artichokes (40-60 mg/100 g), and at lower levels in tea, orange juice, lemon juice and olive oil. Finally, flavanones are present at relatively high levels in grape juice (46 mg/100 mL), orange juice (38 mg/100 mL) and lemon juice (33 mg/100 mL), and at trace levels in beer, wine or tomatoes (Pérez-Jiménez *et al.*, 2010).

1.4.4.3. Occurrence of tannins, lignans and other phenolic compounds in food

Tannins are mainly present in food and beverages as high-molecular-weight polymers (Nutrient Data Laboratory. USDA, 2004). Dimeric tannins are mainly present in chocolate (>300 mg/100 g) and baking chocolate (>200 mg/100 g), while trimers are found in spices (>1200 mg/100 g). Tannins with 4-6 condensed units are mainly present in cocoa beans (>2700 mg/100 g), spices (>2600 mg/100 g), grain bran (>650 mg/100 g), chocolate (>400 mg/100 g), baking chocolate (332 mg/100 g) and beans (125 mg/100 g). Tannins with 7-10 condensed units are mainly present in cocoa beans (>2200 mg/100 g), spices (>1400 mg/100 g), grain bran (>780 mg/100 g) and beans (135 mg/100 g). Finally, tannins with more than 10 condensed units are mainly present in grain bran (>2900 mg/100 g), spices (>2500 mg/100 g), cocoa beans (>1500 mg/100 g), grain (>1400 mg/100 g), choke berries (>540 mg/100 g), baking chocolate (>500 mg/100 g), beans (>450 mg/100 g), nuts (80-320 mg/100 g) and blueberries (130-260 mg/100 g).

Lignans are mainly present in sesame seed oil (1295 mg/100 g), flaxseed meal (867 mg/100 g) and sesame seed meal (776 mg/100 g), while in all other sources they do not exceed 3 mg/100 g. In sesame seed meal and oil, and in olives and olive oil lignans are found in the free form, while in other sources they are bound to complex organic molecules and do not exceed 1 mg/100 g (Pérez-Jiménez *et al.*, 2010).

Hydroxyphenylpropenes are mainly present in spices (Neveu *et al.*, 2010) and the most abundant are eugenol (*e.g.* >12000 mg/100 g in cloves), anethole (*e.g.* 5400 mg/100 g in star anise), acetyl eugenol (*e.g.* 2075 mg/100g in cloves) and gingerol (*e.g.* 187 mg/100 g in ginger). Curcuminoids, such as curcumin and methoxyderivatives, are mainly present in spices (285-2213 mg/100 g). Finally, phenolic terpenes are mainly present in seasoning herbs and the most common are carnosic acid (*e.g.* 672 mg/100 g in rosemary and 526 mg/100 g in common sage), rosmanol and carnosol (*e.g.* 124 and 53 mg/100 g respectively in rosemary).

1.4.5. Nutraceutical and biological effects of phenolic compounds

Phenolic compounds are considered to be fundamental constituents of the human diet, since their high occurrence in fruits and vegetables is correlated with staying healthy and disease prevention (Gutiérrez-Grijalva *et al.*, 2016). At low levels, they mainly act as anti-ageing, anti-inflammatory, antioxidant and anti-proliferative agents, while at high concentrations they can interact with proteins, carbohydrates and minerals, reducing their adsorption (Karakaya, 2004).

The involvement of phenolic compounds in the prevention of degenerative diseases, such as cardiovascular disease, cancer and chronic inflammation, is ascribable to their antioxidant activity and their role as radical scavengers or chelators of metal ions (Bravo, 1998). Phenolic compounds

prevent the oxidation of other molecules by intercepting radicals with transfer of a hydrogen atom (Figure 1.20. *a*-*b*) or by acting as terminators of the propagation route in the form of phenoxy radicals (Figure 1.20. *c*-*d*). However, if they occur at high levels and in the presence of iron and high pH, phenolic compounds can lead to auto-oxidation and act as pro-oxidants (Shahidi *et al.*, 1992).

$$ROO\cdot + PPH \rightarrow ROOH + PP\cdot \quad (a)$$
$$RO\cdot + PPH \rightarrow ROH + PP\cdot \quad (b)$$
$$ROO\cdot + PP\cdot \rightarrow ROOPP \quad (c)$$
$$RO\cdot + PP\cdot \rightarrow ROPP \quad (d)$$

Figure 1.20. Mechanism of reaction of phenolic compounds as radical scavengers (ROO·, RO· and PP· = radicals; PPH = phenolic compounds).

The antioxidant properties of phenolic compounds depend closely on their chemical structure. In particular, the number of hydroxyl groups and their relative position (*e.g. ortho*- and *para*-diphenolic compounds), together with the substitution of hydrogens with ethyl- or butyl groups, generally positively affect antioxidant activity. Therefore, hydroxycinnamic acids are more effective antioxidants than hydroxybenzoic acids (Shahidi *et al.*, 1992). In the case of flavonoids, the antioxidant effects increase in particular with the presence of *o*-diphenolic groups in the B ring, a 4-oxo-conjugated double bond in the C ring and hydroxyl groups in positions 3 and 5, while they decrease with the number of sugar residues in the glycosylated derivatives (Bravo, 1998).

As regards oligo- and polymeric phenolic compounds with a high degree of hydroxylation, the main property is the capacity to bind and precipitate proteins through the formation of complexes kept together by hydrogen bonds and hydrophobic interactions, rather than by covalent or ionic bonds (Hagerman, 1992). As a consequence, the presence of high-molecular-weight phenolic compounds in food is associated with reduced digestibility of proteins and with enhanced elimination of endogenous ones, such as the enzymes involved in digestion (Bravo, 1998). Although this behavior generally leads to labelling of high-molecular-weight phenolic compounds as anti-nutrients in the human diet, when enzymatic inhibition affects glucosidases and amylases, the risk of metabolic syndrome and type 2 diabetes can be considerably reduced. Indeed, when the enzymes involved in dietary carbohydrate digestion are inhibited, the post-prandial glycemic response decreases and the lipid metabolism is modulated. Pancreas β-cell function, insulin secretion and adipose tissue metabolism are improved, hyperglycemia, dyslipidemia and insulin resistance are attenuated, and oxidative stress and inflammatory processes are alleviated. Furthermore, the ingestion of high-molecular-weight phenolic compounds through the diet reduces the risk of long-term diabetes complications, such as cardiovascular disease, neuropathy, nephropathy and retinopathy (Lin *et al.*,

2016). As regards lipid metabolism, dietary intake of phenolic compounds induces an increase in the plasma level of high-density lipoprotein (HDL) cholesterol and a decrease in that of low-density lipoprotein (LDL) cholesterol. This is principally due to reduced intestinal absorption of cholesterol and enhancement of reverse-cholesterol transport and bile acid excretion (Bravo, 1998).

Finally, phenolic compounds, and in particular hydroxycinnamic acids, can stop the synthesis of leukotrienes acting in immunoregulatory diseases and allergic reactions, have antitumoral effects on colon carcinogenesis, and can inhibit retroviral integrase produced by human immunodeficiency virus type 1 (HIV-1) and an activator protein involved in the control of inflammation, cell differentiation and proliferation (Robbins, 2003).

1.4.6. Analysis of phenolic compounds

Analysis of phenolic compounds is hindered by the high heterogeneity of their chemical properties (*e.g.* size, polarity and solubility) and the concentration levels at which they occur in natural samples, as well as by the variability of samples analyzed (Motilva *et al.*, 2013). Consequently, numerous methods (Dai, & Mumper, 2010) have been described for the determination of phenolic compounds and none of them is all-encompassing.

Some analytical approaches, such as the Folin-Denis method, Folin-Ciocalteu method, permanganate titration, colorimetry with iron salts, ultraviolet absorbance and protein binding (for both condensed and hydrolysable tannins), allow determination of total phenol content. They are widely employed for quality control by industry, because they are simple and time-saving approaches, use inexpensive reagents, show high repeatability and reproducibility, do not require sophisticated equipment and can be used for different matrices (Granato *et al.*, 2016). However, these methods can lead to different and often non-comparable results, due to the different reactivity of phenolic compounds towards assay reagents (Dai, & Mumper, 2010).

The recent development of chromatographic techniques, both gas and liquid chromatography, and their hyphenation with modern HRMS offers new tools for improving the analysis of complex matrices and for quantifying even a single phenolic compound. GC is rarely used due to the low volatility of phenolic compounds, which is principally due to their hydrogen bonding capability, which increases the melting point (Stalikas, 2007). On the other hand, LC is the most suitable approach for qualitative and quantitative analysis of phenolic compounds, since it is able to simultaneously detect all compounds of interest and their potential sub-products (Dai, & Mumper, 2010). Depending on the number and chemical properties of the phenolic compounds studied and the nature of the matrices, different stationary phases, solvents and elution gradients can be used (Motilva *et al.*, 2013). Reversed-phase columns are the most widely employed, although they can be

modified with different embedded or end-capping groups. Acetonitrile (ACN) and methanol (MeOH), or a mixture of these, are the most common organic modifiers, while water is usually acidified with a small amount of formic, acetic or trifluoroacetic acid at a concentration generally ranging from 0.05% to 0.2%. As regards detection, the approaches described in the literature include UV/VIS, photodiode array (PDA), UV-fluorescence, electrochemical detector (ECD) and electro-array detector (EAD), together with the voltammetry technique, chemical reaction detection technique, MS and NMR (Dai, & Mumper, 2010). However, in the last few years the LC-MS approach has gained widespread acceptance thanks to its high selectivity and sensitivity of detection, and because it allows detailed structural elucidation of phenolic compounds (Granato *et al.*, 2016).

When analyzing phenolic compounds using LC-MS methods, ESI source is the most commonly used and ionization is usually performed in negative ion mode, since the molecular ion generated is more intense than in the positive polarity (Motilva *et al.*, 2013). The only exceptions are anthocyanins, in themselves positive ions, and isoflavones, which are generally methoxylated and thus lack hydroxyl groups ionizable in negative ion mode.

In the case of quantitative analysis, it is necessary to use the corresponding calibration curve for each compound, since ionization can vary with the molecular structure, and to reduce the matrix effect. The latter causes suppression or enhancement of analyte ionization, is attributed to co-elution of matrix interference with the analytes of interest and strongly affects the linearity and accuracy of the method. The matrix effect can be evaluated by comparing the analyte response in standard solution and spiked samples at the same concentration, and can be reduced with efficient LC separation or adequate clean-up procedures for samples. Alternative approaches for reducing signal variability and improving method accuracy are the use of stable isotope-labelled internal standards (IS), which have similar ionization properties to the analytes studied, and the use of matrix-calibration curves (Motilva *et al.*, 2013).

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2. Experimental section and results

2.1. Non-targeted screening analysis

2.1.1. Non-targeted glycosidic profiling of international wines using Neutral Loss-high resolution mass spectrometry.

SECTION 2.1.1.

Non-targeted glycosidic profiling of international wines using Neutral Loss-high resolution mass spectrometry

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Aim of the work

Many metabolites naturally occur as glycosides, since sugar moieties can be crucial for their biological activity, can temporarily reduce their chemical reactivity, as in the case of stored toxins, and can increase their water solubility (Xu *et al.*, 2016). In the plant kingdom they can occur as glycosides or sugar esters, depending on the precursor's chemical structure (Harborne, 1964), and in wine they have traditionally attracted attention due to their organoleptic properties, such as astringency and bitterness, and because they affect the color and aroma of wines (Hjelmeland, & Ebeler, 2014).

The aim of this work was to investigate Neutral Loss experiments as an instrument for non-targeted screening analysis of glycosides, using liquid chromatography coupled to high resolution tandem mass spectrometry (Q-Orbitrap). Furthermore, the applicability of this approach to detailed glycosidic profiling of a wide selection of monovarietal wines was evaluated.

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Non-targeted glycosidic profiling of international wines using neutral loss-high resolution mass spectrometry

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ABSTRACT

Many metabolites naturally occur as glycosides, since sugar moieties can be crucial for their biological activity and increase their water solubility. In the plant kingdom they may occur as glycosides or sugar esters, depending on precursor chemical structure, and in wine they have traditionally attracted attention due to their organoleptic properties, such as astringency and bitterness, and because they affect the colour and aroma of wines.

A new approach directed at detailed description of glycosides in a large selection of monovarietal wines (8 samples each of Pinot Blanc, Muller Thurgau, Riesling, Traminer, Merlot, Pinot Noir and Cabernet Sauvignon) was developed by combining high performance liquid chromatography with high resolution tandem mass spectrometry. Analytical separation was performed on an Accucore™ Polar Premium LC column, while mass analysis was performed in negative ion mode with an non-targeted screening approach, using a Full MS/AIF/NL dd-MS^2 experiment at a resolving power of 140,000 FWHM.

Over 280 glycoside-like compounds were detected, of which 133 (including low-molecular weight phenols, flavonoids and monoterpenols) were tentatively identified in the form of pentose (6), deoxyhexose (17), hexose (73), hexose-pentose (16), hexose-deoxyhexose (7), dihexose (5) and hexose ester (9) derivatives. It was not possible to univocally define the corresponding chemical structure for the remaining 149 glycosides. Non-parametric statistical analysis showed it was possible to well characterise the glycosylated profile of all red and Traminer wines, while the identified glycosides were almost entirely lacking in Pinot Blanc, Riesling and Muller Thurgau wines. Also Tukey's Honestly Significant Difference test ($p < 0.05$) and Principal Component Analysis confirmed that it was possible to almost entirely distinguish the selected red wines from each other according to their glycosylated profile.

1. Introduction

Glycosides are organic compounds with semi-acetal linkage between the reducing end of a sugar and a nucleophilic group of another organic molecule known as aglycone [1]. When the linkage involves an alcoholic or phenolic hydroxyl group in the aglycone, they are called *O*-glycosides, whereas when it involves a carboxyl group, as in the case of acid aglycones, they are called sugar esters [2]. It is difficult to define the specific role of glycosides, because their biological activity depends on the aglycones and sugars making them up [3]. Generally, glycosylation enhances the water solubility of many aglycones, protects hydroxyl and phenolic groups from oxidation, reduces the reactivity of toxic compounds or produces intermediate products in the biosynthesis of several metabolites [2,4].

In *Vitis vinifera*, secondary metabolites normally responsible for wine aroma, taste and colour can occur as glycosides [5–8]. These compounds represent in part the potential character of wine, whose expression can be enhanced by enzymatic treatment or pH dependent hydrolysis occurring during wine ageing [9]. Consequently, these precursors represent a natural stock able to extend the shelf-life of products. Furthermore, several compounds, such as phenols, have an important role in physiological and biochemical processes, being involved in maintaining health and disease prevention [10–12]. Consequently, a high concentration of their glycosides can also improve the quality of fruit and vegetables, since many nutraceutical effects dispatched by the corresponding aglycone are enhanced by greater mobility inside the human body [4].

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Glycoside analysis generally involves complex and time-consuming procedures combining preliminary extraction, acid or enzymatic hydrolysis, aglyconic form separation and, finally, gas-chromatography detection of the latter [13]. Nonetheless, the recent availability of liquid chromatography coupled with high resolution mass spectrometry (HRMS) allows rapid detection and identification of organic components of complex biological mixtures without any derivatization step [14,15]. Furthermore, in the case of hybrid instruments, high-resolution tandem mass spectrometry (HRMS/MS) can be performed and both precursor and product ions can be detected with four-decimal figure accurate mass [15]. Recently, these instruments were implemented in dd-MS/MS Neutral Loss mode, which triggers MS/MS fragmentation of every molecular ion characterised by the neutral loss of one or more mass-selected fragments [16,17]. However, unlike GC–MS, few databases are freely available for the interpretation of mass spectra extracted using LC–MS methods.

The aim of this work was to develop an enhanced and systematic non-targeted analytical method for the tentative identification of glycosides, using a liquid chromatography-high resolution tandem mass approach (LC-Q-Orbitrap) in Neutral Loss mode. A detailed glycosylated profile of wines from international varieties was then defined.

2. Methods and materials

2.1. Chemicals and reagents

Acetonitrile (ACN; LC–MS grade, 99.9%) and formic acid (MS grade, 98%) were supplied by Fluka (St. Louis, MO, USA). Methanol (MeOH; LC grade, 99.9%), dichloromethane anhydrous (LC grade, 99.8%), 4-allylsyringol (99%), quercetin 3-β-D-glucoside (90%), rutin hydrate (94%) and aesculetin-glucoside (aesculetin-6-*O*-β-D-glucoside, 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Vanillic acid-glucoside (vanillic acid-4-*O*-β-D-glucoside, 99%), acetovanillone-glucoside (acetovanillone-4-*O*-β-D-glucoside, 99%) and scopoletin-glucoside (scopoletin-7-*O*-β-D-glucoside, 99%) were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Salicylic acid-glucoside (salicylic acid-2-*O*-β-D-glucoside, 98%) and orcinol-glucoside (98%) were custom synthesized and supplied by TransMIT (Giessen, Germany). Mass calibration solution (Pierce® ESI Negative Ion Calibration Solution) was purchased from Thermo Fischer Scientific Inc. (Waltham, MA, USA). Deionised water was produced using an Arium® Pro Lab Water System (Sartorius AG, Goettingen, Germany).

2.2. Sample preparation

Fifty-six samples of international monovarietal wines (24 wines with skin-contact maceration: Merlot, Pinot Noir, Sangiovese; 32 without skin-contact maceration: Muller Thurgau, Pinot Blanc, Riesling and Traminer; 8 samples each) were collected from Italian producers that guaranteed their variety and vintage, together with the absence of any exogenous contribution to glycosidic profile (e.g. use of pectolitic enzyme, tannins or wood ageing). In particular, 4 Merlot wines were of vintage 2009 and 4 of 2015, 4 Sangiovese of 2008 and 4 of 2010, all Pinot Noir of 2015, 3 Muller Thurgau of 2012, 3 of 2013 and 2 of 2014, 4 Pinot Blanc of 2013 and 4of 2016, 3 Riesling of 2013 and 5 of 2016, and 4 Traminer of 2012 and 4 of 2013.

Before analysis, glycosidic compounds were extracted with solid phase extraction (SPE) using an ISOLUTE® ENV+ SPE cartridge (1 g/6 mL, 90 μm, Biotage, Uppsala, Sweden), according to the method proposed by Boido et al. [18]. After cartridge activation with MeOH (15 mL) and water (20 mL), 100 mL of samples diluted 2 times in water were loaded. Interference and non-glycosylated compounds were eluted with water (15 mL) and dichloromethane (30 mL), while glycosides were recovered with 30 mL of MeOH. An aliquot of the methanolic extract was then filtered with 0.45 μm PTFE filter cartridges, diluted 2 times with water and added of the internal standard (4-allylsyringol, 0.5 mg/L).

2.3. LC-HRMS analysis

The analysis was performed on a Thermo Ultimate™ 3000 HPLC (Thermo Scientific, Sunnyvale, CA, USA) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive™; Thermo Scientific, Bremen, Germany), equipped with heated electrospray source (HESI-II). Analytical separation was performed on an Accucore™ Polar Premium LC column (150 mm × 3 mm, 2.6 μm particle size; Thermo Fischer Scientific, Waltham, MA, USA), using water-acetonitrile at a flow rate of 0.3 mL min^{-1}. In order to maximize the chromatographic separation of potential isomeric compounds, the organic solvent gradient was set as follows: 0–4 min, isocratic elution at 6% of ACN; 4–8 min, linear ramp to 7%; 8–10 min, linear ramp to 10%; 10–14 min, isocratic elution at 10%; 14–16 min, linear ramp to 15%; 16–24 min, isocratic elution at 15%; 24–26 min, linear ramp to 20%; 26–30 min, linear ramp to 25%; 30–32 min, isocratic elution at 25%; 32–36 min, linear ramp to 35%; 36–38 min, isocratic elution at 35%; 38–42 min, linear ramp to 50%; 42–44 min, isocratic elution at 50%; 44–48 min, linear ramp to 100%; 48–50 min, isocratic elution at 100%; 50–51 min, linear ramp to 6%. Column equilibration (51–55 min) was performed with ACN and aqueous formic acid (0.1%, v/v), set at 6:94 (v/v). Ten μL of samples were injected. The autosampler was held at 5 °C and the column compartment was heated to 40 °C.

Mass analysis was performed in negative ion mode using a Full MS/AIF/NL dd-MS^2 experiment (full mass/all ion fragmentation/Neutral Loss data dependent-MS^2). Mass resolving power was set at 140,000 full width at half-maximum (FWHM, calculated for *m/z* 200, 1.5 Hz) for full MS spectra, at 70,000 FWHM (3 Hz) for AIF spectra and at 17,500 FWHM (12 Hz) for dd-MS^2. The scan range was *m/z* 100–1000 for full MS mode and *m/z* 50–1000 for AIF and dd-MS^2 mode. The intensity threshold required to trigger the NL experiment was set at $1.6 \cdot 10^5$ (arbitrary unit). Glycoside precursor ions, positive in the NL experiment, were fragmented with stepped normalised collision energy (NCE), set at 25, 35 and 45 arbitrary units. dd-MS^2 experiments, once performed, were repeated after 6 s.

The neutral losses considered were *m/z* 132.04225 for pentose loss [$C_5H_8O_4$], *m/z* 146.05790 for deoxyhexose loss [$C_6H_{10}O_4$], *m/z* 162.05282 for hexose loss [$C_6H_{10}O_5$], *m/z* 264.08451 for pentose–pentose loss [$C_{10}H_{16}O_8$], *m/z* 294.09508 for hexose-pentose loss [$C_{11}H_{18}O_9$] and *m/z* 324.10564 for hexose–hexose loss [$C_{12}H_{20}O_{10}$].

The HESI source was set as follows: spray voltage, 2.80 kV; sheath gas flow rate at 30 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; capillary temperature, at 310 °C; capillary gas heater temperature, 280 °C [19].

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software was used for timing of chromatography and mass acquisition. Thermo Fisher Scientific Xcalibur and TraceFinder™ software (Thermo Scientific, Waltham, MA, USA) were used for data processing and evaluation.

2.4. Tentative identification of glycosides

Ion mapping of potential glycosides was carried out based on detection of all precursor ions losing a mass equal to neutral loss masses ± 0.01 Da, excluding isotopes and false positives. Isotopes can be distinguished from the corresponding precursor ions by

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considering the ratio between the relative product ion intensities, which remain constant. False positives were identified if the difference between the precursor and product ion masses did not correspond to one of considered neutral losses in the MS/MS spectrum, with an error lower than 5 ppm.

Ion mapping produced a list of glycoside masses, repeated as many times as the isomers, without specifying molecular formulas and retention times. In order to proceed with compound identification, potential matching of experimental MS/MS spectra with the fragmentation patterns of glycosides known in the literature [20–28] was searched for, alongside manual interpretation of spectra. Further evidences were obtained by comparing experimental isotope spacing and relative abundance to theoretical values based on chemical formulae. Consequently, each putative glycoside was related to a specific retention time.

2.5. Method testing

The proposed non-targeted screening approach was tested using 8 commercially available glycosylated phenolic compounds (acetovanillone-glucoside, aesculetin-glucoside, orcinol-glucoside, quercetin-glucoside, rutin, salicylic acid-glucoside, scopoletin-glucoside and vanillic acid-glucoside) at 5 different concentration levels (1, 0.5, 0.1, 0.01 and 0.001 mg/L).

Samples were randomly processed in a single analytical batch, and a quality control sample containing the internal standard was repeated every 10 wine samples to evaluate carry-over and the narrow repeatability of the analyte's normalised area (R.S.D. always <11%, for both wine samples and quality control samples).

2.6. Statistical analysis

Statistical analysis was performed with Statistica 13.1 Software (StatSoft, 2016), considering only analytical compounds detectable in 90% of wines of at least one variety and using ionisation intensity expressed as the peak area, as adopted in a similar metabolome approach [29]: The peak areas were normalised as relative areas (%) in comparison to the sum of glycosidic precursor areas. Non-detectable data were replaced with a random value between zero and the intensity threshold required to trigger the NL experiment.

Separated statistical analysis was performed on white and red wines: Kruskal-Wallis test ($p < 0.05$) was limited to white wines and performed on the entire variable dataset, while Tukey's Honestly Significant Difference HSD test ($p < 0.05$) and Principal Component Analysis (PCA) were limited to red wines and performed on normally distributed data or normalised by applying Box-Cox transformation.

3. Results and discussion

3.1. Method testing

The proposed non-targeted method made it possible to identify the neutral loss of glucose at a concentration of 1 mg/L for 7 out of 8 commercially available phenolic glucosides (aesculetin-glucoside, orcinol-glucoside, quercetin-glucoside, rutin, salicylic acid-glucoside, scopoletin-glucoside and vanillic acid-glucoside), since the full MS signal intensity of acetovanillone-glucoside was lower than the intensity threshold set to trigger the Neutral Loss experiment. However, decreasing standard concentration, the Neutral Loss experiment was still performed at 0.500 mg/L for aesculetin-glucoside, orcinol-glucoside, quercetin-glucoside, rutin, salicylic acid-glucoside and vanillic acid-glucoside, and at 0.100 mg/L for aesculetin-glucoside, salicylic acid-glucoside and vanillic acid-glucoside. At lower concentration levels (0.010 and 0.001 mg/L), the Neutral Loss experiments could not be performed for each tested standard.

In the case of glycosides, the possibility of carrying out Neutral Loss experiments contemporary depends on their ionisation yield and their stability in the ESI source. Indeed, when glycosides partially lose their sugar moieties in the source, their full MS signal intensities can be strongly affected. Consequently, the proposed method allowed the detection of particularly abundant or highly ionisable and chemical stable glycosides with the set source conditions.

As regards the real samples analysis, the normalisation of each peak areas as relative areas (%) in comparison to the sum of glycosidic precursor areas, according the approach proposed by Cuadros-Inostroza et al. [29], allowed to reduce any possible matrix effects as demonstrated by the narrow repeatability of the internal standard's normalised area (R.S.D. always <11%) in all wine samples and quality control samples.

3.2. Analysis of glycosides

The non-targeted approach described allowed the detection of 282 glycosides, of which 133 were tentatively identified as glycosylated derivatives of alkylphenols (N = 5), hydroxyalkylphenols (5), hydroxybenzoic acids and derivatives (26), hydroxycinnamic acids and derivatives (26), hydroxycinnamoyltartaric acid (3), hydroxyphenylacetic acids (4), hydroxymethoxyacetophenones and derivatives (6), stilbenes (2), flavones (2) and isoflavones (5), flavonols (14), flavanones (6), flavanonols (9), flavan-3-ols (4) and monoterpenols (16). Based on neutral loss and the characteristic sugar ring fragmentations [30] (Fig. 1), these glycosides were tentatively identified in the form of pentose (6), deoxyhexose (17), hexose (73), hexose-pentose (16), hexose-deoxyhexose (7), dihexose (5) and hexose ester (9) derivatives. The fragment ion Y_0 (Fig. 1) was produced from the neutral loss of all the sugar moieties, while Y_1 resulted from the neutral loss only of the distal moiety. The fragment ion B_i resulted when neutral loss of Y_0, the aglycone, occurred. Fragments C_i and Z_j can occur if cleavage of the glycosidic bond leads to the retention of glycosidic oxygen by the terminal sugar residue, but they were never detected. Table 1 summarises the molecular formulas, accurate masses $[M-H]^-$, retention times and fragmentation patterns of the tentatively identified glycosides.

The remaining glycosides (N = 149) were not identified due to the lack of comparable spectral data in the literature and were reported as unknown in Table 2, together with their accurate mass and retention time.

3.2.1. Alkylphenol glycosides

Four compounds with $[M-H]^-$ ions at m/z 401.1453 were possibly methylphenol hexose-pentose conjugates (compounds 40, 45, 52 and 56), since the ion at m/z 269.1031 corresponded to fragment Y_1 and the ion at m/z 161.0455 (B_2-B_1) was produced by subsequent neutral loss of fragment Y_0. Furthermore, sugar ring fragments ($^{k,l}A_i$; Fig. 1) were detected for both hexose and pentose moieties. The Y_0 fragmentation pattern confirmed what has already been reported for alkylphenols by Barnaba et al. [31].

The compound with the $[M-H]^-$ ion at m/z 285.0979 was possibly a methylbenzenediol hexose conjugate (compound 21), since neutral losses of B_1 and methyl (15.0234 u) fragments were detected, together with sugar ring fragments ($^{k,l}A_i$; Fig. 1).

3.2.2. Hydroxyalkylphenol and derivative glycosides

Three compounds with $[M-H]^-$ ions at m/z 315.1085 were possibly hydroxyalkylphenols hexose conjugates, since neutral losses of B_1 and CH_2O (30.0105 u; [32]) fragments were detected, together with sugar ring fragments ($^{k,l}A_i$; Fig. 1). Further neutral loss of a methyl was detected for only one compound, making it possible

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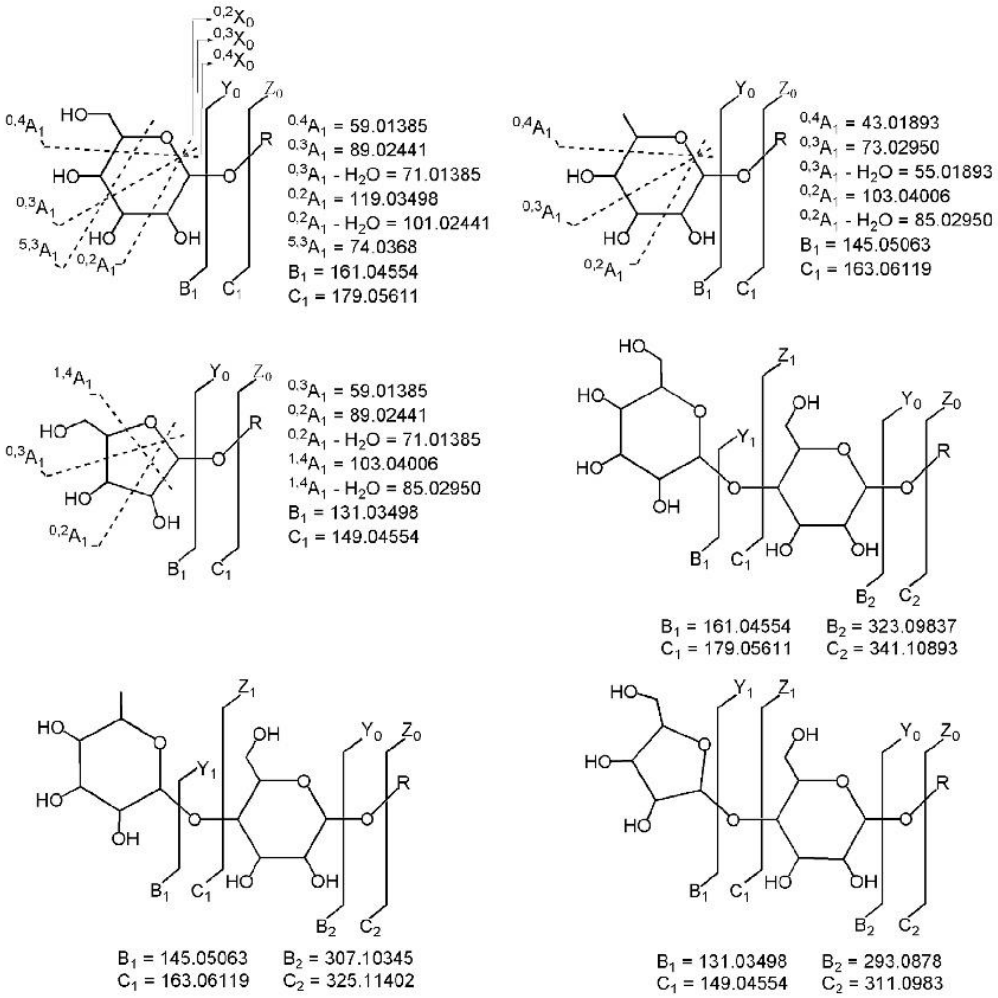

Fig. 1. Glycoside and sugar ring fragmentation according to the nomenclature reported by Domon and Costello (1988) and the accurate mass of the fragment produced.

to distinguish between one hydroxymethylmethoxyphenol (compound 5) and two hydroxyethylbenzenediol (compounds 6 and 12) hexose conjugates.

Two compounds were possibly ethoxymethylmethoxyphenol glycosides, since neutral losses of B_1, methyl (15.0234 u), C_2H_4O (44.0266 u) and C_3H_6O (58.0418 u) fragments were detected for both. Compound 63 was possibly ethoxymethylmethoxyphenol in the form of a hexose, while compound 90 was in the form of a deoxyhexose conjugate.

The Y_0 fragmentation pattern confirmed what has already been reported for hydroxyalkylphenols and derivatives by Barnaba et al. [31], and sugar ring fragments ($^{k,l}A_i$; Fig. 1) were detected for all sugar moieties.

3.2.3. Hydroxybenzoic acid and derivative glycosides

Hydroxybenzoic acid glycosides were characterised by the neutral loss of fragments B_i and CO_2 (43.9899 u). In the event of the additional presence of one or more methoxy groups, further neutral loss of a methyl (15.0234 u) was detected.

Four monohydroxybenzoic acids (compounds 4, 11, 15 and 22), six dihydroxybenzoic acids (compounds 3, 9, 10, 17, 37 and 38) and two monohydroxymethoxybenzoic acids (compounds 13 and 57) were detected in samples in the form of hexose conjugates. One dihydroxybenzoic acid (compound 44) was detected in the form of a hexose-deoxyhexose conjugate. One monohydroxydimethoxybenzoic acid (compound 36) was detected in the form of a pentose and four (compounds 16, 23, 32 and 50) in the form of hexose conjugates. Three trihydroxybenzoic acids (compounds 2, 8 and 14) were detected in the form of hexoses, one (compound 47) in the form of a hexose-deoxyhexose and one (compound 1) in the form of a dihexose conjugate. The Y_0 fragmentation pattern confirmed what has already been reported in the literature for hydroxybenzoic acids and derivatives [21,27,31], and corresponding ring fragments ($^{k,l}A_i$; Fig. 1) were detected for all sugar moieties.

In the event of detection of $^{k,l}X_j$ fragments (Fig. 1), sugar esters were distinguished, as reported by Jaiswal et al. [33], and three monohydroxymethoxybenzoylhexoses (compounds 19, 28 and 30) were putatively identified.

3.2.4. Hydroxycinnamic acid and derivative glycosides

Hydroxycinnamic acid glycosides were characterised by the neutral loss of fragments B_i and CO_2 (43.9899 u). In the event of the additional presence of several hydroxy or methoxy groups, fur-

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Table 1
Retention times, exact mass [M-H] −, difference between exact and accurate masses (Δ *m/z*) and characteristic fragmentation profile (all expressed as *m/z*) of the 133 glycosides tentatively identified in wine samples.

i.d.	Compounds*	RT (min)	Molecular formula	$[M-H]^-$ (*m/z*)	Δ** (*m/z*)	MS/MS fragments***
1	trihydroxybenzoic acid dihexose conjugate	2.87	C19H26O15	493.1199	2.21	331.0671,169.0143,125.0244
2	trihydroxybenzoic acid hexose conjugate 1	3.00	C13H16O10	331.0671	1.92	169.0143,125.0244
3	dihydroxybenzoic acid hexose conjugate 1	3.79	C13H16O9	315.0722	1.87	153.0193,109.0295
4	monohydroxybenzoic acid hexose conjugate 1	3.80	C13H16O8	299.0772	2.02	137.0244,93.0646
5	hydroxymethylmethoxyphenol hexose conjugate	3.98	C14H20O8	315.1085	2.08	153.0557,123.0437,108.0203
6	hydroxyethylbenzenediol hexose conjugate 1	4.02	C14H20O8	315.1085	2.09	153.0193,123.0437
7	monohydroxydimethoxyacetophenone hexose conjugate	4.16	C16H22O9	357.1191	0.28	195.0663,180.0426,165.019
8	trihydroxybenzoic acid hexose conjugate 2	4.50	C13H16O10	331.0671	1.70	169.0143,125.0244
9	dihydroxybenzoic acid hexose conjugate 2	4.70	C13H16O9	315.0722	2.00	153.0193,109.0295,108.0217
10	dihydroxybenzoic acid hexose conjugate 3	4.75	C13H16O9	315.0722	2.00	153.0193,109.0295,108.0217
11	monohydroxybenzoic acid hexose conjugate 2	4.76	C13H16O8	299.0772	2.21	137.0244,93.0646
12	hydroxyethylbenzenediol hexose conjugate 2	5.11	C14H20O8	315.1085	−4.32	153.0193,123.0437
13	monohydroxymethoxybenzoic acid hexose conjugate 1	5.13	C14H18O9	329.0878	2.03	167.035,152.0114,123.0452
14	trihydroxybenzoic acid hexose conjugate 3	5.50	C13H16O10	331.0671	1.61	169.0143,125.0244
15	monohydroxybenzoic acid hexose conjugate 3	5.54	C13H16O8	299.0772	2.16	137.0244,93.0344
16	monohydroxydimethoxybenzoic acid hexose conjugate 1	5.85	C15H20O10	359.0984	2.22	197.0456,182.0216,166.9984
17	dihydroxybenzoic acid hexose conjugate 4	6.00	C13H16O9	315.0722	1.82	153.0193,109.0295,108.0217
18	monohydroxymethoxyphenylacetic acid hexose conjugate 1	6.10	C15H20O9	343.1035	2.26	181.0506,137.0617,122.0373
19	monohydroxymethoxybenzoylhexose 1	6.31	C14H18O9	329.0878	2.39	269.0666,239.0561,209.0455,167.035,152.0114,123.0452,119.0349
20	tetrahydroxyflavan-3-ol hexose conjugate (2A+2B) 1	6.42	C21H24O11	451.1246	2.41	179.035,167.035,151.0401,137.0244,121.0295,109.0295
21	methylbenzenediol hexose conjuagate	6.60	C13H18O7	285.098	1.74	123.0452,108.0214,90.0591
22	monohydroxybenzoic acid hexose conjugate 4	6.65	C13H16O8	299.0772	2.22	137.0244,93.0344
23	monohydroxydimethoxybenzoic acid hexose conjugate 2	6.68	C15H20O10	359.0984	2.42	197.0456,182.0216,166.9984
24	monohydroxymethoxycinnamic acid hexose-pentose conjugate	7.20	C21H28O13	487.1457	2.10	325.0929,193.0506,178.0268,149.0608
25	monohydroxycinnamic acid dihexose conjugate 1	7.36	C21H28O13	487.1457	2.10	325.0928,163.04,119.0502
26	monohydroxymethoxyacetophenone hexose conjugate 1	7.46	C15H20O8	327.1085	1.67	165.0557,121.0659
27	monohydroxymethoxyacetophenone hexose conjugate 2	7.60	C15H20O8	327.1085	1.75	165.0557,150.0321,122.0371
28	monohydroxymethoxybenzoylhexose 2	7.70	C14H18O9	329.0878	2.07	269.0666,239.0561,209.0455,167.035,152.0114,123.0452,119.0349
29	monohydroxymethoxyphenylacetic acid hexose conjugate 2	7.90	C15H20O9	343.1035	1.54	181.0506,137.0617,122.0373
30	monohydroxymethoxybenzoylhexose 3	8.36	C14H18O9	329.0878	2.11	269.0666,239.0561,209.0455,167.035,152.0114,123.0452,119.0349
31	dihydroxycinnamic acid hexose conjugate 1	8.62	C15H18O9	341.0878	2.21	179.035,135.0452
32	monohydroxydimethoxybenzoic acid hexose conjugate 3	8.85	C15H20O10	359.0984	2.26	197.0456,182.0216,166.9984
33	monohydroxymethoxyphenylacetic acid hexose conjugate 3	9.15	C15H20O9	343.1035	2.07	181.0506,137.0617,122.0373
34	monohydroxycinnamic acid hexose conjugate 1	9.43	C15H18O8	325.0929	1.95	163.0402,119.0502,93.1266
35	dihydroxycinnamic acid hexose conjugate 2	9.64	C15H18O9	341.0878	2.30	179.035,135.0452
36	monohydroxydimethoxybenzoic acid pentose conjugate	9.68	C14H18O9	329.0878	1.53	197.0456,182.0216,166.9984
37	dihydroxybenzoic acid hexose conjugate 5	9.91	C13H16O9	315.0722	1.76	153.0193,109.0295
38	dihydroxybenzoic acid hexose conjugate 6	10.03	C13H16O9	315.0722	1.49	153.0193,109.0295,108.0217
39	tetrahydroxyflavan-3-ol hexose conjugate (2A+2B) 2	10.05	C21H24O11	451.1246	1.60	179.035,167.035,151.0401,137.0244,121.0295,109.0295
40	methylphenol hexose-pentose conjugate 1	10.16	C18H26O10	401.1453	2.05	269.1031,161.0455,119.0349,101.0244,89.0244,71.0138,59.0138
41	dihydroxycinnamoylhexose 1	10.26	C15H18O9	341.0878	2.46	281.0672,251.0577,221.0458,179.035
42	monohydroxycinnamic acid hexose conjugate 2	10.54	C15H18O8	325.0929	1.34	163.04,119.0502,93.1266
43	dihydroxycinnamic acid hexose conjugate 3	10.56	C15H18O9	341.0878	2.43	179.035,135.0452
44	dihydroxybenzoic acid hexose-deoxyhexose conjugate	10.94	C19H26O13	461.1301	1.95	153.0193,109.0295,108.0217
45	methylphenol hexose-pentose conjugate 2	11.38	C18H26O10	401.1453	1.06	269.1031,161.0455,119.0349,101.0244,89.0244,71.0138,59.0138
46	monohydroxymethoxyacetophenone hexose conjugate 3	11.40	C15H20O8	327.1085	2.30	165.0557,150.0321,122.0371
47	trihydroxybenzoic acid hexose-deoxyhexose conjugate	11.43	C19H26O14	477.1249	1.81	331.0671,169.0143,151.0038,125.0244
48	monohydroxymethoxyacetophenone hexose conjugate 4	11.58	C15H20O8	327.1085	2.30	165.0557,147.0453
49	monohydroxymethoxycinnamic acid hexose conjugate 1	11.92	C16H20O9	355.1035	2.20	193.0506,178.0268,149.0608
50	monohydroxydimethoxybenzoic acid hexose conjugate 4	12.00	C15H20O10	359.0984	2.25	197.0456,182.0216,166.9984
51	dihydroxycinnamic acid pentose conjugate 1	12.11	C14H16O8	311.0772	0.23	179.035,135.0452
52	methylphenol hexose-pentose conjugate 3	12.30	C18H26O10	401.1453	2.60	269.1031,161.0455,119.0349,101.0244,89.0244,71.0138,59.0138
53	tetrahydroxyflavan-3-ol hexose conjugate (2A+2B) 3	12.30	C21H24O11	451.1246	1.92	179.035,167.035,151.0401,137.0244,121.0295,109.0295
54	monohydroxymethoxycinnamic acid hexose conjugate 2	12.58	C16H20O9	355.1035	2.13	193.0506,178.0268,149.0608,134.0375

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Table 1 (*Continued*)

i.d.	Compounds*	RT (min)	Molecular formula	$[M-H]^-$ (*m/z*)	Δ** (*m/z*)	MS/MS fragments***
55	dihydroxycinnamoylhexose 2	12.69	C15H18O9	341.0878	1.84	281.0672,251.0577,221.0458,179.035
56	methylphenol hexose-pentose conjugate 4	13.15	C18H26O10	401.1453	2.25	269.1031,161.0455,119.0349,101.0244,89.0244,71.0138,59.0138
57	monohydroxymethoxybenzoic acid hexose conjugate 2	13.30	C14H18O9	329.0878	−0.84	167.035,152.0114,123.0452
58	dihydroxycinnamic acid hexose conjugate 4	13.33	C15H18O9	341.0878	1.65	179.035,135.0452
59	monohydroxymethoxycinnamic acid hexose conjugate 3	13.36	C16H20O9	355.1035	2.12	193.0506,178.0268,149.0608
60	dihydroxycinnamic acid pentose conjugate 2	13.41	C14H16O8	311.0772	1.54	179.035,135.0452
61	hydroxydimethoxyacetophenone deoxyhexose conjugate	13.81	C16H22O8	341.1242	2.17	195.0663,180.0426,165.019
62	dihydroxycinnamic acid pentose conjugate 3	14.16	C14H16O8	311.0772	1.79	179.035,135.0452
63	ethoxymethylmethoxyphenol hexose conjugate	14.42	C16H24O8	343.1398	2.37	181.087,166.0633,153.0656
64	monohydroxymethoxycinnamic acid pentose conjugate	14.50	C15H18O8	325.0929	2.12	193.0506,178.0268,149.0608
65	tetrahydroxyflavan-3-ol hexose conjugate (2A + 2B) 4	14.70	C21H24O11	451.1246	2.46	179.035,167.035,151.0401,137.0244,121.0295,109.0295
66	monohydroxycinnamic acid hexose conjugate 3	15.04	C15H18O8	325.0929	1.85	163.0402,119.0502,93.1266
67	monohydroxymethoxycinnamic acid hexose conjugate 4	15.51	C16H20O9	355.1035	4.07	193.0506,178.0268,149.0608
68	monohydroxycinnamoylhexose 1	16.50	C15H18O8	325.0929	1.81	265.0717,235.0612,205.0506,163.04,119.0349,101.0244,89.0244
69	monohydroxycinnamoyldihexose 1	16.96	C21H28O13	487.1457	2.50	325.0928,265.072,235.0615,205.0509
70	monohydroxycinnamic acid dihexose conjugate 2	17.00	C21H28O13	487.1457	2.37	325.0928,163.04,119.0502
71	dimethyloctadienediol hexose-pentose conjugate 1	17.49	C21H36O11	463.2184	2.66	331.1762,161.0455
72	monohydroxycinnamoylhexose 2	19.06	C15H18O8	325.0929	2.16	265.0717,235.0612,205.0506,163.04,119.0349,101.0244,89.0244
73	trihydroxyflavanonol hexose conjugate (1A + 2B)	19.50	C21H22O11	449.1089	2.51	177.0193,151.0401,151.0036,125.0244,107.0138
74	trihydroxymethoxyflavanonol pentose conjugate (2A + 2B, 1 CH_3O on B)	19.55	C21H22O11	449.1089	2.54	179.035,167.0349,149.0244,121.0295,107.0138,93.0346
75	monohydroxycinnamoyldihexose 2	19.79	C21H28O13	487.1457	2.71	325.0928,265.072,235.0615,205.0509
76	monohydroxycinnamic acid dihexose conjugate 3	20.29	C21H28O13	487.1457	2.08	325.0928,163.04,119.0502
77	trihydroxyflavanonol hexose conjugate (1A + 2B)	20.30	C21H22O11	449.1089	2.59	181.0142,151.0401,151.0036,135.0088,123.045,121.0295,109.0295
78	monohydroxycinnamic acid dihexose conjugate 4	20.68	C21H28O13	487.1457	2.50	325.0928,163.04,119.0502
79	monohydroxymethoxyphenylacetic acid hexose conjugate 4	20.90	C15H20O9	343.1035	0.69	181.0506,137.0617,122.0373
80	dimethyloctadienediol hexose-pentose conjugate 2	21.02	C21H36O11	463.2184	2.01	331.1762,161.0455
81	monohydroxycinnamoyltartaric hexose conjugate 1	21.07	C19H22O13	457.0988	−0.71	295.0459,163.04,149.0092,119.0502,87.0087,93.1266
82	dimethyloctadienediol hexose-pentose conjugate 3	21.37	C21H36O11	463.2184	1.95	331.1762,161.0455
83	dimethyloctadienediol hexose-pentose conjugate 4	22.31	C21H36O11	463.2184	2.02	331.1762,161.0455
84	monohydroxycinnamoyltartaric hexose conjugate 2	22.79	C19H22O13	457.0988	3.12	295.0459,163.04,149.0092,119.0502,87.0087,93.1266
85	dimethyloctadienediol hexose-pentose conjugate 5	23.09	C21H36O11	463.2184	1.51	331.1762,161.0455
86	tetrahydroxydimethoxyisoflavone hexose conjugate (2A + 2B, 1 CH_3O on A and B) 1	23.37	C23H24O13	507.1144	2.25	492.0917,477.0685,193.0142,165.0193,163.04,149.0244,137.0244
87	tetrahydroxydimethoxyisoflavone hexose conjugate (2A + 2B, 1 CH_3O on A and B) 2	24.45	C23H24O13	507.1144	2.13	178.9985,151.0394,151.0036,149.0244,107.0138
88	dimethyloctadienediol hexose-pentose conjugate 6	24.53	C21H36O11	463.2184	2.57	331.1762,161.0455
89	dimethyloctadienediol hexose-pentose conjugate 7	24.82	C21H36O11	463.2184	2.25	331.1762,161.0455
90	ethoxymethylmethoxyphenol deoxyhexose conjugate	25.17	C16H24O7	327.1449	2.42	181.087,166.0633,153.0656
91	monohydroxycinnamoyltartaric hexose conjugate 3	25.25	C19H22O13	457.0988	2.65	295.0459,163.04,149.0092,119.0502,87.0087,93.1266
92	trihydroxyflavanone hexose conjugate (2A + 1 B) 1	28.98	C21H22O10	433.114	1.60	151.0036,135.0452,123.0088,107.0138
93	trihydroxyflavanone hexose conjugate (2A + 1B) 2	30.60	C21H22O10	433.114	2.21	151.0036,135.0452,123.0088,107.0138
94	pentahydroxyflavonol hexose conjugate (3B + 2A) 1	30.89	C21H20O13	479.0831	2.36	178.9985,151.0036,137.0244,107.0138
95	trihydroxystilbene hexose conjugate 1	31.43	C20H22O8	389.1242	2.26	227.0713,185.0608,159.0815,143.0502
96	pentahydroxyflavonol hexose conjugate (3B + 2A) 2	31.48	C21H20O13	479.0831	2.15	178.9985,151.0036,151.0036,137.0244,107.0138
97	trihydroxystilbene hexose conjugate 2	32.86	C20H22O8	389.1242	2.14	227.0713,185.0608,159.0815,143.0502
98	pentahydroxyflavanone deoxyhexose conjugate (2A + 3B) 1	33.80	C21H22O11	449.1089	2.45	177.0193,151.0401,151.0036,125.0244,107.0138
99	tetrahydroxyflavanonol deoxyhexose conjugate (2A + 2B) 1	33.91	C21H22O11	449.1089	2.15	177.0193,151.0401,151.0036,125.0244,107.0138
100	pentahydroxyisoflavone deoxyhexose conjugate	34.24	C21H20O11	447.0933	2.09	178.9985,151.0036,107.0138
101	pentahydroxyflavanone deoxyhexose conjugate (2R + 3R) 2	34.72	C21H22O11	449.1089	2.44	181.0142,177.0193,151.0401,151.0036,125.0244,109.0295,107.0138
102	tetrahydroxyflavone hexose conjugate (2A + 2B) 1	34.75	C21H20O11	447.0933	1.93	178.9985,151.0036,145.0295,121.0293,107.0138
103	tetrahydroxyflavanonol deoxyhexose conjugate (2A + 2B) 2	34.80	C21H22O11	449.1089	2.56	177.0193,151.0401,151.0036,125.0244,107.0138
104	tetrahydroxymethoxyflavonol hexose conjugate (2A + 3B, 1 CH_3O on B)	34.87	C22H22O13	493.0988	1.91	193.0142,178.9985,163.0036,151.0036,107.0138
105	pentahydroxyflavanone deoxyhexose conjugate (2A + 3B) 3	35.01	C21H22O11	449.1089	2.58	177.0193,151.0401,151.0036,107.0138

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Table 1 (*Continued*)

i.d.	Compounds*	RT (min)	Molecular formula	$[M-H]^-$ (*m/z*)	Δ** (*m/z*)	MS/MS fragments***
106	tetrahydroxyflavanonol deoxyhexose conjugate (2A + 2B) 3	35.11	C21H22O11	449.1089	2.09	177.0193,151.0401,151.0036,125.0244,107.0138
107	tetrahydroxyflavonol hexose conjugate (2A + 2B) 1	35.39	C21H20O12	463.0882	1.25	178.9985,151.0036,121.0295,107.0138
108	pentahydroxyflavonol deoxyhexose conjugate (3B + 2A)	35.41	C21H20O12	463.0882	1.21	178.9985,151.0036,137.0244,107.0138
109	dimethyloctadienol hexose-pentose conjugate 1	35.49	C21H38O10	447.2236	−0.06	315.1813,161.0455
110	tetrahydroxyflavonol hexose conjugate (2A + 2B) 2	35.90	C21H20O12	463.0882	1.70	178.9985,151.0036,121.0295,107.0138
111	tetrahydroxyflavone hexose conjugate (2A + 2B) 2	35.99	C21H20O11	447.0933	2.69	178.9985,151.0036,145.0295,121.0293,107.0138
112	tetrahydroxyflavanonol deoxyhexose conjugate (2A + 2B) 4	36.00	C21H22O11	449.1089	2.44	177.0193,151.0401,151.0036,125.0244,107.0138
113	pentahydroxyflavanone deoxyhexose conjugate (2A + 3B) 4	36.02	C21H22O11	449.1089	2.51	181.0142,177.0193,151.0401,151.0036,109.0295,107.0138
114	dimethyloctadienol hexose-pentose conjugate 2	37.07	C21H38O10	447.2236	−1.31	315.1813,161.0455
115	dimethyloctadienol hexose-deoxyhexose conjugate 1	37.20	C22H38O10	461.2392	2.65	315.1813,161.0455
116	tetrahydroxyflavonol hexose conjugate (2A + 2B) 3	37.36	C21H20O12	463.0882	0.82	178.9985,177.0193,151.0036
117	tetrahydroxydimethoxyisoflavone hexose conjugate (3A + 1B, 2 CH_3O on B)	37.43	C23H24O13	507.1144	2.16	492.0917,477.0685,193.0142,165.0193,163.04,149.0244,137.0244
118	dimethyloctadienol hexose-deoxyhexose conjugate 2	37.47	C22H38O10	461.2392	2.55	315.1813,161.0455
119	trihydroxyflavonol hexose coniugate (2A + 1B) 1	37.48	C21H20O11	447.0933	2.52	177.0193,163.0036,151.0036,121.0295,107.0138
120	dimethyloctadienol hexose-pentose conjugate 3	37.98	C21H38O10	447.2236	2.75	315.1813,161.0455
121	trihydroxyflavonol hexose coniugate (2A + 1B) 2	38.35	C21H20O11	447.0933	2.35	178.9985,151.0036,107.0138
122	dimethyloctadienol hexose-pentose conjugate 4	38.50	C21H38O10	447.2236	2.13	315.1813,161.0455
123	dimethyloctadienol hexose-deoxyhexose conjugate 3	38.53	C22H38O10	461.2392	0.75	315.1813,161.0455
124	tetrahydroxyflavonol hexose conjugate (2A + 2B) 4	38.64	C21H20O12	463.0882	2.48	193.0142,178.9985,151.0036,149.0244,121.0295,107.0138
125	trihydroxyflavanonol deoxyhexose conjugate (2A + 1B) 1	38.69	C21H22O10	433.114	2.54	177.0193,151.0036,119.0502,107.0138,93.0346
126	dimethyloctadienol hexose-deoxyhexose conjugate 4	39.06	C22H38O10	461.2392	2.06	315.1813,161.0455
127	tetrahydroxyflavonol deoxyhexose conjugate (2A + 2B)	39.56	C21H20O11	447.0933	2.20	177.0193,163.0036,151.0036,121.0295,107.0138
128	trihydroxyflavanonol deoxyhexose conjugate (2A + 1B) 2	39.58	C21H22O10	433.114	2.28	177.0193,151.0036,119.0502,107.0138,93.0346
129	trihydroxyflavonol hexose coniugate (2A + 1B) 3	39.60	C21H20O11	447.0933	2.56	178.9985,151.0036,107.0138
130	dimethyloctadienol hexose-deoxyhexose conjugate 5	40.06	C22H38O10	461.2392	2.74	315.1813,161.0455
131	tetrahydroxyflavonol hexose conjugate (2A + 2B) 5	40.29	C21H20O12	463.0882	1.52	193.0142,178.9985,151.0036,149.0244,121.0295,107.0138
132	tetrahydroxydimethoxyisoflavone deoxyhexose conjugate	41.93	C23H24O12	491.1195	1.82	178.9995,165.0193,153.0193,151.0036
133	trihydroxyflavonol deoxyhexose conjugate (2A + 1B)	42.69	C21H20O10	431.0984	1.96	178.9985,151.0036,107.0138

Note: i.d. = identification number; * = In brackets, the number and the positions of hydroxyl/methoxyl groups in flavonoids; ** Δ *m/z* = difference between expected and experimental masses (ppm). ***$^{k,l}A_i$ sugar ring fragments are not reported for each compounds, they are principally: $^{0,4}A_1$ = 59.01385, $^{0,3}A_1$ = 89.02441, $^{0,2}A_1$ = 119.03498 for hexose; $^{0,4}A_1$ = 43.01893, $^{0,3}A_1$ = 73.02950, $^{0,2}A_1$ = 103.04006 for deoxyhexose; $^{0,3}A_1$ = 59.01385, $^{0,2}A_1$ = 89.02441, $^{1,4}A_1$ = 103.04006 for pentose.

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ther neutral losses of water (18.0106 u) or methyl (15.0234 u) were detected respectively.

Three monohydroxycinnamic acids (compounds 34, 42 and 66) were detected in the form of hexoses and four (compounds 25, 70, 76 and 78) in the form of dihexose conjugates. Four dihydroxycinnamic acids (compounds 31, 35, 43 and 58) were detected in the form of hexoses and three (compounds 51, 60 and 62) in the form of pentose conjugates. One monohydroxymethoxycinnamic acid (compound 64) was detected in the form of a pentose, four (compounds 49, 54, 59 and 67) in the form of hexoses and one (compound 24) in the form of a hexose-pentose conjugate. The Y_0 fragmentation pattern confirmed what has already been reported in the literature for hydroxycinnamic acids and derivatives [21,27,31], and corresponding in-ring fragments ($^{k,l}A_i$; Fig. 1) were detected for all sugar moieties.

In the event of detection of $^{k,l}X_j$ fragments (Fig. 1), sugar esters were distinguished, as reported by Jaiswal et al. [33]. Two monohydroxycinnamoylhexoses (compounds 68 and 72), two monohydroxycinnamoyldihexoses (compounds 69 and 75) and two dihydroxycinnamoylhexoses (compounds 41 and 55) were putatively identified.

3.2.5. Hydroxycinnamoyltartaric acid glycosides

Three compounds with $[M-H]^-$ ions at m/z 457.0987 were possibly monohydroxycinnamoyltartaric acid hexose conjugates (compounds 81, 84 and 91), since ions at m/z 325.0928 corresponded to neutral loss of the tartaryl moiety and $^{k,l}X_j$ fragments were detected in the MS/MS spectra.

3.2.6. Hydroxyphenylacetic acid glycosides

Four compounds with $[M-H]^-$ ions at m/z 343.1035 were possibly monohydroxyphenylacetic acid hexose conjugates (compounds 18, 29, 33 and 79), since neutral losses of B_1, CO_2 (m/z 43.9899) and methyl (m/z 15.0234) fragments were detected, together with sugar ring fragments ($^{k,l}A_i$; Fig. 1). The Y_0 fragmentation pattern confirmed what has already been reported for hydroxyphenylacetic acids by Barnaba et al. [31].

3.2.7. Hydroxymethoxyacetophenone and derivative glycosides

Hydroxymethoxyacetophenone glycosides were characterised by the neutral loss of B_i, methyl (15.0234 u) and CO (27.9949 u) fragments. In the event of the additional presence of several methoxy groups, further neutral losses of methyl (15.0234 u) were detected. Deoxyhexose (compound 61) and hexose (compound 7) conjugates were putatively identified for hydroxydimethoxyacetophenone, while four hydroxymethoxyacetophenone (compounds 26, 27, 46 and 48) were detected in the form of hexose conjugates. The Y_0 fragmentation pattern confirmed what has already been reported for hydroxymethoxyacetophenones and derivatives by Barnaba et al. [31], and sugar ring fragments ($^{k,l}A_i$; Fig. 1) were detected for both deoxyhexose and hexose moieties.

3.2.8. Stilbene glycosides

Two compounds with $[M-H]^-$ ions at m/z 389.1242 were possibly trihydroxystilbene hexose conjugates (compounds 95 and 97), since neutral losses of B_1, C_2H_2O (42.0105 u) and C_3O_2 (67.9898 u) fragments were detected. The Y_0 fragmentation pattern confirmed what has already been reported for stilbenes [23], and the corresponding in-ring fragments ($^{k,l}A_i$; Fig. 1) were detected for hexose moieties.

3.2.9. Flavonoid glycosides

Flavonoid glycosides were characterised by the neutral loss of fragments B_i, CO (27.9949 u), CO_2 (43.9899 u), C_2H_2O (42.0105 u) and C_3O_2 (67.9898 u) [20]. In the event of the additional presence of one or more methoxy groups, further neutral losses of methyl (15.0234 u) fragments were detected. Furthermore, flavonoid aglycones could be distinguished in three groups on the basis of the number of hydrogen atoms in the chemical formula. Flavones and flavonols were characterised by 10 hydrogen atoms, flavanones and flavanonols by 12 and flavan-3-ols by 14. Two more hydrogen atoms and one oxygen atom were considered for every additional methoxy group.

Starting from data reported in the literature [20,34,35], a more detailed description of the fragmentation pattern of flavonoid glycosides is provided here for the first time (Fig. 2 and Table 3).

3.2.9.1. Flavone and flavonol glycosides.

Two tetrahydroxyflavones (compounds 102 and 111), with two hydroxyl groups on ring A and two on ring B, were identified in the form of hexose conjugates.

One pentahydroxyisoflavone (compound 100) was identified in the form of a deoxyhexose conjugate. One tetrahydroxydimethoxyisoflavone (compound 132) was identified in the form of a deoxyhexose conjugate and three in the form of hexoses. Of the latter, two (compounds 86 and 87) showed two hydroxyl groups on both ring A and ring B and one methyl group on both rings, and one (compound 117) showed three hydroxyl groups on ring A, one on ring B and two methyl groups on the ring B.

One trihydroxyflavonol (compound 133), with two hydroxyl groups on ring A and one on ring B, was identified in the form of a deoxyhexose conjugate and three (compounds 119, 121 and 129) in the form of hexose conjugates. One tetrahydroxyflavonol (compound 127), with two hydroxyl groups on ring A and two on ring B, was identified in the form of a deoxyhexose conjugate and five (compounds 107, 110, 116, 124 and 131) in the form of hexose conjugates. One pentahydroxyflavonol (compound 108), with two hydroxyl groups on ring A and three on ring B, was identified in the form of a deoxyhexose and two (compounds 94 and 96) in the form of hexose conjugates. One tetrahydroxymethoxyflavonol (compound 104), with two hydroxyl groups on ring A, two on ring B and one methyl group on the ring B, was detected in the form of hexose conjugate.

3.2.9.2. Flavanone and flavanonol glycosides.

Two trihydroxyflavanones (compounds 92 and 93), with two hydroxyl groups on ring A and one on ring B, were identified in the form of hexose conjugates. Four pentahydroxflavanones (compounds 98, 101, 105 and 113), with two hydroxyl groups on ring A and three on ring B, were identified in the form of deoxyhexose conjugates.

Two trihydroxyflavanonols (compounds 125 and 128), with two hydroxyl groups on ring A and one on ring B, were identified in the form of deoxyhexoses and two (compounds 73 and 77), with one hydroxyl group on ring A and two on ring B, in the form of hexose conjugates. Four tetrahydroxyflavanonols (compounds 99, 103, 106 and 112), with two hydroxyl groups on ring A and two on ring B, were detected in the form of deoxyhexose conjugates. One trihydroxymethoxyflavanonol (compound 74), with two hydroxyl groups on ring A, one on ring B and one methyl group on ring B, was identified in the form of a pentose conjugate.

3.2.9.3. Flavan-3-ol glycosides.

Four tetrahydroxyflavan-3-ols (compounds 20, 39, 53 and 65), with two hydroxyl groups on ring A and two on ring B, were detected in the form of hexose conjugates.

3.2.10. Monoterpenol glycosides

Sixteen glycosides can be tentatively ascribed to the class of monoterpenols, since neutral losses of fragments Y_1 were detected in the MS/MS spectra, together with fragments B_i an $^{k,l}A_i$ (Fig. 1) as reported by Hjelmeland et al. [28].

Four compounds with $[M-H]^-$ ions at m/z 447.2236 (compounds 109, 114, 120 and 122) were possibly dimethyloctadienols

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Table 2
Retention times and accurate masses of the 149 unknown glycosides found in wine samples.

i.d.	Compounds	$[M-H]^-$ (m/z)	RT (min)
pentose derivatives			
1	unknown pentose conjugate 1	293.0687	1.85
2	unknown pentose conjugate 2	221.0668	2.53
3	unknown pentose conjugate 3	382.1035	3.90
4	unknown pentose conjugate 4	243.0510	4.50
5	unknown pentose conjugate 5	231.0879	5.07
6	unknown pentose conjugate 6	231.0879	5.07
7	unknown pentose conjugate 7	219.0502	5.36
8	unknown pentose conjugate 8	216.0872	6.10
9	unknown pentose conjugate 9	321.1223	6.69
10	unknown pentose conjugate 10	393.0505	7.25
11	unknown pentose conjugate 11	329.0883	9.03
12	unknown pentose conjugate 12	283.0480	10.06
13	unknown pentose conjugate 13	575.2404	10.82
14	unknown pentose conjugate 14	327.1085	11.54
15	unknown pentose conjugate 15	403.1539	12.27
16	unknown pentose conjugate 16	312.0844	12.69
17	unknown pentose conjugate 17	309.0644	12.77
18	unknown pentose conjugate 18	323.0799	14.50
19	unknown pentose conjugate 19	381.1803	15.09
20	unknown pentose conjugate 20	336.1089	17.83
21	unknown pentose conjugate 21	336.1113	17.95
22	unknown pentose conjugate 22	211.0610	18.25
23	unknown pentose conjugate 23	561.2611	19.22
24	unknown pentose conjugate 24	349.1533	19.57
25	unknown pentose conjugate 25	225.0785	20.45
26	unknown pentose conjugate 26	537.2023	20.82
27	unknown pentose conjugate 27	537.2023	21.31
28	unknown pentose conjugate 28	361.1510	21.85
29	unknown pentose conjugate 29	309.0997	21.92
30	unknown pentose conjugate 30	361.0942	23.88
31	unknown pentose conjugate 31	551.2179	24.10
32	unknown pentose conjugate 32	395.1963	24.54
33	unknown pentose conjugate 33	551.2179	24.59
34	unknown pentose conjugate 34	395.1927	25.09
35	unknown pentose conjugate 35	521.2060	25.28
36	unknown pentose conjugate 36	435.0938	29.96
37	unknown pentose conjugate 37	553.2333	30.10
38	unknown pentose conjugate 38	225.0769	31.46
39	unknown pentose conjugate 39	359.1686	32.37
40	unknown pentose conjugate 40	373.0952	36.27
41	unknown pentose conjugate 41	257.1396	36.81
42	unknown pentose conjugate 42	367.0988	37.31
43	unknown pentose conjugate 43	225.0769	39.24
44	unknown pentose conjugate 44	488.1009	45.97
deoxyhexose derivatives			
45	unknown deoxyhexose conjugate 1	219.0502	5.36
46	unknown deoxyhexose conjugate 2	274.0924	7.34
47	unknown deoxyhexose conjugate 3	241.0737	9.38
48	unknown deoxyhexose conjugate 4	217.0717	14.73
49	unknown deoxyhexose conjugate 5	281.1395	16.39
50	unknown deoxyhexose conjugate 6	435.1308	21.21
51	unknown deoxyhexose conjugate 7	350.1285	21.21
52	unknown deoxyhexose conjugate 8	435.1334	21.28
53	unknown deoxyhexose conjugate 9	333.0980	25.10
54	unknown deoxyhexose conjugate 10	241.1082	26.21
55	unknown deoxyhexose conjugate 11	339.0722	28.71
56	unknown deoxyhexose conjugate 12	323.0772	28.94
57	unknown deoxyhexose conjugate 13	243.1238	30.23
58	unknown deoxyhexose conjugate 14	323.0772	30.30
59	unknown deoxyhexose conjugate 15	323.0772	31.66
60	unknown deoxyhexose conjugate 16	243.1226	32.15
61	unknown deoxyhexose conjugate 17	271.1662	32.78
62	unknown deoxyhexose conjugate 18	448.1008	35.97
63	unknown deoxyhexose conjugate 19	461.0775	36.68
64	unknown deoxyhexose conjugate 20	433.1179	38.63
65	unknown deoxyhexose conjugate 21	463.1302	39.37
66	unknown deoxyhexose conjugate 22	433.1181	39.50
hexose derivatives			
67	unknown hexose conjugate 1	329.0885	9.97
68	unknown hexose conjugate 2	327.1111	11.29
69	unknown hexose conjugate 3	382.1144	11.34
70	unknown hexose conjugate 4	355.0675	11.47
71	unknown hexose conjugate 5	373.0714	12.71

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Table 2 (*Continued*)

i.d.	Compounds	$[M-H]^-$ (*m/z*)	RT (min)
72	unknown hexose conjugate 6	359.0934	13.16
73	unknown hexose conjugate 7	249.1347	13.56
74	unknown hexose conjugate 8	431.1595	14.01
75	unknown hexose conjugate 9	259.1187	14.12
76	unknown hexose conjugate 10	423.1894	14.44
77	unknown hexose conjugate 11	323.0799	14.50
78	unknown hexose conjugate 12	363.1698	15.14
79	unknown hexose conjugate 13	307.1401	15.16
80	unknown hexose conjugate 14	359.0994	15.50
81	unknown hexose conjugate 15	366.1198	15.76
82	unknown hexose conjugate 16	509.1373	15.86
83	unknown hexose conjugate 17	363.1692	16.02
84	unknown hexose conjugate 18	382.1178	16.03
85	unknown hexose conjugate 19	366.1225	16.06
86	unknown hexose conjugate 20	307.1402	16.21
87	unknown hexose conjugate 21	281.1395	16.39
88	unknown hexose conjugate 22	323.1243	17.10
89	unknown hexose conjugate 23	329.0883	17.50
90	unknown hexose conjugate 24	379.1618	17.59
91	unknown hexose conjugate 25	365.1824	18.05
92	unknown hexose conjugate 26	429.2170	18.05
93	unknown hexose conjugate 27	429.2171	18.12
94	unknown hexose conjugate 28	509.1742	18.74
95	unknown hexose conjugate 29	365.1823	19.38
96	unknown hexose conjugate 30	245.1033	19.69
97	unknown hexose conjugate 31	347.1742	20.24
98	unknown hexose conjugate 32	611.2695	20.90
99	unknown hexose conjugate 33	611.2617	21.30
100	unknown hexose conjugate 34	427.2011	21.59
101	unknown hexose conjugate 35	350.1612	22.64
102	unknown hexose conjugate 36	405.1814	22.90
103	unknown hexose conjugate 37	521.2066	22.97
104	unknown hexose conjugate 38	521.2075	22.99
105	unknown hexose conjugate 39	321.1560	23.31
106	unknown hexose conjugate 40	449.2081	23.47
107	unknown hexose conjugate 41	335.1741	23.48
108	unknown hexose conjugate 42	335.1716	23.69
109	unknown hexose conjugate 43	403.1043	23.94
110	unknown hexose conjugate 44	347.1737	24.73
111	unknown hexose conjugate 45	339.0722	25.39
112	unknown hexose conjugate 46	509.1341	25.47
113	unknown hexose conjugate 47	405.1797	25.58
114	unknown hexose conjugate 48	357.1191	25.59
115	unknown hexose conjugate 49	333.0980	26.76
116	unknown hexose conjugate 50	465.1082	26.93
117	unknown hexose conjugate 51	465.1082	26.93
118	unknown hexose conjugate 52	369.1198	28.22
119	unknown hexose conjugate 53	433.1158	28.99
120	unknown hexose conjugate 54	523.2227	29.02
121	unknown hexose conjugate 55	474.1116	29.20
122	unknown hexose conjugate 56	429.2169	29.34
123	unknown hexose conjugate 57	523.2224	30.03
124	unknown hexose conjugate 58	613.1617	30.25
125	unknown hexose conjugate 59	601.1647	31.04
126	unknown hexose conjugate 60	431.1951	33.21
127	unknown hexose conjugate 61	449.0725	33.60
128	unknown hexose conjugate 62	445.2088	35.45
129	unknown hexose conjugate 63	459.1338	36.98
130	unknown hexose conjugate 64	535.3164	37.70
131	unknown hexose conjugate 65	433.0752	37.76
132	unknown hexose conjugate 66	533.3018	37.76
133	unknown hexose conjugate 67	431.2320	38.23
134	unknown hexose conjugate 68	537.3319	38.59
135	unknown hexose conjugate 69	671.3714	39.65
136	unknown hexose conjugate 70	489.2740	39.68
137	unknown hexose conjugate 71	537.3325	40.64
138	unknown hexose conjugate 72	489.2751	41.46
139	unknown hexose conjugate 73	549.1290	42.04
140	unknown hexose conjugate 74	493.3059	42.38
141	unknown hexose conjugate 75	491.2902	42.71
142	unknown hexose conjugate 76	491.2894	43.12
143	unknown hexose conjugate 77	519.3215	43.40
144	unknown hexose conjugate 78	519.3216	44.20
145	unknown hexose conjugate 79	515.2904	44.82

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Table 2 (*Continued*)

i.d.	Compounds	$[M-H]^-$ (*m/z*)	RT (min)
146	unknown hexose conjugate 80	669.3922	45.93
dipentose derivatives			
147	unknown dipentose conjugate 1	457.1767	6.64
148	unknown dipentose conjugate 2	621.1862	38.54
dihexose derivatives			
149	unknown dihexose conjugate	485.1805	15.60

Note: i.d. = identification number.

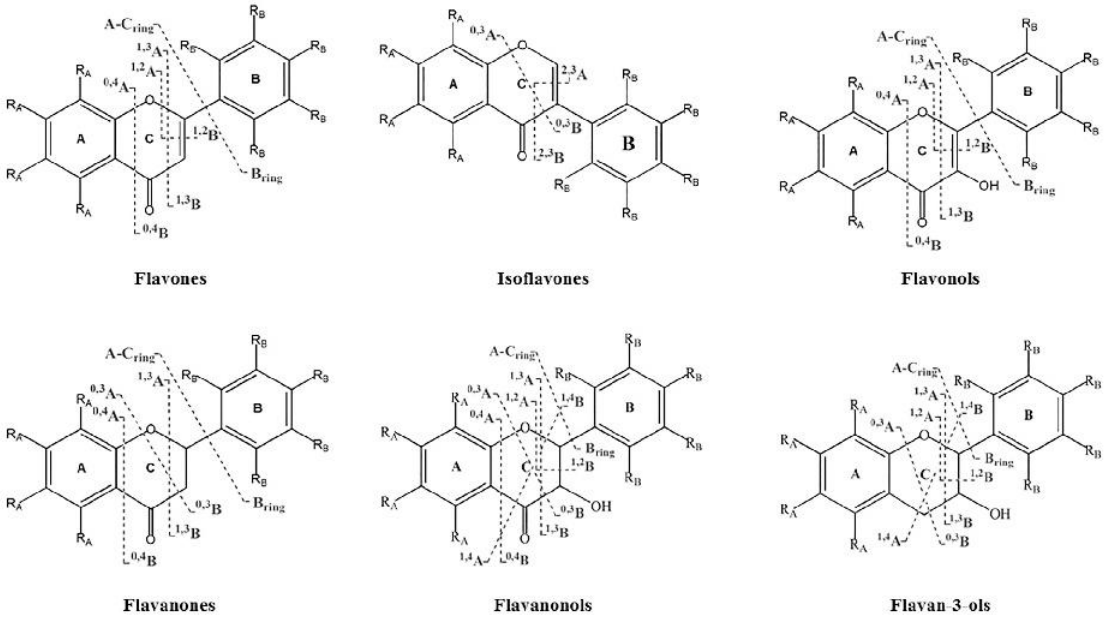

Fig. 2. Possible fragmentation pathways of flavones, isoflavones, flavonols, flavanones, flavanonols and flavan-3-ols.

in the form of hexose-pentose conjugates, since the ion at *m/z* 315.1813 (Y_1) was produced by the neutral loss of a pentose moiety and the ion at *m/z* 161.0455 (B_2-B_1) was produced by subsequent neutral loss of fragment Y_0.

Five compounds with $[M-H]^-$ ions at *m/z* 461.2392 (compounds 115, 118, 123, 126 and 130) were possibly dimethyloctadienols in the form of hexose-deoxyhexose conjugates, since the ion at *m/z* 315.1813 (Y_1) was produced by the neutral loss of a deoxyhexose moiety and the ion at *m/z* 161.0455 (B_2-B_1) was produced by subsequent neutral loss of fragment Y_0.

Seven compounds with $[M-H]^-$ ions at *m/z* 463.2184 (compounds 71, 80, 82, 83, 85, 88 and 89) were possibly dimethyloctadienediols in the form of hexose-pentose conjugates, since the ion at *m/z* 331.1762 (Y_1) was produced by the neutral loss of a pentose moiety and the ion at *m/z* 161.0455 (B_2-B_1) was produced by subsequent neutral loss of fragment Y_0.

3.3. Glycosidic profile in international monovarietal wines

Of the 133 tentatively identified glycosides, compounds 24, 40, 44–45, 51–52, 56, 60–64, 73–74, 77, 90, 98, 100–102, 105, 107, 110–111, 113, 116, 124–125, 128, 131 and 133 have never been reported in wines, as far as we know.

Considering that the presence of glycosides in wines may be affected by the technological process and in particular by the occurrence of skin-contact maceration during winemaking, the glycosidic profiles of white and red wines were separately studied.

As regards white wines, only non-parametric statistical test (Kruskal-Wallis test; $p < 0.05$) was performed, since data cannot be normalised despite the application of Box-Cox transformation. The Kruskal-Wallis test highlighted significant differences between Traminer wines and the other white varieties considered (Table 4), with particular focus on Muller Thurgau and Riesling wines. In particular, Traminer wines differed from the other white wine varieties for the ionisation response of glycosides belonging to the chemical classes of monoterpenols, hydroxybenzoic acids, hydroxycinnamic acids, alkylphenols, hydroxyalkylphenols, hydroxymethoxyacetophenones, flavanonols, flavanones, flavones, flavanones, flavan-3-ols or derivatives. Pinot Blanc wines can be distinguished only by Muller Thurgau ones by the response of three glycosides belonging to the chemical class of alkylphenols, hydroxyalkylphenols and flavanones, while Riesling and Muller Thurgau wines can never be distinguished one another.

As regards red wines, Tukey's test and PCA were performed on the entire dataset of variables, with the exception of compounds 7 and 110, not normalised by applying Box-Cox transformation. According to Tukey's test, Sangiovese wines were significantly different from both Pinot Noir and Merlot wines for compounds 2–3, 9–10, 18, 30, 35, 37–38, 44, 54–55, 59, 67, 71, 73–74, 79, 83, 85, 92–93, 106–107, 124, 126 and 131, from alone Pinot Noir wines for compounds 1, 13, 19, 26, 27, 36, 87, 95 and 96, and from alone Merlot wines for compound 50. In particular, Sangiovese wines were distinguished by the ionisation response of glycosides belonging to the chemical classes of hydroxyben-

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Table 3
Molecular formula and exact mass of fragments deliverable from different fragmentation pathways of flavonoids. Different substitution patterns are considered.

Fragmentation	Substituents	FLAVONES		ISOFLAVONES		FLAVONOLS		FLAVANONES		FLAVANONOLS		FLAVAN-3-OLS	
		formula	$[M-H]^-$ (m/z)	formula	$[M-H]^-$ (m/z)	formula	$[M-H]^-$ (m/z)	formula	$[M-H]^-$ (m/z)	formula	$[M-H]^-$ (m/z)	formula	$[M-H]^-$ (m/z)
$\mathbf{B}_{ring}$	5B = H	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H5]^-$	77.0396	$[C6H5]^-$	77.0396	$[C6H5]^-$	77.0396
	1B = OH	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H5O]^-$	93.0346	$[C6H5O]^-$	93.0346	$[C6H5O]^-$	93.0346
	2B = OH	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H5O2]^-$	109.0295	$[C6H5O2]^-$	109.0295	$[C6H5O2]^-$	109.0295
	3B = OH	$[C6H3O3]^-$	123.0082	$[C6H3O3]^-$	123.0082	$[C6H3O3]^-$	123.0082	$[C6H5O3]^-$	125.0244	$[C6H5O3]^-$	125.0244	$[C6H5O3]^-$	125.0244
	4B = OH	$[C6H3O4]^-$	139.0036	$[C6H3O4]^-$	139.0036	$[C6H3O4]^-$	139.0036	$[C6H5O4]^-$	141.0193	$[C6H5O4]^-$	141.0193	$[C6H5O4]^-$	141.0193
	5B = OH	/	/	/	/	/	/	/	/	$[C6H5O5]^-$	157.0142	$[C6H5O5]^-$	157.0142
A-C$_{ring}$	4A = H	$[C9H5O2]^-$	145.0295	$[C9H5O2]^-$	145.0295	$[C9H5O3]^-$	161.0244	$[C9H5O2]^-$	145.0295	$[C9H5O2]^-$	161.0244	$[C9H7O2]^-$	147.0451
	1A = OH	$[C9H5O3]^-$	161.0244	$[C9H5O3]^-$	161.0244	$[C9H5O4]^-$	177.0193	$[C9H5O3]^-$	161.0244	$[C9H5O3]^-$	177.0193	$[C9H7O3]^-$	163.0400
	2A = OH	$[C9H5O4]^-$	177.0193	$[C9H5O4]^-$	177.0193	$[C9H5O5]^-$	193.0142	$[C9H5O4]^-$	177.0193	$[C9H5O4]^-$	193.0142	$[C9H7O4]^-$	179.0350
	3A = OH	$[C9H5O5]^-$	193.0142	$[C9H5O5]^-$	193.0142	$[C9H5O6]^-$	209.0092	$[C9H5O5]^-$	193.0142	$[C9H5O5]^-$	209.0092	$[C9H7O5]^-$	195.0299
	4A = OH	$[C9H5O6]^-$	209.0092	$[C9H5O6]^-$	209.0092	$[C9H5O7]^-$	*225.0041*	$[C9H5O6]^-$	209.0092	$[C9H5O6]^-$	225.0041	$[C9H7O6]^-$	211.0248
$^{1,2}\mathbf{B}$	5B = H	$[C7H5]^-$	89.0397	$[C7H5]^-$	89.0397	$[C7H5]^-$	89.0397	/	/	$[C7H5]^-$	89.0397	$[C7H5]^-$	89.0397
	1B = OH	$[C7H5O]^-$	105.0345	$[C7H5O]^-$	105.0345	$[C7H5O]^-$	105.0345	/	/	$[C7H5O]^-$	105.0345	$[C7H5O]^-$	105.0345
	2B = OH	$[C7H5O2]^-$	121.0295	$[C7H5O2]^-$	121.0295	$[C7H5O2]^-$	121.0295	/	/	$[C7H5O2]^-$	121.0295	$[C7H5O2]^-$	121.0295
	3B = OH	$[C7H5O3]^-$	137.0244	$[C7H5O3]^-$	137.0244	$[C7H5O3]^-$	137.0244	/	/	$[C7H5O3]^-$	137.0244	$[C7H5O3]^-$	137.0244
	4B = OH	$[C7H5O4]^-$	153.0193	$[C7H5O4]^-$	153.0193	$[C7H5O4]^-$	153.0193	/	/	$[C7H5O4]^-$	153.0193	$[C7H5O4]^-$	153.0193
	5B = OH	$[C7H5O5]^-$	169.0142	$[C7H5O5]^-$	169.0142	$[C7H5O5]^-$	169.0142	/	/	$[C7H5O5]^-$	169.0142	$[C7H5O5]^-$	169.0142
$^{1,2}\mathbf{A}$	4A = H	$[C8H3O2]^-$	131.0138	$[C8H3O2]^-$	131.0138	$[C8H3O3]^-$	147.0087	/	/	$[C8H5O3]^-$	149.0244	$[C8H7O2]^-$	135.0452
	1A = OH	$[C8H3O3]^-$	147.0087	$[C8H3O3]^-$	147.0087	$[C8H3O4]^-$	163.0036	/	/	$[C8H5O4]^-$	165.0193	$[C8H7O3]^-$	151.0401
	2A = OH	$[C8H3O4]^-$	163.0036	$[C8H3O4]^-$	163.0036	$[C8H3O5]^-$	178.9985	/	/	$[C8H5O5]^-$	181.0142	$[C8H7O4]^-$	167.0349
	3A = OH	$[C8H3O5]^-$	178.9985	$[C8H3O5]^-$	178.9985	$[C8H3O6]^-$	194.9935	/	/	$[C8H5O6]^-$	197.0092	$[C8H7O5]^-$	183.0299
	4A = OH	$[C8H3O6]^-$	194.9935	$[C8H3O6]^-$	194.9935	$[C8H3O7]^-$	210.9884	/	/	$[C8H5O7]^-$	213.0041	$[C8H7O6]^-$	199.0248
$^{1,3}\mathbf{B}$	5B = H	$[C8H5]^-$	101.0396	$[C8H5]^-$	101.0396	$[C8H5O]^-$	117.0346	$[C8H7]^-$	103.0553	$[C8H7O]^-$	119.0502	$[C8H7O]^-$	119.0502
	1B = OH	$[C8H5O]^-$	117.0346	$[C8H5O]^-$	117.0346	$[C8H5O2]^-$	133.0295	$[C8H7O]^-$	119.0502	$[C8H7O2]^-$	135.0452	$[C8H7O2]^-$	135.0452
	2B = OH	$[C8H5O2]^-$	133.0295	$[C8H5O2]^-$	133.0295	$[C8H5O3]^-$	149.0244	$[C8H7O2]^-$	135.0452	$[C8H7O3]^-$	151.0401	$[C8H7O3]^-$	151.0401
	3B = OH	$[C8H5O3]^-$	149.0244	$[C8H5O3]^-$	149.0244	$[C8H5O4]^-$	165.0193	$[C8H7O3]^-$	151.0401	$[C8H7O4]^-$	167.0349	$[C8H7O4]^-$	167.0349
	4B = OH	$[C8H5O4]^-$	165.0193	$[C8H5O4]^-$	165.0193	$[C8H5O5]^-$	181.0142	$[C8H7O4]^-$	167.0349	$[C8H7O5]^-$	183.0299	$[C8H7O5]^-$	183.0299
	5B = OH	$[C8H5O5]^-$	181.0142	$[C8H5O5]^-$	181.0142	$[C8H5O6]^-$	197.0092	$[C8H7O5]^-$	183.0299	$[C8H7O6]^-$	199.0248	$[C8H7O6]^-$	199.0248
$^{1,3}\mathbf{A}$	4A = H	$[C7H3O2]^-$	119.0138	$[C7H3O2]^-$	119.0138	$[C7H3O2]^-$	119.0138	$[C7H3O2]^-$	119.0138	$[C7H3O2]^-$	119.0138	$[C7H5O]^-$	105.0345
	1A = OH	$[C7H3O3]^-$	135.0038	$[C7H3O3]^-$	135.0038	$[C7H3O3]^-$	135.0038	$[C7H3O3]^-$	135.0038	$[C7H3O3]^-$	135.0038	$[C7H5O2]^-$	121.0295
	2A = OH	$[C7H3O4]^-$	151.0036	$[C7H3O4]^-$	151.0036	$[C7H3O4]^-$	151.0036	$[C7H3O4]^-$	151.0036	$[C7H3O4]^-$	151.0036	$[C7H5O3]^-$	137.0244
	3A = OH	$[C7H3O5]^-$	166.9985	$[C7H3O5]^-$	166.9985	$[C7H3O5]^-$	166.9985	$[C7H3O5]^-$	166.9985	$[C7H3O5]^-$	166.9985	$[C7H5O4]^-$	153.0193
	4A = OH	$[C7H3O6]^-$	182.9935	$[C7H3O6]^-$	182.9935	$[C7H3O6]^-$	182.9935	$[C7H3O6]^-$	182.9935	$[C7H3O6]^-$	182.9935	$[C7H5O5]^-$	169.0142
$^{0,3}\mathbf{B}$	5B = H	/	/	$[C8H5O]^-$	117.0346	/	/	$[C8H7O]^-$	119.0502	$[C8H7O2]^-$	135.0452	$[C8H7O2]^-$	135.0452
	1B = OH	/	/	$[C8H5O]^-$	133.0295	/	/	$[C8H7O]^-$	135.0452	$[C8H7O3]^-$	151.0401	$[C8H7O3]^-$	151.0401
	2B = OH	/	/	$[C8H5O2]^-$	149.0244	/	/	$[C8H7O2]^-$	151.0401	$[C8H7O4]^-$	167.0349	$[C8H7O4]^-$	167.0349
	3B = OH	/	/	$[C8H5O3]^-$	165.0193	/	/	$[C8H7O3]^-$	167.0349	$[C8H7O5]^-$	183.0299	$[C8H7O5]^-$	183.0299
	4B = OH	/	/	$[C8H5O4]^-$	181.0142	/	/	$[C8H7O4]^-$	183.0299	$[C8H7O6]^-$	199.0248	$[C8H7O6]^-$	199.0248
	5B = OH	/	/	$[C8H5O5]^-$	197.0092	/	/	$[C8H7O5]^-$	199.0248	$[C8H7O7]^-$	215.0197	$[C8H7O7]^-$	215.0197
$^{0,3}\mathbf{A}$	4A = H	/	/	$[C7H3O]^-$	*103.0189*	/	/	$[C7H3O]^-$	103.0189	$[C7H3O]^-$	103.0189	$[C7H5]^-$	89.0397
	1A = OH	/	/	$[C7H3O2]^-$	119.0138	/	/	$[C7H3O2]^-$	119.0138	$[C7H3O2]^-$	119.0138	$[C7H5O]^-$	105.0345
	2A = OH	/	/	$[C7H3O3]^-$	135.0038	/	/	$[C7H3O3]^-$	135.0038	$[C7H3O3]^-$	135.0088	$[C7H5O2]^-$	121.0295
	3A = OH	/	/	$[C7H3O4]^-$	151.0036	/	/	$[C7H3O4]^-$	151.0036	$[C7H3O4]^-$	151.0036	$[C7H5O3]^-$	137.0244
	4A = OH	/	/	$[C7H3O5]^-$	166.9985	/	/	$[C7H3O5]^-$	166.9985	$[C7H3O5]^-$	166.9985	$[C7H5O4]^-$	153.0193
$^{0,4}\mathbf{B}$	5B = H	$[C9H5O2]^-$	145.0295	$[C9H5O2]^-$	145.0295	$[C9H5O3]^-$	161.0244	$[C9H7O2]^-$	147.0451	$[C9H7O3]^-$	163.04	$[C9H9O]^-$	133.0658
	1B = OH	$[C9H5O3]^-$	161.0244	$[C9H5O3]^-$	161.0244	$[C9H5O4]^-$	177.0193	$[C9H7O3]^-$	163.04	$[C9H7O4]^-$	179.035	$[C9H9O2]^-$	149.0608
	2B = OH	$[C9H5O4]^-$	177.0193	$[C9H5O4]^-$	177.0193	$[C9H5O5]^-$	193.0142	$[C9H7O4]^-$	179.035	$[C9H7O5]^-$	195.0299	$[C9H9O3]^-$	165.0557
	3B = OH	$[C9H5O5]^-$	193.0142	$[C9H5O5]^-$	193.0142	$[C9H5O6]^-$	209.0092	$[C9H7O5]^-$	195.0299	$[C9H7O6]^-$	211.0248	$[C9H9O4]^-$	181.0506

Table 3 (*Continued*)

Fragmentation	Substituents	FLAVONES		ISOFLAVONES		FLAVONOLS		FLAVANONES		FLAVANONOLS		FLAVAN-3-OLS	
		formula	$[M-H]^-$ (*m/z*)	formula	$[M-H]^-$ (*m/z*)	formula	$[M-H]^-$ (*m/z*)	formula	$[M-H]^-$ (*m/z*)	formula	$[M-H]^-$ (*m/z*)	formula	$[M-H]^-$ (*m/z*)
	4B = OH	$[C9H5O6]^-$	209.0092	$[C9H5O6]^-$	209.0092	$[C9H5O7]^-$	225.0041	$[C9H7O6]^-$	211.0248	$[C9H7O7]^-$	227.0197	$[C9H9O5]^-$	197.0455
	5B = OH	$[C9H5O7]^-$	225.0041	$[C9H5O7]^-$	225.0041	$[C9H5O8]^-$	*240.999*	$[C9H7O7]^-$	227.0197	$[C9H7O8]^-$	243.0146	$[C9H9O6]^-$	213.0405
^{0,4}**A**	4A = H	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H3]^-$	75.0240	$[C6H3O]^-$	91.0189
	1A = OH	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H3O]^-$	91.0189	$[C6H3O2]^-$	107.0138
	2A = OH	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H3O2]^-$	107.0138	$[C6H3O3]^-$	123.0088
	3A = OH	$[C6H3O3]^-$	123.0088	$[C6H3O3]^-$	123.0088	$[C6H3O3]^-$	123.0088	$[C6H3O3]^-$	123.0088	$[C6H3O3]^-$	123.0088	$[C6H3O4]^-$	139.0037
	4A = OH	$[C6H3O4]^-$	139.0037	$[C6H3O4]^-$	139.0037	$[C6H3O4]^-$	139.0037	$[C6H3O4]^-$	139.0037	$[C6H3O4]^-$	139.0037	$[C6H3O5]^-$	154.9986
^{1,4}**B**	5B = H	/	/	/	/	/	/	/	/	$[C9H7O2]^-$	147.0451	/	/
	1B = OH	/	/	/	/	/	/	/	/	$[C9H7O3]^-$	163.0400	/	/
	2B = OH	/	/	/	/	/	/	/	/	$[C9H7O4]^-$	179.0350	/	/
	3B = OH	/	/	/	/	/	/	/	/	$[C9H7O5]^-$	195.0299	/	/
	4B = OH	/	/	/	/	/	/	/	/	$[C9H7O6]^-$	211.0248	/	/
	5B = OH	/	/	/	/	/	/	/	/	$[C9H7O7]^-$	227.0197	/	/
^{1,4}**A**	4A = H	/	/	/	/	/	/	/	/	$[C6H3O]^-$	91.0189	/	/
	1A = OH	/	/	/	/	/	/	/	/	$[C6H3O2]^-$	107.0138	/	/
	2A = OH	/	/	/	/	/	/	/	/	$[C6H3O3]^-$	123.0088	/	/
	3A = OH	/	/	/	/	/	/	/	/	$[C6H3O4]^-$	139.0037	/	/
	4A = OH	/	/	/	/	/	/	/	/	$[C6H3O5]^-$	154.9986	/	/

NOTE: substituents show how the free positions of rings A and B are substituted.

Table 4
Significant differences among white wine varieties according to the Kruskal-Wallis test ($p < 0.05$).

i.d.	Traminer	Muller Thurgau	Riesling	Pinot Blanc
2	a	b	b	ab
3	a	b	b	ab
5	a	b	b	ab
6	a	b	ab	ab
9	a	b	b	ab
10	a	b	b	ab
15	a	b	ab	a
19	a	b	b	ab
21	a	b	b	ab
24	a	b	b	ab
25	a	b	b	ab
41	a	b	b	ab
42	a	b	b	b
46	a	b	b	ab
48	a	b	b	ab
52	a	b	b	a
55	a	b	b	ab
58	a	b	b	ab
71	a	b	b	b
77	a	b	b	ab
80	a	b	b	ab
82	a	b	b	b
83	a	b	b	b
85	a	b	b	b
88	a	b	b	b
89	a	b	b	ab
98	a	b	b	b
99	a	b	ab	ab
100	a	b	b	ab
101	a	b	b	b
102	a	b	b	ab
103	a	b	b	ab
104	a	ab	ab	b
105	a	b	b	b
106	a	ab	b	b
109	a	b	b	ab
111	a	ab	b	ab
113	a	b	ab	a
115	a	b	b	ab
117	a	b	ab	ab
118	a	ab	b	ab
120	a	b	b	b
125	a	b	b	ab
126	a	b	b	ab
130	a	b	b	b

zoic acids, hydroxycinnamic acids, hydroxyphenylacetic acids, monoterpenols, stilbenes, hydroxymethoxyacetophenones, flavanonols, flavanones, flavonols, flavones or derivatives. Pinot Noir wines were significantly different from both Sangiovese and Merlot wines for compounds 15–16, 23, 28, 31, 69, 86, 101, 103–105, 112–113, 125, 128 and 132, and from alone Merlot wines for compounds 102 and 127. In particular, Pinot Noir wines were distinguished by the ionisation response of glycosides belonging to the chemical classes of hydroxybenzoic acids, hydroxycinnamic acids, flavanonols, flavanones, flavonols, flavones or derivatives. Finally, Merlot wines were significantly different from both Sangiovese and Pinot Noir wines for compounds 41, 43, 58, 64, 76, 78 and 117, belonging to the chemical classes of hydroxycinnamic acids, flavones or derivatives. All varieties were significantly different from each other for the remaining compounds (24–25, 68, 70, 72 and 108).

Finally, in Principal Component Analysis compounds 112, 113 and 132 were the variables with the highest positive component loadings (0.84, 0.83 and 0.78 respectively), while compounds 24, 25 and 92 were those with the highest negative component loadings (−0.93, −0.92 and −0.91 respectively) of the first component (Factor 1), which explained 31.08% of total variability. Compounds 82, 128 and 127 were the variables with the highest positive compo-

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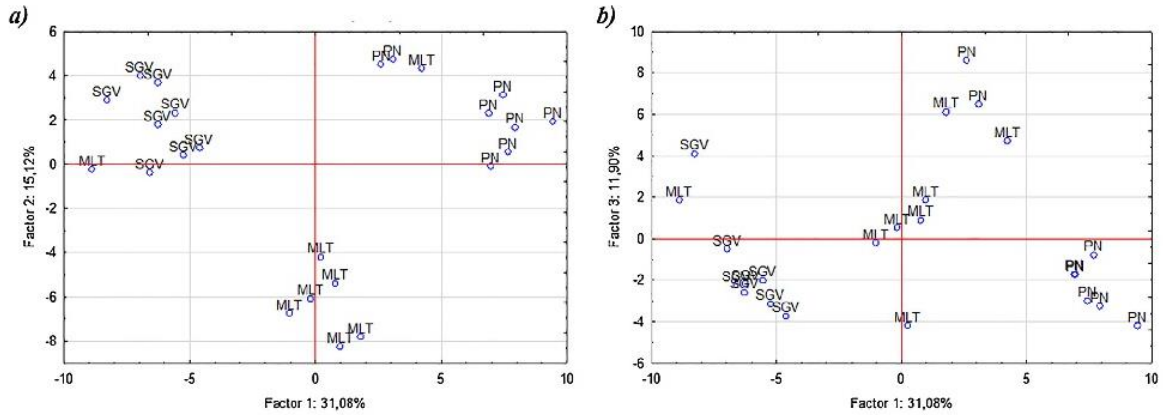

Fig. 3. Principal Component Analysis' score plot (Factor 1 and Factor 2, ***a***; Factor 1 and Factor 3, ***b***) for the selected monovarietal red wines (**SGV =** Sangiovese, N = 8; **MLT =** Merlot, N = 8; **PN =** Pinot Noir, N = 8), based on the ionisation intensities of the tentatively identified glycosilated phenolic compounds.

nent loadings (0.75, 0.69 and 0.62 respectively), while compounds 72, 68 and 58 were those with the highest negative component loadings (−0.84, −0.82 and −0.81 respectively) of the second component (Factor 2), which explained 15.12% of total variability. Compounds 21, 57 and 22 were the variables with the highest positive component loadings (0.83, 0.82 and 0.76 respectively), while compounds 99, 98 and 97 were those with the highest negative component loadings (−0.61, −0.60 and −0.57 respectively) of the third component (Factor 3), which explained 11.9% of total variability. With the exception of two Merlot samples, one grouped with Sangiovese wines and the other with Pinot Noir, all the monovarietal wines could be distinguished from each other (Fig. 3).

4. Conclusion

An innovative non-targeted high-resolution tandem mass approach was developed to tentatively identify glycosides in wines. By using the Neutral Loss experiment and matching the accurate mass, isotope pattern and fragmentation profile with spectral data reported in the literature, over 280 glycosides were detected and more than 130 were tentatively identified in selected samples. The ionisation profile of glycosides was able to more specifically characterise red and Traminer wines, since the other white wines had few of the identified glycosides.

In conclusion, the proposed non-targeted high-resolution mass-NL approach represents a promising tool for detailed description of the glycosidic profiles of wines and their varietal characterisation, in the event of availability of commercially available standards.

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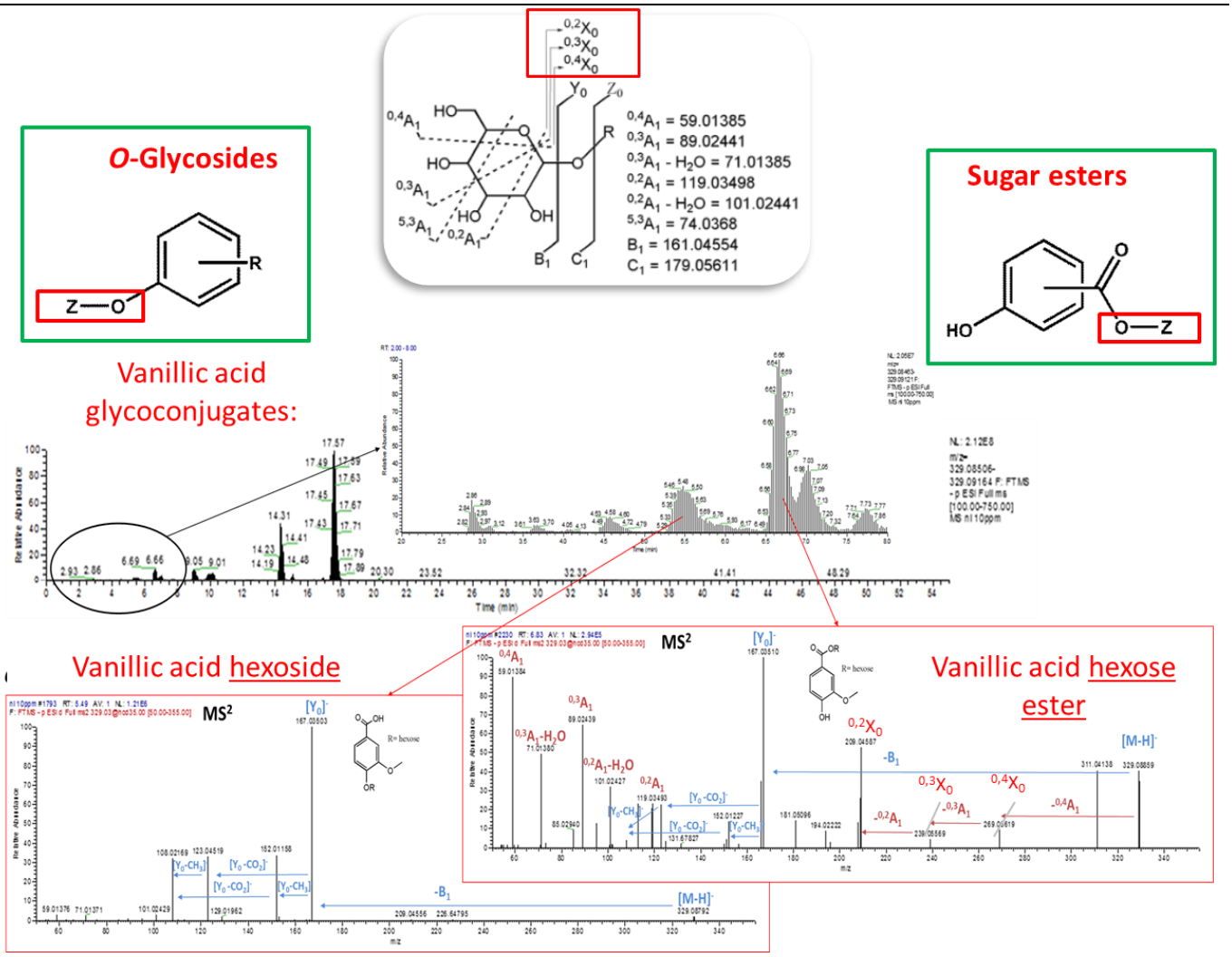
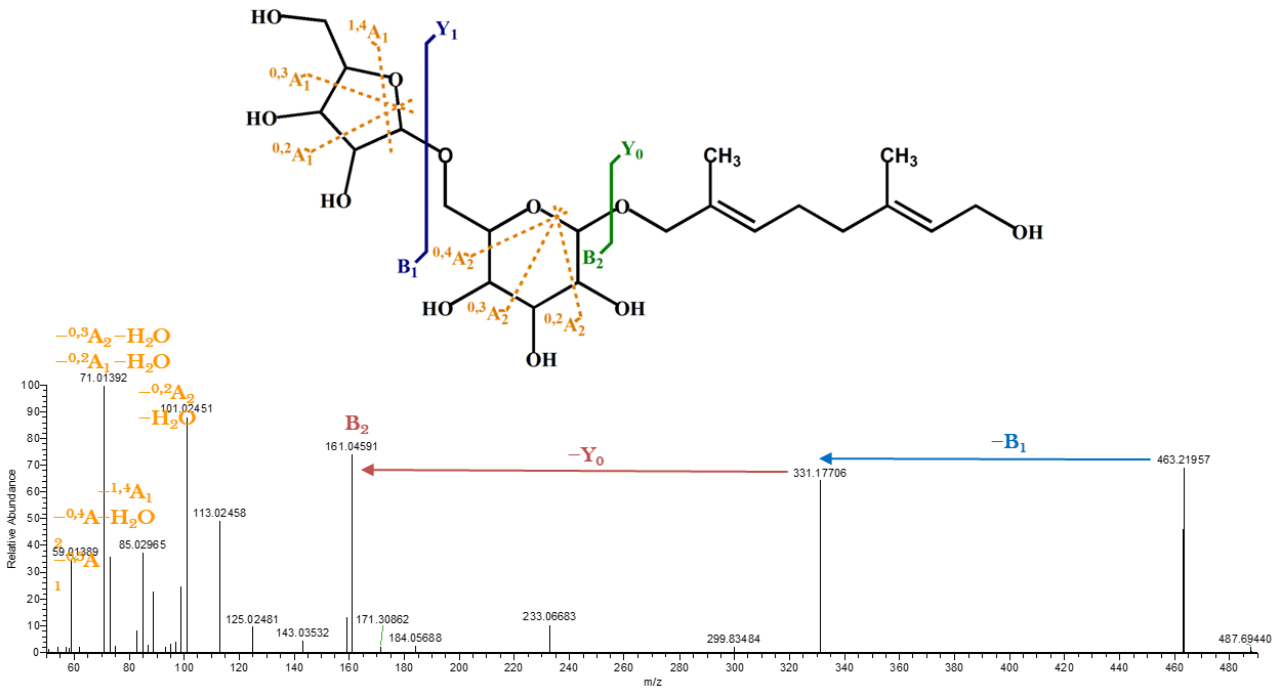


Supplementary Fig. 1: Example of distinction between *O*-glycosides and sugar esters, through MS/MS spectrum

Isomers of dimethyloctadienol hexose-pentose



Supplementary Fig. 2: Example of terpinol-glycosides tentative identification through a specific MS/MS spectrum.

Conclusion

In this work, NL experiments proved to be an efficient tool for tentative identification of glycosides with a non-targeted approach. Indeed, false positives attributed to the neutral loss of sugar-isobaric fragments could certainly be excluded by the absence of $^{k,l}A_i$ fragments in the MS/MS spectrum of the selected precursor ion, while those caused by low-resolution isolation of the quadrupole were rejected due to an inappropriate mass difference between the precursor and product ion. Furthermore, the occurrence of false positives was also strongly reduced by isolation of the glycosidic fraction during SPE sample pretreatment.

However, the greatest limit of the NL approach was the impossibility of detecting glycosides if either the precursor or product ion did not ionize, or if the precursor ion was chemically unstable in source conditions, undergoing hydrolysis. Indeed, in the first case, it was impossible to associate precursor and product ions by comparing full MS and AIF scans if one of them was not detected. In the second case, the precursor ion could be reduced and its ionization intensity in the full MS could be lower than the intensity threshold required to trigger the NL dd-MS/MS experiment. Obviously, the impossibility of defining sugar stereochemistry is not a limitation of the NL approach, but is closely related to the inability of LC-MS analysis to assign regio- and stereochemistry to sugars.

Finally, despite the difficulty in defining the chemical structure of a compound using only its MS/MS spectrum, almost half of the detected glycosides were tentatively identified, allowing good characterization of the wines studied.

2.2. Suspect and targeted screening analysis and technological applications of all HRMS approaches

2.2.1. Color expression of grape polyphenols from unusual *Vitis vinífera* varieties present in Uruguay.

2.2.2. Identification and quantification of 56 targeted phenols in wines, spirits, and vinegars by online solid-phase extraction – ultrahigh-performance liquid chromatography – quadrupole-orbitrap mass spectrometry.

2.2.3. Targeted and untargeted high resolution mass approach for a putative profiling of glycosylated simple phenols in hybrid grapes.

2.2.4. Glycosylated simple phenolic profiling of food tannins using high resolution mass spectrometry (Q-Orbitrap).

2.2.5. The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine.

2.2.6. Free and glycosylated simple phenol profiling in Apulian Italian wines.

2.2.7. Targeted and untargeted characterization of free and glycosylated simple phenols in cocoa beans using high resolution-tandem mass spectrometry (Q-Orbitrap).

2.2.8. Polyphenolic profile, palynological analysis, mineral content and antioxidant properties of bee honeys from Uruguayan native plants.

2.2.9. Targeted and untargeted profiling of alkaloids in herbal extracts using online solid-phase extraction and high-resolution mass spectrometry (Q-Orbitrap).

2.2.10. Cissampelos pareira extract effects in envenomation induced by Bothropsdiporus snake venom.

SECTION 2.2.1.

Color expression of grape polyphenols from unusual *Vitis vinífera* varieties present in Uruguay

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Aim of work

Among the Uruguayan wines, those elaborated from *Vitis vinifera* cv Tannat are the most known, being this varietal representative of the country wines between international consumer. Nevertheless, work is being carried out to improve quality in order to obtain premium wines, and other less frequent grape varieties are now being introduced and employed in the wine production because, for example, of their colour contribution to wines.

To our knowledge, only few previous studies have been focused on the polyphenolic profile for these varieties, even when they are cultivated by their color contribution to wines. In addition, most of the studies were performed on the wines produced. However, in order to evaluate the color expression on grapes, the study of the corresponding wines, even monovarietal ones, should not substitute the study of the polyphenolic profile in the corresponding grapes.

The aim of this study was to determine, by means of HPLC-DAD and HPLC-MS (Orbitrap), the polyphenolic profiles of five red *Vitis vinifera* L. grape varieties cultivated in small vineyards in southern Uruguay in the 2016 and 2017 vintages. The selected varieties were: Ancellota, Aspiran Bouschet (Aramon × Teinturier du Cher × Aspiran), Marselan (Grenache × Cabernet Sauvignon), Arinarnoa (Tannat × Cabernet Sauvignon), Egiodola (Abouriou × Tinta Negra Mole) and Caladoc (Malbec × Grenache).

Conclusion

This work allowed defining for the first time, as far as we know, the profile and the abundance of anthocyanins in the selected color-rich *Vitis vinifera* red grapes potentially important for Uruguayan vitiviniculture. Furthermore, it confirmed the efficiency of NL experiment in tentatively identifying glycosides of low molecular-weight phenolic compounds and its compatibility with suspect screening analysis of free phenols. The occurrence of false positives was prevented by comparing the MS/MS spectra of selected precursor ions with those reported in literature for other matrices and by verifying the presence of the $^{k,l}A_i$ fragments in the case of glycosidic compounds.

However, the greatest limit of this approach was the impossibility of detecting possible free and glycosylated phenolic compounds that did not ionize very well or that were chemically unstable in source conditions. In this case in fact, due to the low ionization intensity of precursor ion or to its complete absence in the full scan spectrum respectively, the MS/MS experiment cannot be performed and no enough spectral information were available to proceed with the tentative identification of phenolic compounds.

SECTION 2.2.2.

Identification and quantification of 56 targeted phenols in wines, spirits, and vinegars by online solid-phase extraction – ultrahigh-performance liquid chromatography – quadrupole-orbitrap mass spectrometry

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Aim of work

Natural phenolic compounds constitute a wide and complex group of plant secondary metabolites, known for their major contribution to the color and aroma of fruit and plant derivatives and for their anti-inflammatory, antioxidant, cardio-protective and many other remarkable physiological effects (Bravo *et al.*, 1998).

The aim of this study was to develop a new comprehensive method to analyze a larger number of low-molecular-weight phenolic compounds using ultra high-performance liquid chromatography coupled with quadrupole/high-resolution mass spectrometry (Q-Orbitrap), since many of them are particularly appreciated because they act as antioxidant agents and reduce LDL-C oxidation. Furthermore, online SPE clean-up was evaluated as an instrument preventing the matrix effect on analyte ionization, in order to allow the method to be applied to different matrices. Finally, the occurrence of free phenolic compounds in wine, vinegar and distillates was investigated.

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Identification and quantification of 56 targeted phenols in wines, spirits, and vinegars by online solid-phase extraction – ultrahigh-performance liquid chromatography – quadrupole-orbitrap mass spectrometry

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ABSTRACT

Phenolic compounds seriously affect the sensory and nutritional qualities of food products, both through the positive contribution of wood transfer in barrel-aged products and as off-flavours. A new targeted analytical approach combining on-line solid-phase extraction (SPE) clean-up to reduce matrix interference and rapid chromatographic detection performed with ultrahigh-performance liquid chromatography coupled with quadrupole/high-resolution mass spectrometry (Q-Orbitrap), was developed for the quantification of 56 simple phenols. Considering the advantages of using on-line SPE and a resolving power of 140,000, the proposed method was applied to define phenolic content in red ($N = 8$) and white (8) wines, spirits (8), common (8) and balsamic (8) vinegars. The final method was linear from the limits of quantification (0.0001–0.001 $\mu g\,mL^{-1}$) up to 10 $\mu g\,mL^{-1}$ with R^2 of at least 0.99. Recovery, used to define method accuracy, ranged from 80 to 120% for 89% of compounds. The method was suitable for analytical requirements in the tested matrices being able to analyse 46 phenols in red wines, 41 phenols in white wines and in spirits, 42 phenols in common vinegars and 44 phenols in balsamic vinegars.

1. Introduction

For many years natural phenolic compounds have attracted the attention of scientists due to the role they play in plant morphology (i.e., pigmentation), physiology (growth and reproduction) and protection (resistance to pathogens and predators) [1], as well as due to their major contribution to the colour and the aroma of fruit and plant derivatives [2]. In the last few years, interest in food phenolic compounds has increased owing to their anti-inflammatory, anti-oxidant, anti-microbial [3], anti-allergic, anti-atherogenic, anti-thrombotic, cardioprotective and vasodilatatory properties and many other effects [4]. These compounds are currently widely used in the food, cosmetic and pharmaceutical industries for countless applications [1,5].

Phenols are powerful anti-oxidant compounds because they can scavenge free radicals, donate hydrogen atoms or electrons and chelate metal cations [6]. However all these activities are strictly related to the phenolic chemical structure considered. This is the case of phenolic acids, where anti-oxidant activity increases with the hydroxylation level and decreases when the hydroxyl group is substituted by a methoxyl group [7]. Of phenolic acids, hydroxycinnamic acids appear to be more anti-oxidant than hydroxybenzoic compounds [8], probably thanks to the greater H-donating ability and radical stabilization of the —CH=CH—COOH [7].

Structurally, natural phenolic compounds reveal a great structural diversity ranging from simple molecules, such as phenolic acids, to complex high-molecular weight polymers, such as tannins [1,8]. Furthermore, many of them appear in conjugated form with mono-, di- or oligosaccharidic residues or occur as ester and methoxy derivatives [9].

Among low molecular weight phenolic compounds, most plant phenols can be found as simple phenols (phenol and cresol), hydroxybenzoic acids (gallic, protocatechuic, vanillic, and syringic) or their aldehydic derivatives (vanillin, syringaldehyde, and protocatechuic aldehyde), hydroxycinnamic acids (ferulic, caffeic, and *p*-coumaric), cinnamyl alcohols (guaiacol, syringol, and coniferyl alcohol) and flavonoids (catechin and epicatechin) [1]. They can be present in vegetables, cereals, legumes, fruits, nuts and in transformed plant products such as wine, cider, beer, tea and cocoa, at levels that can range over several orders of magnitude [1]. In wine,

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phenolic content varies greatly in reds and whites and in young and aged products [9].

Phenolic content strongly affects the sensory and nutritional qualities of plant foods, such as astringency and bitterness, while phenolic propensity to oxidize during processing and storage can result in either beneficial or undesirable characteristics in food products [10]. This is the case of cocoa browning or tea polyphenol polymerization, both resulting in the development of new distinctive organoleptic characteristics. Conversely, the browning reaction in fresh fruits and vegetables causes undesirable colours and flavours [9].

Plant phenolic compounds have been extensively studied using spectrophotometric and chromatographic methods [11]. Spectrophotometric assays make it possible to recognize various functional groups present in phenolic compounds. The most widely used assays are the Folin-Denis and the Folin-Ciocalteau. However, both reagents are not specific in detecting all phenolic groups, including those present in extractable proteins. Chromatographic methods are instead used for both separation and quantification of single phenolic compounds. Gas chromatographic techniques require some phenolic compounds to be transformed into more volatile derivatives by methylation, trifluoroacetylation or conversion to trimethylsilyl derivatives. High-performance liquid chromatographic (HPLC) techniques do not require the derivatization step and food phenolic compounds can be detected using common detectors such as UV–vis, photodiode array and UV-fluorescence. Other detection systems include electrochemical coulometric array or voltammetric detectors.

Structural elucidation of phenolic compounds is generally performed using HPLC coupled to mass spectrometry (MS). Over time, the ionization technique applied to phenol analysis has evolved from fast atom bombardment, to matrix assisted laser desorption ionization, electrospray ionization (ESI) and atmospheric pressure chemical ionization. These sources can be coupled to several MS analysers, including quadrupole (Q), ion trap, time of flight and Fourier-transform ion cyclotron resonance, all characterized by specific available resolution and mass range.

Our aim in the study was the development of a new targeted analytical approach for the quantification of a large selection of simple phenols, using automatic on-line SPE clean-up to reduce matrix interference, combined with the rapid chromatographic detection provided by ultrahigh-performance liquid chromatography (UHPLC) coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap).

2. Materials and methods

2.1. Reagents and solutions

LC–MS grade acetonitrile (99.9%), LC–MS grade methanol (99.9%), MS grade formic acid (98%) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol, 99.5%) were purchased from Fluka (St. Louis, MO, USA), while acetic acid (99–100%), L-glutathione reduced (99%) and *p*-nitrophenol (99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Mass calibration was performed using a standard mixture of sodium dodecyl sulfate and sodium taurocholate (Pierce® ESI Negative Ion Calibration Solution, Rockford, IL, USA). Deionized water was produced using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany). The target phenolic compounds, grouped into 19 classes according to their chemical structure [12], are summarized in Table 1.

Eleven water–methanol stock solutions of almost 4–6 phenols, each of 200 mg L^{-1} (Table 1) were prepared. L-Glutathione reduced and DL-dithiothreitol (2.5 g L^{-1}) were added to the previous solutions as antioxidants. Methanol content ranged from 15 to 55% according to the components solubility. The stock solutions were then combined in a single intermediate solution (water–methanol mixture; 80:20, v/v), with a concentration of 10 mg L^{-1} for each phenol. The calibration solutions were prepared in the range 0.0001–10 μg mL^{-1}. Stock solutions were stored at −4 °C.

2.2. Samples and sample preparation

Eight red wines, 8 white wines, 8 spirits (brandy, rum, calvados, armagnac, whisky, cognac and 2 types of grappa), 8 common vinegars and 8 balsamic vinegars were sampled on the Italian markets and used to test the efficacy of the proposed method.

Before analysis the samples were filtered using 0.45 μm PTFE filter cartridges (Sartorius AG, Goettingen, Germany), diluted 10 times and added of the internal standard (*p*-nitrophenol, 0.495 μg mL^{-1}) and formic acid (0,1%, v/v).

2.3. On-line SPE-LC set up and chromatographic conditions

Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with two pumps and a Rheodyne 6-port automated switching valve, able to control two independent fluidic systems. The first system was dedicated to on-line SPE sample processing, while the second controlled chromatographic separation on the analytical column. Chromeleon™ 7.2 Chromatography data system software (Thermo Scientific™ Dionex™) was used to automatically control the switching valve and the chromatographic separation gradient. The autosampler was set at 5 °C and the column oven at 40 °C.

As shown in Fig. 1, the analytical procedure began with the switching valve in position 1–6 (liquid connection between 1st and 6th port). In this configuration the SPE cartridge, connected to pump 1 which was delivering deionized water (eluent A) at a flow rate of 0.250 mL min^{-1}, could be loaded by the autosampler with 2 μL of sample. Meanwhile, pump 2 conditioned the analytical column with an initial 95:5 (v/v) eluent of deionized water (eluent A) and acetonitrile (eluent B) at a flow rate of 0.400 mL min^{-1}.

After 4 min the valve was switched to position 1–2 and the mobile phase of pump 2 (95% A and 5% B, flow rate of 0.400 mL min^{-1}) flowed through the SPE cartridge, progressively removing the retained analytes and transferring them to the analytical C18 UHPLC column. Chromatographic separation was performed by managing the solvent B concentration as follows: from 4.0 to 5.5 min eluent B at 5%, from 5.5 to 17 min a linear increase to 60%, from 17.0 to 18.5 min a linear increase to 100%, then column equilibration from 18.5 to 22.0 min at 5%. Meanwhile the flow rate of pump 1, connected now through the SPE to the waste, decreased to 0.100 mL min^{-1}.

During the column equilibration step, the valve was again switched to the initial 1–6 position and pump 1 flushed with 0.1% of formic acid at 1 mL min^{-1} the SPE cartridge in order to re-equilibrate and reactivate it before the next analysis.

Different types of SPE columns were evaluated: Zorbax Eclipse Plus-C18 (2.1 mm × 12.5 mm ID, 5 μm; Agilent, Santa Clara, CA, USA), Zorbax Eclipse Plus-C8 (2.1 mm × 12.5 mm ID, 5 μm; Agilent, Santa Clara, CA, USA), Acclaim Trinity P1 (2.1 mm × 10 mm, 5 μm, Dionex ThermoFisher, Sunnyvale, CA, USA), SolEx HRP (2.1 mm × 20 mm, 12–14 μm, hydrophilic divinylbenzene; ThermoFisher, Sunnyvale, CA, USA), HyperSep™ Retain PEP spe cartridge (3.0 mm × 10 mm, 40–60 μm, Thermo Scientific, Sunnyvale, CA, USA).

Furthermore, 4 chromatographic columns were tested to achieve the best chromatographic separation and peak shape, including Acclaim HILIC-10 (2.1 mm × 150 mm, 3 μm particle size; Dionex ThermoFisher, Sunnyvale, CA, USA), Raptor Biphenyl

Table 1
Technical characteristics of target phenolic compounds.

Nomenclature		Purity	CAS No.	Supplier*	Stock solution	
Common	IUPAC				I.D.	MeOH** (%)
Hydroxybenzoic acids						
Ellagic acid	2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione	≥96%	476-66-4	a	1	55
Gallic acid	3,4,5-Trihydroxybenzoic acid	≥97.5%	149-91-7	b	2	15
Gentisic acid	2,5-Dihydroxybenzoic acid	≥98%	490-79-9	a	2	15
Lithium salicylate	Lithium-2-hydroxybenzoate	≥100%	552-38-5	c	3	15
p-Carboxyphenol	4-Hydroxybenzoic acid	≥99%	99-96-7	a	2	15
Protocatechuic acid	3,4-Dihydroxybenzoic acid	≥97%	99-50-3	a	3	15
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	≥97%	530-57-4	a	2	15
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	≥97%	121-34-6	a	2	15
Hydroxycinnamic acids						
Caffeic acid	3,4-Dihydroxycinnamic acid	≥95%	331-39-5	a	2	15
trans-Ferulic acid	(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid	≥98%	1135-24-6	a	2	15
trans-*p*-Coumaric acid	(E)-3-(4-hydroxyphenyl)-2-propenoic acid	≥98%	501-98-4	b	2	15
Sinapinic (sinapic) acid	3-(4-Hydroxy-3,5-dimethoxyphenyl)prop-2-	≥97%	530-59-6	a	2	15
Hydroxyphenylacetic acids						
Homovanillic acid	2-(4-Hydroxy-3-methoxy-phenyl)acetic acid	≥98%	306-08-1	b	2	15
Hydroxybenzaldehydes						
o-Vanillin	2-Hydroxy-3-methoxybenzaldehyde	≥99%	148-53-8	b	4	40
Protocatechuic aldehyde	3,4-Dihydroxybenzaldehyde	≥97%	139-85-5	b	5	40
Syringaldehyde	4-Hydroxy-3,5-dimethoxybenzaldehyde	≥98%	134-96-3	d	5	40
Vanillin	4-Hydroxy-3-methoxybenzaldehyde	≥99%	121-33-5	b	5	40
Hydroxycinnamaldehydes						
Coniferylaldehyde	3-(4-Hydroxy-3-methoxyphenyl)prop-2-enal	≥98%	458-36-6	b	1	55
Sinapinaldehyde	3-(4-Hydroxy-3,5-dimethoxyphenyl)prop-2-	≥98%	4206-58-0	b	1	55
Simple phenols						
Phenol	Phenol	≥99%	108-95-2	b	6	22
Pyrocatechol (catechol)	Benzene-1,2-diol	≥99%	120-80-9	b	6	22
Alkylphenols						
3,4-Xylenol	3,4-Dimethylphenol	≥98%	95-65-8	a	7	55
4-Ethylcatechol	4-Ethyl-1,2-benzenediol	≥98%	1124-39-6	e	7	55
4-Ethylphenol	4-Ethylphenol	≥97%	123-07-9	a	8	55
4-Methylcatechol	4-Methyl-1,2-benzenediol	≥95%	452-86-8	a	6	22
4-Vinylphenol	4-Ethenylphenol	n.d.	2628-17-3	d	8	55
m-Cresol	3-Methyphenol	≥98%	108-39-4	b	7	55
o-Cresol	2-Methyphenol	≥99%	95-48-7	b	9	35
p-Cresol	4-Methyphenol	≥99.9%	106-44-5	f	8	55
Methoxy and alkylmethoxyphenols						
4-Ethylguaiacol	4-Ethyl-2-methoxyphenol	≥98%	2785-89-9	d	8	55
4-Methylguaiacol (creosol)	2-Methoxy-4-methylphenol	≥99%	93-51-6	b	8	55
4-Vinylguaiacol	4-Ethenyl-2-methoxyphenol	≥98%	7786-61-0	d	1	55
Guaiacol	2-Methoxyphenol	≥99%	90-05-1	b	7	55
Dimethoxyphenol and alkyldimethoxyphenols						
4-Methylsyringol	2,6-Dimethoxy -4-methylphenol	≥97%	5/7/6638	d	8	55
Syringol	1,3-Dimethoxy-2-hydroxybenzene	≥99%	91-10-1	b	9	35
Alkylphenyl alcohols and alkylphenylmethoxy alcohols						
Coniferyl alcohol (coniferol)	4-(3-Hydroxy-1-propenyl)-2-methoxyphenol	≥98%	458-35-5	b	6	22
Homovanillyl alcohol	4-(2-Hydroxyethyl)-2-methoxyphenol	≥99%	2380-78-1	b	9	35
Hydroxytyrosol	4-(2-Hydroxyethyl)-1,2-benzenediol	≥98%	10597-60-1	g	6	22
Tryptophol	2-(*1H*-indol-3yl)ethanol	≥98%	526-55-6	a	7	55
Tyrosol	4-(2-Hydroxyethyl)phenol	≥99.5%	501-94-0	b	6	22
Hydroxyphenylpropenes						
4-Allyl syringol	4-Allyl-2,6-dimethoxyphenol	≥90%	6627-88-9	b	8	55
Eugenol	4-Allyl-2-methoxyphenol	≥99%	97-53-0	a	7	55
Isoeugenol	*Cis* + *trans*, 2-methoxy-4-(prop-1-en-1-yl)phenol	≥98%	97-54-1	b	9	35
Hydroxybenzoketones and derivatives						
Acetosyringone	4′-Hydroxy-3′,5′-dimethoxyacetophenone	≥97%	2478-38-8	b	10	40
Acetovanillone (apocynin)	1-(4-Hydroxy-3-methoxyphenyl)ethanone	≥98%	498-02-2	b	10	40
Ethyl vanillate	ethyl 4-hydroxy-3-methoxybenzoate	n.d.	617-05-0	b	9	35
Isoacetosyringone	2′,4′-Dimethoxy-3′-hydoxyacetophenone	n.d.	23133-83-7	h	4	40
Isoacetovanillone	4-Hydroxy -3,5-dimethoxyphenyl acetone	≥97%	6100-74-9	b	4	40
Isopropiosyringone	4-Hydroxy-3-methoxy-phenylacetone	n.d.	19037-58-2	h	10	40
Isopropiovanillone	4-Hydroxy-3-methoxy-phenylacetone	≥96%	2503-46-0	b	10	40
Methyl vanillate	Methyl 4-hydroxy-3-methoxybenzoate	≥99%	3943-74-6	d	10	40
Hydroxybenzoether						
Vanillyl ethyl ether	4-(Ethoxymethyl)-2-methoxyphenol	n.d.	13184-86-6	h	9	35
Flavanols						
(−)-Epicatechin	(2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol	≥90%	490-46-0	b	11	30
(+)-Cathechin	(2R,3S)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-Triol	≥99%	154-23-4	a	6	22
Hydroxycoumarins						
Aesculetin	6,7-Dihydroxy-2-chromenone	≥98%	305-01-1	b	11	30
Scopoletin	7-Hydroxy-6-methoxychromen-2-one	≥98.5%	92-61-5	a	11	30

n.d., not detected.

* a = Fluka (St. Louis, MO, USA); b = Sigma Aldrich (St. Louis, MO, USA); c = Aldrich (St. Louis, MO, USA); d = SAFC (St. Louis, MO, USA); e = Alfa Aesar (Karlsruhe, Germany); f = Supelco (St. Louis, MO, USA); g = CHEMOS GmbH (Regenstauf, Germany); h = TransMIT (Gießen, Germany).

** % MeOH in water-methanol standard mixture.

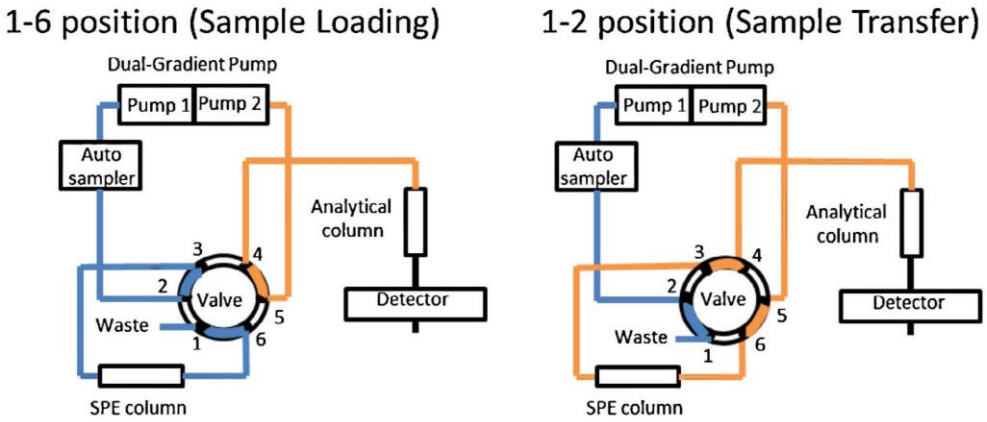

Fig. 1. Automated on-line solid-phase extraction – ultrahigh-performance liquid chromatography (UHPLC) configuration.

(3 mm × 150 mm, 2.7 μm particle size; Restek, Bellefonte, PA, USA), Poroshell 120 EC-C18 (3 mm × 100 mm, 2.7 μm particle size; Agilent, Santa Clara, CA, USA) and Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm particle size; Waters, Milford, MA, USA).

2.4. HRMS/MS conditions

Identification and quantification of phenolic compounds were performed using a Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (HQ-OMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II) and operating in negative ion mode. Mass spectra were acquired in profile mode through full MS-data dependent MS/MS analysis (full MS-dd MS/MS).

Full mass spectra were recorded at mass resolving power of 140,000 full width at half-maximum (FWHM, calculated for m/z 200, 1.5 Hz), automatic gain control (AGC) target of 5×10^5 ions, maximum inject time (IT) 150 ms. Data-dependent mass spectra were recorded at a mass resolving power of 17,500 FWHM (defined for m/z 200, 12 Hz), AGC target of 2×10^4 ions, IT of 50 ms. The MS/MS experiment started when the target precursor ion was detected in a defined time window (analyte retention time ± 0.30 min) and once performed, it was not repeated again for a period of 6 s.

The mass spectrometer operated using the following parameters: spray voltage, 2.80 kV; sheath gas flow rate, at 30 arbitrary units; auxiliary gas flow rate, at 20 arbitrary units; capillary temperature, at 310 °C; S lens RF level, at 50 arbitrary units; capillary gas heater temperature, 280 °C.

Accurate mass calibration was performed before each analysis using a solution of sodium dodecyl sulfate (2.6 mg L^{-1}) and sodium taurocholate (4.9 mg L^{-1}), with the addition of formic and acetic acids at a concentration of 5 mg L^{-1} for each. Calibration was performed using a customized mass list: m/z 59.01385 (acetate, $[M-H]^-$), m/z 112.98560 (formiate dimer, $[M_2+Na-2H]^-$), m/z 265.14690 (lauryl sulfate, $[M-H]^-$), m/z 514.28440 (taurocholate, $[M-H]^-$). Mass instrument control was performed using Thermo Fisher Scientific Xcalibur 2.2 software, data processing with Thermo Fisher Scientific TraceFinder™ software (Thermo Scientific, San Jose, CA, USA.

Full mass spectral data were used for identification and quantification of analytes. The presence of analytes in real matrices was identified through data-dependent mass spectral results, by matching MS/MS spectra with those obtained from previous experiments performed on standard solutions and collected as a spectral library in the Thermo Library Manager Application (Thermo Scientific, San Jose, CA, USA).

2.5. Calibration and method validation

High resolution m/z values, mass tolerance < 5 ppm [13], made it possible to identify almost univocally target compounds in real samples, but in the case of isobaric interferences further evidence came from the comparison of peak retention times with those observed for calibration standards.

Analytes were quantified through external solvent calibration curves, using $1/x$ as the weighting factor for linear regression. The working range was determined through 7 repeated measurements of solvent standard solutions at 13 concentration levels (0.0001, 0.0005, 0.001, 0.005, 0.01, 0.1, 0.5, 1, 2, 3, 4, 5, 10 μg mL^{-1}). Linearity range was defined considering only standard concentration levels allowing a regression coefficient of determination (R^2) of at least 0.990.

Limits of detection (LODs) were determined as reported by Mol et al. [14]. Limits of quantitation (LOQs) were established according to EURACHEM [15]. Method accuracy was estimated in terms of relative recovery by spiking 40 natural samples of 5 different matrices with 1 μg mL^{-1} of each phenol. Method precision (R.S.D.%) was evaluated through standard deviation of 7 repetitions carried out at 13 concentration levels inside the working range.

Inter day method precision and standard stability were investigated at two concentration levels (0.1 and 1 μg mL^{-1}), repeating the analysis after 24, 48 and 72 h during the same analytical batch.

The effectiveness of the proposed on-line SPE method in reducing the possible matrix effects that can occur analysing real samples, was evaluated in a white wine spiked at 8 levels with standards (calibration range 0.1–2 μg mL^{-1}), comparing the linearity (R^2) of its calibration curves as compared to those obtained by direct injection. A reduced range of calibration was used in order to ensure greater linearity of the methods.

Finally, since carry over can represent a severe limiting factor for any on-line sample treatment [16], sample loading and eluting conditions were carefully selected in order to avoid significant problems in blank samples analysed after a highly-concentrated standard (5 μg mL^{-1}).

3. Results and discussion

3.1. Method optimization

3.1.1. On-line SPE clean-up conditions

Although UHPLC coupled with HRMS is a very powerful analytical technique, the susceptibility of the ESI source to matrix-related ionization represents the major drawback. Consequently, the analysis of real samples generally requires pretreatment steps aimed

at the removal of unwanted matrix constituents from the sample. These processes are often rate-limiting steps in the analytical protocol because time-consuming and can significantly affect the quality and cost of the analysis. Thus, fully automated clean-up techniques are required for an accurate, reliable and efficient analytical procedure.

A series of experiments was performed to optimize on-line clean-up conditions and maximize holding efficiency, considering various parameters such as column stationary phase, loading solvent, loading flow rate and washing time. Insufficient retention power was the main issue for the first three columns tested (Zorbax Eclipse Plus-C18, Zorbax Eclipse Plus-C8, and Acclaim Trinity P1), with many polar analytes leaking during matrix washing. Using hydrophilic divinylbenzene stationary phases (SolEx HRP), leakage problems decreased and were limited to some hydroxybenzoic acids such as gallic, protocatechuic and gentisic acids, eluted early due to their higher polarity. The HyperSep™ Retain PEP cartridge, packed with porous polystyrene DVB material modified with urea functional groups, showed the most balanced retention capability for polar and non-polar analytes and delayed the elution of hydroxybenzoic acids.

3.1.2. UHPLC separation

Due to major differences in the physicochemical properties of target compounds, in particular in terms of polarity, great attention had to be paid to analytical column type, composition of the mobile phase and eluent pH.

The Acclaim HILIC-10 column was unable to retain a large number of non-polar analytes, and the peaks were broad and irregular. When C18 reverse phase chromatography was used, all analytes were reasonably retained, but the Raptor Biphenyl column produced broad and irregular peaks. Poroshell 120 EC was shown to be less selective and efficient. The Waters C18 column was finally selected for chromatographic separation because it provided suitable results in terms of retention times and peak shape.

As regard mobile phase composition, a gradient of water and acetonitrile was the most reasonable elution compromise. Considering the very high selectivity of high resolution mass detection, perfect separation of all analytes was not required, except for isomers or target compounds with the same m/z value (eugenol-isoeugenol $[M-H]^-$ = 163.0764; protocatechuic-gentisic acids $[M-H]^-$ = 153.01933). With the exception of isomer pairs of acetovanillone-isoacetovanillone and *m*- and *p*-cresol, determined as isomeric sum, all target compounds were individually quantified.

Although the formic acid addition to the aqueous phase could increase acidic compound retention, the decrease in dissociation caused a reduction in ESI ionization performance for many analytes. Therefore, acidifying sample vials instead of mobile phase was considered the right balance to retain mainly acids, without losing sensitivity. In addition, sample acidification also made the acid retention time more reproducible in different matrices.

3.1.3. MS detection

HQ-OMS combines quadrupole mass isolation capability with the high mass resolution of Orbitrap, achieving remarkable results in terms of detection, quantification and unequivocal identification of the target analytes present in complex matrices.

In our study, the full MS-dd-MS/MS method using a list of target analytes (inclusion list) was shown to be the most suitable option. Orbitrap mass resolving power is directly correlated with acquisition time: the higher the mass resolution, the longer the acquisition time and the lower the number of scan points earned. According to the general requirements [17] 12–15 data points are sufficient to accurately define a chromatographic peak, and setting 140,000 FWHM as the resolving power represented a good compromise between spectral resolution and scan speed for full MS mode. Furthermore, during the analysis of real samples the number of data points per peak could be higher than for standards, since not all coeluting analytes may be present in the sample and consequently the number of triggered MS/MS experiments was lower.

As regard MS/MS spectra acquisition, the resolving power of dd-MS/MS mode was set to 17,500 FWHM, since additional selectivity in the isolation of target compounds was granted by the quadrupole analyser. In order to obtain the most informative MS/MS spectra, containing both precursor ion and fragments, normalized collision energy (NCE) for higher-energy collisional dissociation was optimized for each target compound. The optimal NCE values were defined by using solvent standards analysed individually by direct infusion in the mobile phase flow with the same eluent ratio as their relative retention times and by modifying NCE settings. The exact masses of the target compounds, relative retention times, NCE optimized values and an overview of the main fragment ions are summarized in Table 2.

Vaclavick et al. [18] demonstrated the importance of using the peak smoothing procedure to minimize the impact of full MS-dd-MS/MS acquisition mode on both the shape and area of peaks. For this reason, the smoothing degree was set to 7. Instrument mass calibration was performed with a customized mass list, modifying the manufacturer's calibration solution in order to improve mass accuracy over the entire scan range. Indeed, without implementing the standard calibration solution, the $\Delta m/z$ value was higher than 5 ppm for all target compounds with an exact mass lower than 200 m/z.

3.1.4. Method validation

3.1.4.1. Standard solutions. The eleven water–methanol stock solutions grouped target compounds according to similarity in terms of chemical structure and eluent solubility. If stored at −4 °C, stock solutions remained intact for at least 2 months, with the exception of solution 1 (Table 1), which needed to be prepared each month.

3.1.4.2. Calibration. The upper limit of the linear range was the highest standard concentration level that allowed a R^2 value of at least 0.99, considering the compound LOQ as the lower limit. The only exceptions were syringic acid, homovanillic acid and tyrosol with R^2 values of 0.989, 0.988 and 0.984 respectively, vanillic acid with a R^2 value of 0.977 and ellagic acid with a R^2 value of 0.967. The linearity range was 6 orders of magnitude (from 0.0001 or 0.0005 to 10 $\mu g\,mL^{-1}$) for almost 20% of analytes, 5 orders of magnitude (from 0.0001 or 0.0005 to 3–5 $\mu g\,mL^{-1}$ or from 0.001 or 0.005 to 10 $\mu g\,mL^{-1}$) for almost 40% of analytes, 4 orders of magnitude (from 0.001 or 0.005 to 3–5 $\mu g\,mL^{-1}$ or from 0.01 to 10 $\mu g\,mL^{-1}$) for 18% of compounds, 3 orders of magnitude (from 0.01 to 5 $\mu g\,mL^{-1}$ or from 0.1 to 10 $\mu g\,mL^{-1}$) for 12.5% of analytes and 2 orders of magnitude (from 0.1 to 5 $\mu g\,mL^{-1}$) for almost 9% of compounds. Only ellagic acid had a reduced range of linearity. The upper limit varied between 3 and 5 $\mu g\,mL^{-1}$ depending on the analytes considered. Linearity range, expressed without considering sample dilution, and R^2 are summarized in Table 2.

3.1.4.3. Limits of quantitation. Detectability was strongly dependent on the ionization efficiency of compounds in negative mode. Almost all phenolic acids and their aldehydic derivatives had very low limits of quantitation (Table 2, LOQs are expressed without considering sample dilution), usually 0.0001 $\mu g\,mL^{-1}$. Alkylmethoxyphenols and their derivatives were characterized by lower efficiency in terms of HESI ionization, with LOQs that varied mainly in the range of 0.0005–0.01 $\mu g\,mL^{-1}$ (Table 2). With the exception of tyrosol (LOQ, 0.0001 $\mu g\,mL^{-1}$), the highest values were registered for phenol, tryptophol, cresols and 4-ethylphenol

Table 2
UHPLC–MS parameters of phenolic compounds.

Compounds	$[M-H]^-$ (m/z)	RT (min)	NCE	MS/MS fragments	Linearity range[a] ($\mu g\,mL^{-1}$)	LOQ[a] ($\mu g\,mL^{-1}$)
Gallic acid	169.0142	5.60	45	125.0244	0.0001–8.80	0.0001
Protocatechuic acid	153.0193	5.76	50	109.0294	0.0001–5.03	0.0001
p-Carboxyphenol acid	137.0244	6.14	40	93.0646	0.0001–5.28	0.0001
Gentisic acid	153.0193	6.21	45	109.0295, 108.0217	0.0001–5.30	0.0001
Hydroxytyrosol	153.0557	6.28	50	123.0437, 95.0487	0.0005–5.15	0.0005
Vanillic acid	167.0350	6.42	40	152.0114, 123.0452	0.0001–3.04	0.0001
Syringic acid	197.0455	6.57	35	182.0216, 166.9984	0.0001–4.26	0.0001
Caffeic acid	179.0350	6.60	40	135.0452	0.0001–5.33	0.0001
Homovanillic acid	181.0506	6.79	45	137.0617, 122.0373	0.0010–2.97	0.0010
Tyrosol	137.0608	6.79	40	119.0502, 106.0426	0.0001–3.15	0.0001
Protocatechuic aldehyde	137.0244	7.10	60	108.0216, 93.0344	0.0001–5.05	0.0001
Pirocatecolo	109.0295	7.28	80	108.0202, 91.0176	0.0005–8.95	0.0005
p-Coumaric acid	163.0401	7.37	35	119.0502, 93.1266	0.0001–5.20	0.0001
Salicylic acid	137.0244	7.72	60	93.0346, 122.0374	0.0001–8.85	0.0001
Phenol	93.0345	7.73	100	65.0382	0.1050–9.49	0.1050
Catechin	289.0717	7.89	35	245.0805, 221.0812	0.0051–5.12	0.0051
Ferulic acid	193.0506	8.17	40	178.0268, 149.0608	0.0001–6.21	0.0001
Aesculetin	177.0193	8.48	50	133.0296, 105.0345	0.0001–9.72	0.0001
Sinapinic acid	223.0611	8.54	30	208.0373, 179.0714	0.0005–4.99	0.0005
Homovanillic alcohol	167.0714	8.78	35	152.0477, 122.0375	0.0051–5.10	0.0051
Epicatechin	289.0718	9.67	40	245.0805, 221.0812	0.0001–9.02	0.0001
Vanillin	151.0401	9.86	40	136.0152, 108.0202	0.0001–5.36	0.0001
Coniferyl alcohol	179.0714	10.11	35	164.0478, 121.0296	0.0107–5.35	0.0107
4-Methylcatechol	123.0451	10.18	100	108.0214, 90.0591	0.0005–9.36	0.0005
Syringaldehyde	181.0506	10.42	40	166.0269, 151.0035	0.0008–13.5	0.0008
Isopropiovanillone	179.0714	10.55	40	164.0477, 121.0295	0.0054–5.40	0.0054
Scopoletin	191.0350	10.66	40	176.0112, 148.0166	0.0010–9.11	0.0010
Acetovanillone + isoacetovanillone	165.0557	10.69	40	150.0321, 122.0371	0.0001–5.12	0.0001
Isopropiosiringone	209.0819	10.81	35	194.0581, 179.0348	0.0011–4.39	0.0011
Acetosyringone	195.0662	11.00	30	180.0426, 165.0190	0.0001–5.19	0.0001
Isoacetosiringone	195.0662	11.24	30	180.0426, 165.0190	0.0011–9.72	0.0011
Syringol	153.0557	11.32	50	138.0321, 123.0087	0.0129–6.46	0.0129
Coniferylaldehyde	177.0556	11.51	35	162.0320	0.0001–5.07	0.0001
Sinapinaldehyde	207.0663	11.64	35	192.0427, 177.0193	0.0010–5.04	0.0010
Tryptophol	160.0767	11.87	70	142.0659, 130.0660	0.1102–5.51	0.1102
o-Vanillina	151.0401	12.09	40	136.0152, 123.0083	0.0010–4.98	0.0010
Methyl vanillate	181.0506	12.13	40	166.0268, 151.0036	0.0005–9.27	0.0005
(*m* + *p*)-Cresol	107.0502	12.27	60	79.0551, 65.7207	0.1010–5.05	0.1010
4-Ethylcatechol	137.0608	12.30	35	122.0374	0.0005–9.25	0.0005
o-Cresol	107.0502	12.41	60	82.5568	0.1170–5.85	0.1170
Vanillyl ethyl ether	181.0870	12.67	30	166.0633, 153.0656	0.0010–9.16	0.0010
Guaiacol	123.0451	12.85	70	108.0215, 105.0346	0.0110–9.88	0.0110
4-Methylsyringol	167.0713	12.87	20	152.0478, 137.0243	0.0101–5.06	0.0101
4-Vinylphenol	119.0502	13.60	100	91.0550, 93.0346	0.0112–5.60	0.0112
Ethyl vanillate	195.0662	13.69	40	180.0415, 130.9911	0.0006–9.90	0.0006
3,4-Xylenol	121.0658	13.73	90	119.0503, 96.9445	0.0100–4.98	0.0100
4-Vinylguaiacol	149.0608	14.00	20	134.0375, 87.0088	0.0055–9.83	0.0055
Ellagic acid	300.9989	14.00	60	229.0149, 185.0071	3.03–5.05	3.03
4-Ethylphenol	121.0658	14.22	90	106.0423, 83.9854	0.1022–5.11	0.1022
4-Methylguaiacol	137.0608	14.37	35	122.0374	0.0105–9.59	0.0105
4-Ethylguaiacol	151.0764	14.58	10	136.0529, 121.0293	0.0009–8.57	0.0009
4-Allyl syringol	193.0870	14.85	10	178.0632, 163.0399	0.0202–10.1	0.0202
Eugenol	163.0764	15.11	30	148.0529	0.0087–15.6	0.0087
Isoeugenol	163.0764	15.47	30	148.0529, 118.9925	0.0102–9.14	0.0102

Note: RT, retention time; NCE, normalized collision energy; R^2, coefficient of determination; LOQ, limit of quantitation.
[a] Linerity range and LOQs are defined without considering sample dilution.

(LOQ, 0.1 $\mu g\,mL^{-1}$). Focusing on volatile phenols (4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol, 4-ethylguaiacol), detection with coulometric array [19] made it possible to obtain better LOQs for the first three phenols. Only in the case of 4-ethylguaiacol did the UHPLC–MS method give lower LOQ. Hydroxybenzoketones and their derivatives, flavanols and hydroxycoumarins showed less variability than alkylmethoxyphenols, but LOQs still varied in the range of 0.0001–0.005 $\mu g\,mL^{-1}$. In particular, even if isomers, the LOQs of catechin and epicatechin diverged by more than one order of magnitude (0.0051 and 0.0001 $\mu g\,mL^{-1}$ respectively).

With reference to the dilution factor used to analyse the selected matrices (10 dilutions), the proposed method showed limits in the detection of target compounds with a LOQ of over than 0.1 $\mu g\,mL^{-1}$. In particular for ellagic acid (the target compound with the highest LOQ), the defined limit of quantitation agreed with the compound concentration found in red wines and spirits aged in barriques, but was not sensitive enough to allow ellagic acid quantification in white wines and vinegars.

3.1.4.4. Accuracy and method precision. The accuracy of the method was evaluated by recovery: acceptable recovery, ranging from 80 to 120%, was obtained for 89% of compounds, although the five matrices differed widely in terms of composition. For almost 9% of analytes recovery ranged from 72 to 91% and the remaining 2% had recovery ranging from 67 to 132%. Table 3 summarizes these results.

Method precision, measured as R.S.D.% was always lower than 12% for all concentration levels over analyte LOQs (Fig. 2). In

Table 3
Accuracy of the method expressed as recovery in different matrices. Evaluation of the matrix effect on ionization yield by comparison of R^2, measured both for the proposed method (pretreatment with solid-phase extraction, SPE) and for direct injection (without the pretreatment step).

Compounds	Recoveries (%)					Matrix interferences evaluation (as R^2)[a]
	Red wines	White wines	Distillates	Common vinegars	Balsamic vinegars	SPE (direct injection)
Gallic acid	83	90	92	81	80	0.998 (0.999)
Protocatechuic acid	94	95	92	93	98	0.996 (0.997)
p-Carboxyphenol acid	88	90	98	91	94	0.999 (0.994)
Gentisic acid	94	92	96	88	86	0.997 (0.990)
Hydroxytyrosol	83	83	91	84	84	0.873 (0.948)
Vanillic acid	91	99	96	101	100	0.999 (0.983)
Syringic acid	95	98	98	97	99	0.999 (0.991)
Caffeic acid	76	76	85	72	75	0.999 (0.879)
Homovanillic acid	105	98	96	99	100	0.997 (0.970)
Tyrosol	95	96	96	96	98	0.753 (0.949)
Protocatechuic aldehyde	97	97	97	99	102	0.999 (0.981)
Pirocatecolo	95	94	92	93	94	0.999 (0.969)
p-Coumaric acid	88	87	88	85	81	0.999 (0.988)
Salicylic acid	103	99	98	100	102	0.999 (0.954)
Phenol	99	99	99	99	101	0.999 (0.991)
Catechin	100	95	96	93	88	0.999 (0.979)
Ferulic acid	94	87	86	84	84	0.999 (0.980)
Aesculetin	91	98	98	95	96	0.999 (0.978)
Sinapinic acid	89	83	84	80	80	0.999 (0.983)
Homovanillic alcohol	92	96	95	98	98	0.999 (0.928)
Epicatechin	94	97	96	94	84	0.998 (0.975)
Vanillin	96	106	109	111	110	0.999 (0.975)
Coniferyl alcohol	97	100	104	107	112	0.999 (0.958)
4-Methylcatechol	89	89	87	86	86	0.999 (0.981)
Syringaldehyde	89	98	96	103	103	0.999 (0.940)
Isopropiovanillone	93	104	104	107	105	0.999 (0.901)
Scopoletin	83	100	97	99	94	0.999 (0.973)
Acetovanillone + isoacetovanillone	89	100	98	99	97	0.998 (0.938)
Isopropiosiringone	94	103	102	105	102	0.997 (0.888)
Acetosyringone	88	98	96	99	98	0.998 (0.916)
Isoacetosiringone	91	100	98	99	97	0.999 (0.926)
Syringol	90	96	95	96	96	0.998 (0.838)
Coniferylaldehyde	78	82	80	81	78	0.998 (0.991)
Sinapinaldehyde	77	76	76	77	74	0.978 (0.999)
Tryptophol	95	100	95	95	92	0.998 (0.949)
o-Vanillina	89	95	94	96	96	0.999 (0.968)
Methyl vanillate	93	100	97	97	97	0.998 (0.939)
(*m*+*p*)-Cresol	102	104	103	106	109	0.998 (0.910)
4-Ethylcatechol	93	90	87	86	88	0.999 (0.981)
o-Cresol	97	96	98	96	102	0.999 (0.994)
Vanillyl ethyl ether	105	109	106	110	112	0.996 (0.835)
Guaiacol	98	103	113	105	106	0.999 (0.991)
4-Methylsyringol	106	107	101	103	109	0.995 (0.796)
4-Vinylphenol	96	73	83	76	69	0.998 (0.974)
Ethyl vanillate	100	100	97	98	100	0.998 (0.902)
3,4-Xylenol	110	111	109	114	118	0.997 (0.840)
4-Vinylguaiacol	103	94	91	90	95	0.997 (0.957)
Ellagic acid	132	114	164	67	67	0.965 (0.953)
4-Ethylphenol	114	116	112	118	119	0.998 (0.875)
4-Methylguaiacol	94	98	100	100	99	0.999 (0.951)
4-Ethylguaiacol	113	110	110	115	120	0.909 (0.819)
4-Allyl syringol	112	107	106	110	112	0.994 (0.763)
Eugenol	109	109	108	113	119	0.998 (0.824)
Isoeugenol	91	77	76	78	72	0.999 (0.854)

[a] Range of calibration = 0.1–2 μg mL^{-1}.

particular, for almost all phenolic acids, aldehydes, hydroxybenzochetones and their derivatives, flavanols and hydroxycoumarins R.S.D.% was below 5%, while for alkylmethoxyphenols and their derivatives R.S.D.% was mainly between 5 and 10%. Furthermore, even when measurement repeatability improved with an increasing concentration for other target compounds, in the case of alkylmethoxyphenols and derivatives it showed considerable variability.

3.1.4.5. Inter day method precision and standard stability. The relative standard deviations (R.S.D.%) for the two concentration levels tested were always below 10%. As the vials analysed were the same as those used for calibration, maintained at 5 °C for 72 h, solvent standards were considered stable for at least 3 days. Furthermore, no differences in stability were found at the two concentration levels.

3.1.4.6. Carry over. When three calibrations were performed on different days for each target compound, no significant peaks were detected with an area ratio greater than that of the relative limit of detection.

3.1.4.7. Matrix effect. In the case of direct analysis (without a clean-up step) only 18.5% of compounds showed a R^2 of over 0.99, almost 35.2% of analytes showed a R^2 of between 0.951 and 0.988 and for the remaining 46.3%, the R^2 value was between 0.763 and 0.949. On

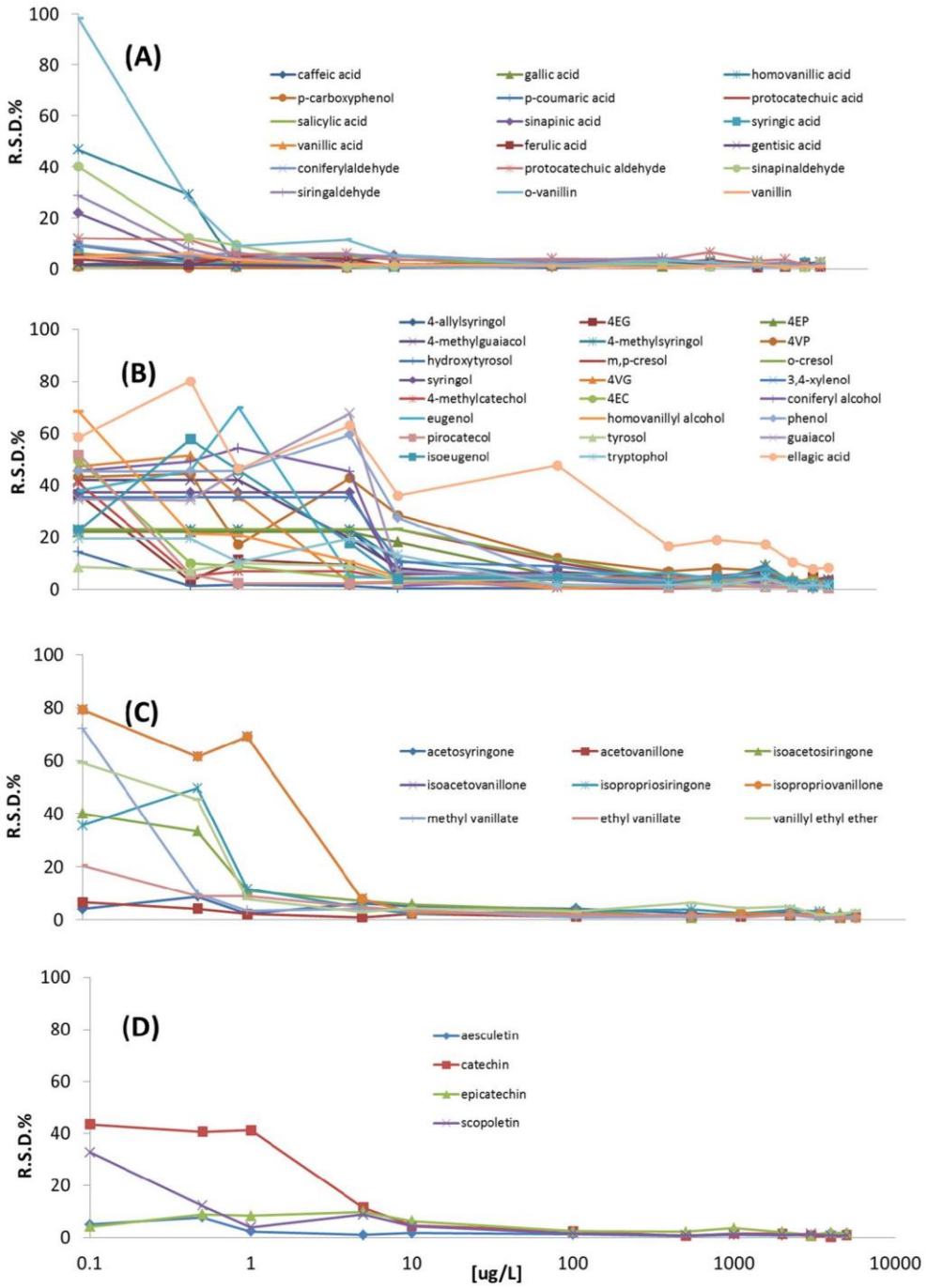

Fig. 2. Precision as R.S.D.% ($N = 7$) of phenolic compounds: (a) phenolic acids and aldehydes; (b) alkylmethoxyphenols and derivatives; (c) hydroxybenzoketones and derivatives; (d) flavanols and hydroxycoumarins.

the other hand, when the proposed on-line SPE method was used, almost 91% of compounds had a R^2 of over 0.994 and consequently, a linear calibration curve. Table 3 summarizes the R^2 values for both experiments.

3.2. Method application

The method developed was applied to determine the phenolic content in red and white wines, common and balsamic vinegars and eight different spirits.

Table 4 summarizes the mean levels of target compounds in the five matrices and the standard deviations.

3.2.1. Hydroxybenzoic acids

In red and white wines hydroxybenzoic acid content complied with the levels described in the literature [20–22]. As regards spirits, as far as we know, the following hydroxybenzoic acids were detected for the first time in the following matrices: gallic acid in calvados and grappa; gentisic acid in cognac, whisky, armagnac, calvados, grappa and rum; *p*-carboxyphenol in grappa;

Table 4
Phenolic compound content (mean, standard deviation; μg mL^{-1}) in selected matrices (red wines, $N = 8$; white wines, 8; spirits, 8; common vinegars, 8; balsamic vinegars, 8).

Compounds	Red wines		White wines		Distillates		Common vinegars		Balsamic vinegars	
	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev	Mean	Std dev
Gallic acid	41.6	15.6	3.76	3.38	4.66	6.13	4.47	4.53	12.4	11.8
Protocatechuic acid	4.58	2.52	0.978	0.684	1.29	2.16	2.00	0.697	6.11	7.14
p-Carboxyphenol acid	1.29	1.04	0.473	0.432	0.238	0.251	1.89	0.929	1.86	2.40
Gentisic acid	0.413	0.311	0.291	0.175	0.022	0.017	0.184	0.132	0.270	0.414
Hydroxytyrosol	2.95	1.90	3.46	5.90	0.024	0.029	1.59	1.47	1.38	1.91
Vanillic acid	6.30	5.60	0.165	0.062	0.727	0.743	0.320	0.126	1.46	1.95
Syringic acid	6.51	2.07	0.099	0.057	1.02	0.886	0.197	0.133	1.05	1.11
Caffeic acid	7.92	4.99	2.47	1.17	0.060	0.044	2.44	4.06	3.14	3.17
Homovanillic acid	0.722	0.247	0.131	0.142	0.025	0.053	12.1	19.3	1.08	1.00
Tyrosol	0.010	0.025	0.001	0.002	0.036	0.058	0.276	0.475	0.273	0.363
Protocatechuic aldehyde	0.560	0.524	0.026	0.025	1.69	2.79	2.07	3.44	6.41	8.35
Pirocatecolo	0.236	0.243	0.013	0.008	0.012	0.008	0.308	0.327	0.176	0.158
p-Coumaric acid	5.27	1.64	1.55	0.591	0.047	0.044	0.770	0.887	2.45	2.52
Salicylic acid	0.542	0.236	0.187	0.118	0.044	0.037	0.156	0.108	0.291	0.261
Phenol	<1.05	n.d.	<1.05	n.d.	<1.05	n.d.	<1.05	n.d.	<1.05	n.d.
Catechin	31.4	9.63	3.23	2.48	0.126	0.176	0.096	0.084	<0.051	n.d.
Ferulic acid	0.262	0.138	0.164	0.171	0.047	0.043	0.068	0.063	0.128	0.139
Aesculetin	0.246	0.164	0.100	0.021	0.142	0.071	0.242	0.351	0.106	0.046
Sinapinic acid	0.054	0.030	0.030	0.024	0.048	0.041	0.040	0.033	0.140	0.163
Homovanillic alcohol	0.180	0.129	0.097	0.065	0.061	0.052	0.071	0.107	0.033	0.014
Epicatechin	20.8	7.17	2.16	2.41	0.011	0.013	0.064	0.079	0.003	0.002
Vanillin	0.523	0.620	0.046	0.037	3.02	3.52	0.049	0.048	5.62	13.3
Coniferyl alcohol	1.89	2.02	0.722	0.746	0.074	0.054	0.353	0.522	4.76	8.47
4-Methylcatechol	0.008	0.006	0.006	0.003	0.005	0.004	0.005	0.003	0.006	0.003
Syringaldehyde	1.25	2.29	0.041	0.042	5.75	6.01	0.088	0.095	0.119	0.161
Isopropiovanillone	0.032	0.012	<0.054	n.d.	<0.054	n.d.	0.034	0.034	0.036	0.025
Scopoletin	0.030	0.026	0.010	0.007	0.069	0.054	0.006	0.002	0.006	0.003
Aceto-/isoacetovanillone	0.074	0.052	0.021	0.015	0.028	0.025	0.022	0.022	0.021	0.017
Isopropiosiringone	0.073	0.069	0.012	0.012	0.026	0.028	0.016	0.009	0.025	0.018
Acetosyringone	0.046	0.052	0.009	0.008	0.059	0.059	0.046	0.045	0.015	0.014
Isoacetosiringone	0.007	0.003	<0.011	n.d.	0.008	0.005	0.069	0.034	0.063	0.077
Syringol	1.95	3.13	3.52	7.65	<0.129	n.d.	4.28	1.82	1.58	2.39
Coniferylaldehyde	0.041	0.046	0.033	0.021	0.863	1.57	0.033	0.013	0.039	0.012
Sinapinaldehyde	0.022	0.028	<0.010	n.d.	0.632	1.10	<0.010	n.d.	0.009	0.011
Tryptophol	1.98	1.04	<1.10	n.d.	<1.10	n.d.	<1.10	n.d.	<1.10	n.d.
o-Vanillina	0.049	0.052	0.052	0.066	0.012	0.019	0.008	0.009	0.007	0.005
Methyl vanillate	1.44	0.923	0.180	0.133	0.080	0.075	0.216	0.345	0.086	0.060
(m + p)-Cresol	<1.01	n.d.	<1.01	n.d.	<1.01	n.d.	<1.01	n.d.	<1.01	n.d.
4-Ethylcatechol	0.035	0.020	0.009	0.006	0.010	0.005	2.48	6.35	0.025	0.017
o-Cresol	<1.17	n.d.	<1.17	n.d.	<1.17	n.d.	<1.17	n.d.	<1.17	n.d.
Vanillyl ethyl ether	<0.010	n.d.	<0.010	n.d.	<0.010	n.d.	<0.010	n.d.	<0.010	n.d.
Guaiacol	<0.110	n.d.	<0.110	n.d.	<0.110	n.d.	<0.110	n.d.	<0.110	n.d.
4-Methylsyringol	0.324	0.219	0.338	0.242	0.312	0.216	0.329	0.230	0.379	0.257
4-Vinylphenol	<0.112	n.d.	0.361	0.182	<0.112	n.d.	0.117	0.062	0.156	0.115
Ethyl vanillate	0.279	0.261	0.010	0.006	0.062	0.042	0.008	0.006	0.008	0.005
3,4-Xylenol	<0.100	n.d.	<0.100	n.d.	<0.100	n.d.	<0.100	n.d.	<0.100	n.d.
4-Vinylguaiacol	0.260	0.593	1.51	1.24	<0.055	n.d.	0.326	0.355	0.170	0.220
Ellagic acid	76.0	118	0.335	0.289	72.0	45.3	4.16	6.49	3.76	3.96
4-Ethylphenol	0.724	0.566	<1.02	n.d.	0.622	0.296	0.814	0.806	<1.02	n.d.
4-Methylguaiacol	0.443	0.379	0.164	0.195	<0.105	n.d.	<0.105	n.d.	0.077	0.065
4-Ethylguaiacol	0.179	0.370	0.199	0.255	0.119	0.157	0.300	0.401	0.179	0.203
4-Allylsyringol	<0.202	n.d.	<0.202	n.d.	<0.202	n.d.	<0.202	n.d.	<0.202	n.d.
Eugenol	0.052	0.021	<0.087	n.d.	0.059	0.042	<0.087	n.d.	0.071	0.050
Isoeugenol	0.066	0.026	0.060	0.024	0.060	0.023	<0.102	n.d.	0.060	0.023

Note: n.d., not detected.

protocatechuic acid in cognac, armagnac, calvados and grappa; salicylic acid in all analysed spirits; syringic acid in calvados and grappa; vanillic acid in calvados and grappa. The other compounds detected have been previously reported in the literature [23–29]. As regards vinegars, gentisic and salicylic acid were detected for the first time in almost all the analysed samples. The same situation was found for *p*-carboxyphenol and vanillic acid in balsamic vinegars, but they had previously been described in common vinegars [30].

3.2.2. Hydroxycinnamic acids

In contrast with our results, the following compounds have not been previously reported in the matrices mentioned: caffeic and ferulic acids in armagnac, calvados and grappa; *p*-coumaric acid in armagnac, calvados, grappa and common vinegars; sinapinic acid in cognac, armagnac, calvados, grappa, common and balsamic vinegars. For the compounds not mentioned, the data were in agreement with the literature [19–22,25,27,30–32].

3.2.3. Hydroxyphenylacetic acids

As far as we know, homovanillic acid has never been described in vinegars and the analysed spirits, except for rum [29], while it has already been reported in both red and white wines [22,34].

3.2.4. Hydroxybenzaldehydes

For the first time vanillin was detected in grappa and balsamic vinegars, while *o*-vanillin was found in all spirits and vinegars.

As concerns their content in other matrices, the results were in agreement with the literature [22–24,29,34–38]. Protocatechuic aldehyde has been reported in red and white wines [39,40] and vinegars [33], but not in spirits, with the exception of brandy [28]. Siringaldehyde was found for the first time in calvados, grappa and balsamic vinegars, while the content in other matrices complied with that described in the literature [22–24,33–35,37].

3.2.5. Hydroxycinnamaldehydes

Coniferylaldehyde was detected at trace levels in red and white wines and almost all vinegars. The concentration levels measured in cognac, whisky, armagnac, brandy and rum agreed with those reported in the literature [24,25,37,41,42]. As far as we know, coniferylaldehyde was detected for the first time in calvados and grappa, while sinapinaldehyde was detected in all spirits except rum [42]. Grappa turned out to be the matrix with the highest content of both hydrocinnamaldehydes.

3.2.6. Simple phenols

Phenol was not detected in any of the analysed samples, probably because it is usually found at trace levels and our limit of quantification was over 0.1 $\mu g\,mL^{-1}$ [43–45]. Pyrocatechol, on the other hand, was detected for the first time in all five matrices.

3.2.7. Alkylphenols

As for phenol, cresols are usually found at trace levels [43–45] but our LOQ was higher than 0.1 $\mu g\,mL^{-1}$, because these compounds show too low a level of ionization efficiency. 4-Ethylphenol was found only in calvados and apple vinegar. 4-Vinylphenol was detected only in white wines and, for the first time, in common vinegars. As far as we know, 4-methylcatechol was found for the first time in cognac, whisky, calvados, grappa and vinegars, while the data collected for red and white wines complied with those reported in the literature [46,47]. As regards 4-ethylcatechol, it was found at trace levels for the first time in vinegars and spirits, apple vinegar being the only one which showed a significant content. Finally, 3,4-xylenol was not found in the analysed samples.

3.2.8. Methoxy and alkylmethoxyphenols

4-Ethylguaiacol, responsible for a wine aroma defect [45], was found for the first time in whisky and grappa. The content of 4-ethylguaiacol in other matrices, as well as those of 4-vinylguaiacol in red and white wines, agreed with the data reported in the literature [19,22,34,38,46–50]. Guaiacol and 4-methylguaiacol are usually found at a concentration of a few $\mu g\,L^{-1}$ [22,43,45,51] and were not detected in many of the analysed samples.

3.2.9. Dimethoxyphenol and alkyldimethoxyphenols

Syringol was not detected in any of the analysed samples, but 4-methylsyringol was detected for the first time in vinegars and calvados, grappa, armagnac, cognac and whisky.

3.2.10. Alkylphenyl and alkylphenylmethoxy alcohols

In contrast with reports in the literature [20,22], tyrosol was not detected in the red and white wines analysed, but was detected in cognac, whisky, calvados, grappa, armagnac and in vinegars at the same concentration levels described in the literature [30,51]. As regards hydroxytyrosol, the content determined in red and white wine was in agreement with the data reported in the literature [20,22]; in vinegars, brandy, rum and grappa, as far as we know, hydroxytyrosol was found for the first time. Homovanillyl alcohol was detected for the first time in whisky, armagnac, grappa and common vinegars, while coniferyl alcohol was found in almost all the analised samples. Since tryptophol had a LOQ higher than 0.1 $\mu g\,mL^{-1}$ it was detected in our experimental conditions only in red wines [22,52]; nevertheless no information was found in the literature about tryptophol content in other analysed matrices.

3.2.11. Hydroxyphenylpropenes

4-Allylsyringol has been reported at very low levels in red and white wines [22,34] but has never been reported in other matrices. In agreement with the data reported in the literature [34], eugenol and isoeugenol were absent or detected at low levels in red and white wines. With the exception of calvados and some balsamic vinegars, eugenol and isoeugenol were not detected in any of the other samples.

3.2.12. Hydroxybenzoketones and derivatives

As far as we know, aceto- and isoacetovanillone were found for the first time in cognac, whisky, armagnac, grappa and vinegars, while their content in red and white wines complied with the literature [34,45]. Acetosyringone, in agreement with the data reported in the literature [36,45], was found at low levels in all wines. Detected for the first time in both spirits and vinegars, acetosyringone was determined at low concentrations in all samples, with the exception of grappa. Only in rum has acetosyringone already been detected [53]. Isoacetosyringone, usually detected in traces [36,45], was not found either in red and white wines or in spirits, but was determined for the first time in almost all vinegars. Isopropiosyringone and isopropiovanillone differed due to the presence of one methoxy group. Isopropiovanillone was never detected in the analysed samples; isopropiosyringone, as far as we know, was found for the first time in cognac, calvados, armagnac, whisky, grappa and vinegars, while its content in red and white wines complied with reported data [22,45]. Methyl and ethyl vanillate were found for the first time at low levels in almost all spirits and vinegars, while the amount found in red and white wines agreed with the literature [34]. Ethyl vanillate was yet found in brandy and rum [53,54]. Vanillyl ethyl ether, a hydroxybenzoether, was never detected in the analysed samples.

3.2.13. Flavanols

(+)-Catechin and (−)-epicatechin are usually detected in large amounts in wine [20,22]. The amounts detected in the analysed wines complied with reported data. As far as we know, catechin and epicatechin were found for the first time in spirits, with the exception of catechin in cognac and rum [23,42] and of epicatechin in rum [42]; the amounts found in common vinegars were in agreement with the literature [30,55] while as regards balsamic vinegars, catechin was not found and epicatechin was detected at low levels.

3.2.14. Hydroxycoumarins

Aesculetin was determined for the first time in spirits and vinegars, its content in brandy, red and white wines agreeing with the results reported in the literature [34,41]. Scopoletin, detected at lower levels than aesculetin, was found for the first time in red wines and in vinegars. For white wines and spirits content, the data obtained complied with the literature [25,42,56,57].

4. Conclusions

In this study we developed a method based on high mass resolution detection, allowing us to identify and quantify over fifty phenolic compounds in different matrices such as wine, vinegar and spirits. The use of a heated electrospray operating in negative polarity provided the sensitivity needed to quantify specific compounds such as phenolic acids and their aldehydic derivatives at a 0.0001 $\mu g\,mL^{-1}$ level. The high selectivity of the mass spectrometer in determining the exact mass of target compounds and the efficiency of SPE pretreatment in reducing matrix interference, made it possible to quantify phenolic compounds in different food

matrices. Phenol, cresols, tryptophol and ellagic acid were the only compounds characterized by a high limit of quantitation.

In short, the proposed method appears to be a valid tool for phenolic determination in beverages and food extracts.

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Conclusion

In this work, ultra high-performance liquid chromatography (UHPLC) coupled with high resolution mass spectrometry proved to be a very powerful analytical technique, because it combined the separation power of UHPLC with the sensitivity and selectivity of MS. HRMS proved to be a solid approach in targeted analysis, since it allowed simultaneous identification of over 50 analytes with different physical-chemical properties and provided the high-resolution MS/MS spectra used for confirmative purposes. Furthermore, thanks to the high selectivity and sensitivity of HRMS, analyte identification was performed with an accuracy of four decimal figures and an error of less than 5 ppm, while quantification was possible up to 0.0001 g/mL.

However, major drawbacks to LC-HRMS were the susceptibility of the electrospray source to matrix-related ionization and the possibility of detecting isobaric interference, which required real samples to undergo time-consuming pretreatment steps. Use of the SPE column and the application of online SPE-LC column switching allowed a fully automated analytical system with less likelihood of sample loss, reducing the exposure of operators to toxic solvents or biohazardous samples, and enhancing reproducibility and the elimination of human error. Furthermore, this clean-up approach allowed the analysis of different matrices, such as red and white wine, spirits and common and balsamic vinegar, showing itself to be an innovative and well-performing method for routine quantification of low-molecular-weight phenolic compounds.

SECTION 2.2.3.

Targeted and untargeted high resolution mass approach for a putative profiling of glycosylated simple phenols in hybrid grapes

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Aim of work

Vitis vinifera is one of the most widely used grapevines around the world for high quality wine production. More resistant interspecific hybrid vine varieties (Burns *et al.*, 2002; Slegers *et al.*, 2015) developed from crosses between *Vitis vinifera* and other *Vitis* species have gained attention since they can be an environmentally-friendly alternative to more unsustainable production (Sun *et al.*, 2011). However, varietal differences between interspecific hybrids and the composition of wine from hybrid grapes have not yet been well defined. Low-molecular-weight phenols in wines, where the glycosylated forms can be transformed into free forms during winemaking, have also attracted wine consumers' interest, due to their role as antioxidants in human health (Middleton *et al.*, 2000).

The aim of this work was to combine targeted and suspect screening approaches in the same analytical method, in order to contemporaneously investigate the occurrence and distribution of free and glycosylated low-molecular-weight phenolic compounds in different parts of the berries – skin, pulp and seeds – of hybrid and *Vitis vinifera* grapes. In particular, it evaluated the possibility of developing a suspect screening approach consisting of searching for and tentative identification of low-molecular-weight phenolic glycosides based on a database of exact masses and fragments collected from spectral data in the literature or surmised theoretically. In the latter case, different combinations of free phenols, phenols detected in the targeted approach, and sugars, in the form of hexose, pentose, dihexose, hexose-pentose, pentose-hexose and dipentose, were used to theoretically calculate exact masses. As regards fragmentation, that observed for free compounds was presumed to be characteristic of the same aglycones, once released by glycosides during collision-induced dissociation. Finally, matching of experimental and theoretical isotope patterns was used for confirmative purposes. These theoretical suppositions were evaluated by studying the mass behavior of 7 glycosylated low-molecular-weight phenols commercially available as standards. Furthermore, the latter were also included in the targeted approach, together with over 50 free phenols.

It is well known that the winemaking process can affect the extraction of phenolic compounds from crushed grapes and their transfer to wines, and that many factors, such as the microbiological, chemical and physical states of the matrix, can induce modification in the structure and concentration of phenolic compounds during fermentation, fining and storage of wine (Giovinazzo, & Grieco, 2015). Consequently, another objective of this work was to investigate the effects of alcoholic fermentation on the free and glycosylated low-molecular-weight phenolic profile of wines produced from grapes of hybrid varieties.

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Targeted and untargeted high resolution mass approach for a putative profiling of glycosylated simple phenols in hybrid grapes

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ABSTRACT

Vitis vinifera is one of the most widespread grapevines around the world representing the raw material for high quality wine production. The availability of more resistant interspecific hybrid vine varieties, developed from crosses between *Vitis vinifera* and other *Vitis* species, has generated much interest, also due to the low environmental effect of production. However, hybrid grape wine composition and varietal differences between interspecific hybrids have not been well defined, particularly for the simple phenols profile. The dynamic of these phenols in wines, where the glycosylated forms can be transformed into the free ones during winemaking, also raises an increasing health interest by their role as antoxidants in wine consumers.

In this work an on-line SPE clean-up device, to reduce matrix interference, was combined with ultra-high liquid chromatography-high resolution mass spectrometry in order to increase understanding of the phenolic composition of hybrid grape varieties. Specifically, the phenolic composition of 4 hybrid grape varieties (red, Cabernet Cantor and Prior; white, Muscaris and Solaris) and 2 European grape varieties (red, Merlot; white, Chardonnay) was investigated, focusing on free and glycosidically bound simple phenols and considering compound distribution in pulp, skin, seeds and wine. Using a targeted approach 53 free simple phenols and 7 glycosidic precursors were quantified with quantification limits ranging from 0.001 to 2 mg Kg^{-1} and calibration R^2 of 0.99 for over 86% of compounds. The untargeted approach made it possible to tentatively identify 79 glycosylated precursors of selected free simple phenols in the form of -hexoside (N = 30), -pentoside (21), -hexoside-hexoside (17), -hexoside-pentoside (4), -pentoside-hexoside (5) and -pentoside-pentoside (2) derivatives on the basis of accurate mass, isotopic pattern and MS/MS fragmentation.

1. Introduction

The European grapevine belongs to the *Vitis vinifera* botanical species which is the most widely used around the world for wine production (about 7.4 million ha; FAO, 2002). Unfortunately, *Vitis vinifera* grapes are susceptible to various diseases, mildew (Reisch, Owens, & Cousins, 2012) and insects' damage, Phylloxera in particular, and despite being grafted onto resistant rootstocks, they need frequent treatments against several pathogens. However, massive use of pesticides is unsustainable from an environmental and economic point of view, as well as leading to pesticide resistance.

An environmentally friendly solution to the problems of pesticide pollution could be represented by hybrid grape varieties crossing *Vitis vinifera* and other *Vitis* spp. (e.g. *V. riparia*, *V. labrusca*, *V. rupestris*; Sun, Gates, Lavin, Acree, & Sacks, 2011), which are selected to obtain higher tolerance or resistance to abiotic stress, e.g. temperature (Burns et al., 2002), and biotic stress (Slegers, Angers, Ouellet, Truchon, & Pedneault, 2015). Due to their low sugar and tannin content (Harbertson et al., 2008; Springer & Gavin, 2014), foxy taste and several off-flavours (Rapp, 1990; Sun et al., 2011), only a reduced number of hybrid varieties are authorised in European countries for the production of wines not included in Protected Designations of Origin (European Community Regulation, 2008; D.Lgs. 61/2010). Despite their potential interest as a result of the low environmental impact, the phenolic profile of hybrid grape varieties has not been extensively evaluated nor have varietal differences between interspecific hybrids been well defined.

The effects on health attributed to wine consumption are principally due to its high content in terms of phenols, which have been demonstrated to have anti-inflammatory, anti-oxidant and cardioprotective effects (Middleton, Kandaswami, & Theoharides, 2000). Furthermore, phenolic compounds, widely accumulated in the skin and seeds (Poudel, Tamura, Kataoka, & Mochioka, 2008), contribute towards defining wine taste (Arnold & Noble, 1978), together with volatile

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compounds produced during fermentation and those transferred from wood during ageing (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006, chap. 7). Structurally, they range from low-molecular weight simple phenols to more complex compounds, and many of them can be found in the form of mono- and disaccharides or as ester and methoxy derivatives (Shahidi & Naczk, 1995). Free phenols can directly affect wine astringency or bitterness, and can be involved in the redox equilibrium of wine (Keller, 2009), while flavourless glycoconjugates are considered aroma precursors, since they can be hydrolysed during winemaking or ageing, releasing the corresponding volatile aglycones (Williams, Strauss, Wilson, & Massy-Westropp, 1982).

Spectrophotometric and chromatographic methods have been developed for phenol analysis (Naczk & Shahidi, 2004), and while the former are rapid but less specific assays, chromatographic approaches are able to identify and quantify single phenols (Larcher et al., 2007; Barnaba et al., 2015). Gas chromatographic methods require some phenolic compounds to be transformed into more volatile derivatives, while high-performance liquid chromatographic approaches do not require a derivatisation step, and are thus less time-consuming. In the case of phenolic glycoconjugates, analyses generally involve complex extraction and hydrolysis procedures aimed at isolating aglycones for subsequent direct analysis (Williams et al., 1982). Recent works (Perestrelo et al., 2012; Di Lecce et al., 2014; Barnaba et al., 2016) have tried to structurally define grape phenolic glycoconjugates through liquid chromatography coupled with tandem mass spectrometry.

The study aims to tentatively define the free and glycosidically simple phenolic composition of 4 hybrid grapes, describing their distribution in skin, pulp and seed in comparison to 2 European varieties, using a targeted and untargeted UHPLC-high resolution tandem mass approach. Wines from hybrid varieties were also investigated in order to define the specific oenological composition of these products.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN; LC-MS grade, 99.9%), methanol (LC-MS grade, 99.9%), formic acid (MS grade, 98%) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol, 99.5%) were supplied by Fluka (St. Louis, MO, USA). Acetic acid (99%), L-glutathione reduced (99%), *p*-nitrophenol (99%), D-(+)-gluconic acid-δ-lactone (99.0%) and sodium azide (99.5%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The targeted phenolic compound suppliers are summarised in Table 1. Deionized water was produced using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Eleven standard stock solutions (200 mg L^{-1} of each phenol) were prepared in water-methanol, with organic solvent ranging from 15 to 55% according to the component's solubility. L-glutathione reduced and DL-dithiothreitol were added as antioxidant agents (2.5 g L^{-1} each). Merging an aliquot from each stock solution, a more diluted solution (10 mg L^{-1} for each phenol) was obtained and used to prepare calibration solutions in the range 0.0001–10 mg L^{-1} (Barnaba et al., 2015). All solutions were stored at −4 °C.

Instrument mass calibration was performed using a standard mixture composed of sodium dodecyl sulphate, sodium taurocholate (2.6 mg L^{-1} and 4.9 mg L^{-1} respectively; Pierce® ESI Negative Ion Calibration Solution, Rockford, IL, USA), formic and acetic acids (5 mg L^{-1} each).

2.2. Samples and sample extraction

This study considered 2 white (Solaris and Muscaris) and 2 red (Cabernet Cantor and Prior) hybrid grape varieties, 2 European ones (Chardonnay and Merlot), and wines from selected hybrid grapes. The Solaris variety, originally known as Fr. 240-75, is a cross between Merzling (mother) and Gm 6493 (Zarya severa x Muskat Ottonel; father), while Muscaris, originally known as Fr. 493-87, is a cross between Solaris (mother) and Muskateller (father). Cabernet Cantor, originally known as Fr. 523-89r, derives from crossing of Seibel 70-53 (mother) with Solaris (father), while Prior, originally known as Fr. 484-87r and then as Fr. 455-83r, derives from the crossing of Bronner (Merzling x Gm 6494; father) and the product of a cross between Joannès-Seyve 23-416 and Pinot Noir (mother) (http://www.wine-searcher.com/grape-varieties.lml).

Solaris, Muscaris and Cabernet Cantor grapes were sampled in triplicate from 2 experimental plots, located in Rovereto (TN, Italy; latitude: 45° 52′ 33.96″, longitude: 11° 1′ 4.12″, altitude: 204 m, AMSL; trellis system, 3 × 0.9 m) and Telve (TN, Italy; latitude: 46° 3′ 42.08″, longitude: 11° 28′ 41.59″, altitude: 548 m, AMSL; guyot, 2 × 0.8 m), respectively. Samples in triplicate were also collected from Telve for Prior, and from Rovereto for Chardonnay and Merlot.

The grape samples were picked at technological ripeness (Muscaris grapes were harvested in the Rovereto plot on 3 September 2015, with sugar content = 23.3°Brix, pH = 3.36, total acidity = 4.8 g tartaric acid L^{-1}, and in the Telve plot on 10 September 2015, with values of 24.8, 3.32 and 4.3 respectively; Solaris: harvested in the Rovereto plot on 28 August 2015 with values of 24.7, 3.30 and 5.2, and in the Telve plot on 31 August 2015 with values of 23.1, 3.30 and 5.8 respectively; Cabernet Cantor: harvested in the Rovereto plot on 22 September 2015 with values of 21.5, 3.40 and 4.7, and in the Telve plot on 16 September 2015 with values of 23.6, 3.26 and 6.3 respectively; Prior: harvested in the Telve plot on 16 September 2015 with values of 20.4, 3.28 and 7.2 respectively; Chardonnay: harvested in the Rovereto plot on 9 September 2015 with values of 21.1, 3.29 and 5.0 respectively; Merlot: harvested in the plot of Rovereto on 10 September 2015 with values of 22.3, 3.40 and 4.6 respectively).

In order to prepare the separate pulp, skin and seed fractions, roughly 600 sound and intact berries from each grape sample (3 *per* plot) were randomly collected from the top, sides, middle and bottom of 15 ripe bunches, and stored at −20 °C until fractions preparation. Before analysis, 25 berries were randomly collected from each one of the 600-berry samples and weighed. Then the frozen berries were separated into skin, pulp and seed fractions using pincers. Two grams of each fraction were extracted with 20 mL of the extraction solvent, dispersing the suspension with Ultra Turrax (24,000 rpm, 30 s; T 25 basic, IKA®-Werke GmbH & Co. KG) and shaken overnight (Rotoshake; C. Gerhardt GmbH & Co. KG). The extraction solvent was a solution of H_2O-MeOH 1:1, added with NaN_3 (0.01%, p/v) as antifermentative agent, L-glutathione reduced and DL-dithiothreitol (0.25% each, p/v) as antioxidant agents, and D-(+)-gluconic acid-δ-lactone (0.50%, p/v) in order to inhibit β-glucosidase (Günata, Biron, Sapis, & Bayonove, 1989). After centrifugation (4000 rpm, 45 min; IEC CL31 Multispeed, Thermo Fisher Scientific), the supernatant was filtered with 0.45 μm PTFE filter cartridges (Sartorius AG, Goettingen, Germany), diluted 2 times with water and added of the internal standard (*p*-nitrophenol, 500 μg Kg^{-1}). Only seed extracts were analysed at two dilution levels (2 and 300 times), in order to cover the very different concentration ranges of the 60 compounds (from a few μg/Kg of aesculetin, methyl vanillate or syringic acid to several mg/Kg of epicatechin, catechin or gallic acid; Di Lecce et al., 2014).

The wines (Solaris, N = 2; Muscaris, 2; Cantor, 2; Prior, 1; 2015 harvest) were produced at the experimental winery of the Mach Foundation from 40 kg of ripe and sound hybrids grapes. After destemming, white varieties were pressed and cold settled for 24 h. The racked must fermented then at 20 °C with EC1118 yeast strain (200 mg L^{-1}; Lallemand, Verona, Italy). At the depletion of sugar content (<1 g L^{-1}), wines were kept at 4 °C until analysis. Red varieties were destemmed and inoculated keeping the same strain and doses as white wines. After 7 days of skin contact maceration, marcs were removed and the resulting wines once the alcoholic fermentation was ascertained, were inoculated with PN4 selected lactic bacteria

Table 1
UHPLC-MS parameters of targeted compounds (free and glycosidically bound phenols).

Compounds	RT (min)	$[M-H]^-$ (m/z)	$\Delta m/z$	NCE	MS/MS fragments	LOQ Grape (mg kg^{-1})	LOQ Wine (mg L^{-1})
Vanillic acid-glu[h]	5.42	329.0878	2.3	20	167.0349; 152.0114	0.4200	0.2100
Gallic acid[b]	5.60	169.0143	1.0	45	125.0244	0.0020	0.0010
Gentisic/protocatechuic acid[a]	5.62	153.0193	1.4	45	109.0295; 108.0217	0.0021	0.0011
p-Carboxyphenol[a]	5.84	137.0244	1.4	40	93.0646	0.0021	0.0011
Salicylic acid-glu[f]	5.84	299.0772	1.2	20	137.0244; 93.0344	0.0200	0.0100
p-Hydroxybenzaldehyde-all[f]	6.08	283.0823	1.6	100	121.0295; 108.0218	0.2000	0.1000
Homovanillic acid[b]	6.23	181.0506	1.0	45	137.0617; 122.0373	0.0198	0.0099
Hydroxytyrosol[e]	6.28	153.0557	0.0	50	123.0437; 95.0487	0.0103	0.0052
Protocatechuic aldehyde[b]	6.30	137.0244	1.3	60	108.0216; 93.0344	0.0020	0.0010
Vanillic acid[a]	6.42	167.0350	1.3	40	152.0114; 123.0452	0.0020	0.0010
Syringic acid[a]	6.57	197.0456	−0.2	35	182.0216; 166.9984	0.0021	0.0011
Caffeic acid[a]	6.60	179.0350	0.0	40	135.0452	0.0021	0.0011
Aesculetin-glu[b]	6.80	339.0722	1.8	35	177.0193; 133.0296	0.2200	0.1100
Orcinol-glu[f]	6.83	285.0980	1.5	40	123.0452; 108.0214	0.1000	0.0500
Salicylic acid[g]	7.06	137.0244	1.2	60	93.0344; 122.0374	0.0020	0.0010
Pyrocatecol[b]	7.28	109.0295	0.7	80	108.0202; 91.0176	0.0099	0.0050
p-Coumaric acid[b]	7.37	163.0401	0.8	35	119.0502; 93.1266	0.0021	0.0010
Tyrosol[b]	7.46	137.0608	1.0	40	119.0502; 106.0426	0.0021	0.0011
Ferulic acid[a]	7.66	193.0506	0.8	40	178.0268; 149.0608	0.0025	0.0012
Phenol[b]	7.73	93.0346	2.6	100	65.0382	2.1000	1.0500
Catechin[a]	7.89	289.0718	1.0	35	245.0805; 221.0812	0.1024	0.0512
Acetovanillone-glu[h]	8.40	327.1085	1.7	20	165.0557; 150.0321	0.5600	0.2800
Aesculetin[b]	8.48	177.0193	0.8	50	133.0296; 105.0345	0.0022	0.0011
Sinapinic acid[a]	8.54	223.0612	1.0	30	208.0373; 179.0714	0.0100	0.0050
Scopoletin-glu[h]	8.60	353.0878	1.5	20	191.03498; 176.0112	0.3600	0.1800
4-Hydroxybenzaldehyde[b]	8.67	121.0295	0.7	130	108.0218; 92.0267	0.2010	0.1005
Orcinol[b]	8.77	123.0452	2.2	60	108.0214; 90.0591	0.2000	0.1000
Homovanillyl alcohol[b]	8.78	167.0714	0.6	35	152.0478; 122.0375	0.1020	0.0510
Epicatechin[b]	9.67	289.0718	1.0	40	245.0805; 221.0812	0.0020	0.0010
Vanillin[b]	9.86	151.0401	1.0	40	136.0152; 108.0202	0.0021	0.0011
Coniferyl alcohol[b]	10.11	179.0714	1.2	35	164.0478; 121.0296	0.2140	0.1070
4-Methylcatechol[a]	10.18	123.0452	2.8	100	108.0214; 90.0591	0.0104	0.0052
Syringaldehyde[d]	10.42	181.0506	0.8	40	166.0268; 151.0036	0.0150	0.0075
Isopropiovanillone[b]	10.55	179.0714	0.8	40	164.0478; 121.0296	0.1080	0.0540
Ethylvanillin[a]	10.65	165.0557	0.6	30	136.0152; 108.0202	0.2000	0.1000
Scopoletin[a]	10.66	191.0350	1.3	40	176.0112; 148.0166	0.0202	0.0101
Isopropiosyringone[f]	10.81	209.0819	−0.9	35	194.0581; 179.0348	0.0220	0.0110
Aceto-/isoacetovanillone[b]	10.82	165.0557	0.6	40	150.0321; 122.0371	0.0020	0.0010
Acetosyringone[b]	11.00	195.0663	0.8	30	180.0426; 165.0190	0.0021	0.0010
Isoacetosyringone[f]	11.24	195.0663	0.8	30	180.0426; 165.0190	0.0216	0.0108
Syringol[b]	11.32	153.0557	1.2	50	138.0321; 123.0087	0.2600	0.1300
Coniferylaldehyde[b]	11.51	177.0557	0.4	35	162.0320	0.0020	0.0010
Sinapinaldehyde[b]	11.64	207.0663	0.3	35	192.0427; 177.0193	0.0202	0.0101
Tryptophol[a]	11.87	160.0768	0.8	70	142.0659; 130.066	2.2040	1.1020
o-Vanillin[b]	12.09	151.0401	0.5	40	136.0152; 123.0083	0.0199	0.0100
Methyl vanillate[d]	12.13	181.0506	0.7	40	166.0268; 151.0036	0.0103	0.0052
o-/*m*-/*p*-Cresol[b]	12.27	107.0502	−0.9	60	79.0551; 65.7207	2.0200	1.0100
4-Ethylcatechol[c]	12.30	137.0608	0.8	35	122.0374	0.0103	0.0051
Vanillyl ethyl ether[f]	12.67	181.0870	0.9	30	166.0633; 153.0656	0.0204	0.0102
Guaiacol[b]	12.85	123.0452	−0.1	70	108.0214; 105.0346	0.2196	0.1098
4-Methylsyringol[d]	12.87	167.0714	−2.5	20	152.0478; 137.0243	0.2024	0.1012
4-Vinylphenol[d]	13.60	119.0502	−1.3	100	91.0550; 93.0346	0.2240	0.1120
Ethyl vanillate[b]	13.69	195.0663	0.4	40	180.0415; 130.9911	0.0110	0.0055
4-Vinylguaiacol[d]	14.00	149.0608	0.2	20	134.0375; 87.0088	0.1092	0.0546
4-Ethylphenol[a]	14.22	121.0659	−2.8	90	106.0423; 83.9854	2.0440	1.0220
4-Methylguaiacol[b]	14.37	137.0608	−2.6	35	122.0374	0.2108	0.1054
4-Ethylguaiacol[d]	14.58	151.0765	−3.0	10	136.0529; 121.0293	0.0171	0.0086
4-Allyl syringol[b]	14.85	193.0870	−0.3	10	178.0632; 163.0399	0.4048	0.2024
Eugenol[a]	15.11	163.0765	0.6	30	148.0529	0.1738	0.0869
Isoeugenol[b]	15.47	163.0765	0.2	30	148.0529; 118.0992	0.2032	0.1016

Note: RT = retention time; $\Delta m/z$ = difference between expected and experimental masses (ppm); NCE = normalized collision energy; LOQ = limit of quantitation; glu = glucoside; all = alloside.
[a] Fluka (St. Louis, MO, USA).
[b] Sigma Aldrich (St. Louis, MO, USA).
[c] Alfa Aesar (Karlsruhe, Germany).
[d] SAFC (St. Louis, MO, USA).
[e] CHEMOS GmbH (Regenstauf, Germany).
[f] TransMIT (Gießen, Germany).
[g] Aldrich (St. Louis, MO, USA).
[h] PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

(Lallemand, Verona, Italy). At the end of the malolactic fermentation and after racking them twice, wines were sampled for analysis. Muscaris wines produced from the Rovereto grapes had an alcohol content of 14.8% vol, pH = 3.40, total acidity of 4.7 g tartaric acid L^{-1} and volatile acidity < 0.10 g acetic acid L^{-1}, while those from Telve showed values of 14.7, 3.13, 6.2 and 0.16 respectively; Solaris from Rovereto had an

alcohol content of 15.1% vol, pH = 3.23, total acidity of 6.0 g tartaric acid L^{-1} and volatile acidity of 0.24 g acetic acid L^{-1}, while Solaris from Telve showed values of 15.2, 3.19, 5.9 and 0.33 respectively; Cabernet Cantor from Rovereto had an alcohol content of 13.7% vol, pH = 3.65, total acidity of 5.1 g tartaric acid L^{-1} and volatile acidity of 0.34 g acetic acid L^{-1}, while the Cabernet Cantor from Telve had values of 12.5, 3.50, 6.0 and 0.40 respectively; Prior from Telve had an alcohol content of 10.9% vol, pH = 3.84, total acidity of 4.5 g tartaric acid L^{-1} and volatile acidity of 0.40 g acetic acid L^{-1}.

As regards analysis, wine samples were filtered with 0.45 μm PTFE filter cartridges, diluted 10 times with water and added of the internal standard.

2.3. Analytical conditions

The basic composition of both musts and wines was measured using a WineScan FT 120 Type 77310 (Foss, Hillerød, Denmark), accurately aligned with official methods (International Organisation of Vine and Wine, 2016).

Chromatographic separation and on-line clean-up were performed using an ultra-high performance liquid chromatograph (Thermo Ultimate R3000; Thermo Scientific, Sunnyvale, CA, USA), equipped with a Rheodyne 6-port automated switching valve. Adapting the approach proposed by Barnaba et al. (2015), on-line clean-up aimed at removing matrix interference was achieved with a HyperSep™ Retain PEP SPE cartridge (3.0 mm × 10 mm, 40–60 μm, Thermo Scientific, Sunnyvale, CA, USA), while chromatographic separation was performed with a Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; Waters, Milford, MA, USA) through a gradient of ACN in water. The SPE cartridge was equilibrated with formic acid aqueous (0.1%, v/v) for 2 min before each analysis, in order to activate ureidic functions and maximise compound retention.

As regards high resolution mass analysis, a tandem mass spectrometer (Q-Exactive™; Thermo Scientific, Bremen, Germany), furnished with a heated electrospray source (HESI-II), was used in negative ion mode, acquiring spectra through full MS-data dependent MS/MS experiments (full MS–dd MS/MS), adapting the method proposed by Barnaba et al. (2016).

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software was used for timing of the injection system, switching valve and chromatographic gradient. Thermo Fisher Scientific TraceFinder™ software (Thermo Scientific, San Jose, CA, USA) was used for data processing and evaluation.

2.4. Targeted analysis

Identification and quantification of free and glycosylated targeted phenolic compounds was carried out using the accurate mass (mass tolerance < 5 ppm; SANCO/12571/2013) of the corresponding deprotonated molecules $[M\text{-}H]^-$, considering peaks extracted from full mass spectra and comparing retention times (RT) and isotopic patterns with those obtained from commercially available standards. Further evidence of the detection of targeted compounds in real samples was obtained from the conformity between the samples' and standards' dd-MS/MS spectra. For free simple phenols, the characteristic fragments of each compound chemical group, such as $[M\text{-}H\text{-}44]^-$ for hydroxybenzoic acids (loss of CO_2), $[M\text{-}H\text{-}15]^-$ for methoxyderivatives (loss of a methyl group) or $[M\text{-}H\text{-}CH_2CHOH]^-$ for catechin and epicatechin (Sánchez-Rabaneda et al., 2003) were detected in the corresponding MS/MS spectra. Table 1 summarises the accurate mass used for identification, the difference between expected and experimental masses, the normalized collision energy (NCE) used for MS/MS fragmentation and typical fragments of targeted compounds. In the case of aesculetin-glucoside (aesculetin-6-*O*-β-D-glucoside) and vanillic acid-glucoside (vanillic acid-4-*O*-β-D-glucoside), ions corresponding to deprotonated molecules $[M\text{-}H]^-$ were used for identification, while ions corresponding to the loss of the glucosidic unit $[M\text{-}H\text{-}C_6H_{10}O_5]^-$, respectively *m/z* 177.0193 and *m/z* 167.0350, and ions at *m/z* 133.0296 (aesculetin-glucoside loss of CO_2 $[M\text{-}H\text{-}C_6H_{10}O_5\text{-}44]^-$) and at *m/z* 152.0114 (vanillic acid-glucoside loss of a methyl group $[M\text{-}H\text{-}C_6H_{10}O_5\text{-}CH_3]^-$) were used for confirmation. As regards acetovanillone-glucoside (acetovanillone-4-*O*-β-D-glucoside) and scopoletin-glucoside (scopoletin-7-*O*-β-D-glucoside), identification was performed on the basis of the corresponding aglyconic forms $[M\text{-}H\text{-}C_6H_{10}O_5]^-$, *m/z* 165.0557 and *m/z* 191.0350 respectively, since due to probable sugar loss in HESI parent ions $[M\text{-}H]^-$ were not isolated. This approach was allowed by different RTs between the corresponding free and bound forms (Table 1). Consequently, only ions at *m/z* 150.0321 $[M\text{-}H\text{-}C_6H_{10}O_5\text{-}CH_3]^-$ and *m/z* 176.0112 $[M\text{-}H\text{-}C_6H_{10}O_5\text{-}CH_3]^-$ were used for confirmation.

2.5. Untargeted analysis

Research into other putative glycosidic precursors was extended to monosaccharidic (hexoside, pentoside) and disaccharidic (hexoside-hexoside, pentoside-hexoside, hexoside-pentoside, pentoside-pentoside) derivatives of all free simple phenols detected with the targeted approach.

Tentative identification was based on checking of the expected accurate mass (Δ *m/z* < 5 ppm), isotopic pattern and fragmentation profile. Specifically, as for aesculetin-glucoside and vanillic acid-glucoside, the two characteristic ions $[M\text{-}H]^-$ and $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ were detected in the mass spectrum of the extracted chromatogram peaks, since the molecules partially lost glucose in HESI, in the same way, the presence of $[M\text{-}H]^-$ and $[M\text{-}H\text{-}S]^-$ was generally required for tentative identification of other monosaccharidic precursors. Depending on whether $[M\text{-}H\text{-}S]^-$ was $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ or $[M\text{-}H\text{-}C_5H_8O_4]^-$, it was possible to distinguish between hexose and pentose derivatives.

The proposed untargeted approach was also extended to putative identification of disaccharidic precursors, requiring the presence of ions $[M\text{-}H]^-$, $[M\text{-}H\text{-}S]^-$, the loss of one sugar unit, and $[M\text{-}H\text{-}S\text{-}S]^-$, the loss of two sugar units, considering the transitions $[M\text{-}H\text{-}C_6H_{10}O_5]^- \rightarrow [M\text{-}H\text{-}C_{12}H_{20}O_{10}]^-$, $[M\text{-}H\text{-}C_6H_{10}O_5]^- \rightarrow [M\text{-}H\text{-}C_{11}H_{18}O_9]^-$, $[M\text{-}H\text{-}C_5H_8O_4]^- \rightarrow [M\text{-}H\text{-}C_{11}H_{18}O_9]^-$ and $[M\text{-}H\text{-}C_5H_8O_4]^- \rightarrow [M\text{-}H\text{-}C_{10}H_{16}O_8]^-$ characteristic of hexoside-hexoside, hexoside-pentoside, pentoside-hexoside and pentoside-pentoside derivatives respectively, in the mass spectrum of the extracted chromatogram peaks.

2.6. Method validation

In real samples, identification was carried out for almost all targeted compounds using their accurate mass (Δ *m/z* < 5 ppm), proving detection using peak retention time, isotopic pattern and the MS/MS fragmentation profile in the case of isobaric interference. Only isomers such as aceto-/isoacetovanillone, gentisic/protocatechuic acids and *o*-/*m*-/*p*-cresol were detected as a sum, since they coeluted and are characterised by the same MS/MS spectrum. Quantification was performed with external solvent calibration, considering standard levels that allowed a regression coefficient (R^2) of at least 0.99. Limits of quantification (LOQs) were established, both for grapes and wines, according to EURACHEM (EURACHEM Secretariat, 1993). The accuracy of the method was estimated in terms of relative recovery by spiking a sample of skin, pulp, seed and wine, both for red and white varieties, at a concentration of 1 mg L^{-1} of each phenol.

As regards the proposed untargeted approach, the robustness of conditions used for tentative identification was tested through custom synthesized standards such as salicylic acid-glucoside, orcinol-glucoside and *p*-hydroxybenzaldehyde-allopyranoside. These phenols were used to confirm the hypothesised retention times and fragmentation.

2.7. Data processing

The concentration of the targeted compounds measured in the grape fractions (pulp, skin and seed) was recalculated, taking into account the relative abundance of the individual fraction in the overall weight of the 25 berries (Cabernet Cantor: 75% pulp, 17% skin, 8% seed; Prior: 87%, 8%, 5%; Merlot: 88%, 8%, 4%; Muscaris: 82%, 13%, 6%; Solaris: 81%, 14%, 5%; Chardonnay: 89%, 7% and 4% respectively; expressed as the average content of samples). Furthermore, limits of quantification for each grape fraction were recalculated by considering the mean contribution of each grape part to the total berry weight.

Concentration data relating to total grapes were expressed as the sum of converted data for the pulp, skin and seed, and limits of quantification were obtained by summing the three grape part LOQs. For each grape fraction, non-detectable data were replaced with a random value between zero and the LOQ.

Statistical analysis was performed using Statistica 9.1 Software (StatSoft, 2010, Tusla, OK, USA), considering only analytical compounds detectable in 90% of samples of at least one grape fraction (pulp, skin, seed) of one variety. Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$) was performed in order to characterise the phenolic profile of each grape part in all selected varieties. Principal Component Analysis and Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$) were performed in order to define the phenolic composition of the total grapes in each variety.

As regards wines, statistical analysis was carried out considering analytical compounds detectable in both samples of at least one variety. Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$) was performed in order to characterise the phenolic profile of red and white wines in comparison to that of the corresponding red and white grapes respectively.

3. Results and discussion

3.1. Optimisation and validation of the analytical method

As regards the targeted approach, with the exception of the isomers aceto-/isoacetovanillone, gentisic/protocatechuic acids and *o*-/*m*-/*p*-cresol, all phenolic compounds were individually detected and quantified through the high selectivity of tandem high-resolution mass detection. The highest sensitive detection was obtained in negative ion mode, tuning source settings and normalized collision energies (NCE, Table 1) for dd-MS/MS experiments with available standards. As regards quantification, R^2 values were at least 0.99, with the exception of 4-methylsyringol, caffeic acid, coniferyl alcohol, homovanillic alcohol, syringic acid and vanillyl ethyl ether (0.98), homovanillic acid (0.96) and phenol (0.88). Acceptable recovery, ranging from 70% to 130%, was obtained in white berry varieties for 70% of compounds in pulp samples, 70% in skin, 40% in seed and 85% in wine samples. As regards red berry varieties, acceptable recovery was obtained for 72% of compounds in pulp samples, over 64% in skin, 60% in seed and 75% in wine samples. All concentration data were corrected with the corresponding recovery.

In the case of the untargeted approach, it was possible to confirm the robustness of the requirements used for putative identification of glycosylated simple phenols by the availability of three custom synthesized standards (orcinol-glucoside, *p*-hydroxybenzaldehyde-allopyranoside and salicylic acid-glucoside). For *p*-hydroxybenzaldehyde-allopyranoside and salicylic acid-glucoside, the two expected characteristic ions $[M-H]^-$ and $[M-H-C_6H_{10}O_5]^-$, at *m/z* 283.0823-121.0295 and *m/z* 299.0772-137.0244 respectively, were detected in the mass spectrum of the extracted chromatogram peaks. The parent ion $[M-H]^-$ from orcinol-glucoside was not isolated, probably because the sugar unit was completely lost in HESI, and both identification and quantification were based on the aglyconic ion $[M-H-C_6H_{10}O_5]^-$ at *m/z* 123.0452, exploiting different retention times for the free and bound forms (8.77 and 6.83 min respectively, Table 1). In fact, orcinol-glucoside provided the evidence of situations where precursors completely fragmented in the source cannot be detected with this untargeted approach.

3.2. Method application

3.2.1. Targeted approach: grape composition

The targeted approach made it possible to quantify 60 simple phenols, 7 of which were glycosidic precursors, and to define the characteristic phenolic profile of the three berry fractions (pulp, skin and seed) for each variety.

As regards simple phenol relative abundance in each fraction, expressed as the mean of all samples, regardless of the variety considered, 4-vinylphenol, isopropiovanillone, hydroxytyrosol, isoacetosyringone, aceto-/isoacetovanillone, orcinol-glu and *o*-vanillin were mostly present in pulp (at least 70% in pulp, <20% in skin or seed); vanillic acid-glu, gallic acid and epicatechin were mainly present in seed (at least 70% in seed, <20% in pulp or skin), confirming what reported by Di Lecce et al. (2014) for European grapes while no compound was predominant in skin. Fig. 1 summarises simple phenol distribution in the pulp, skin and seed.

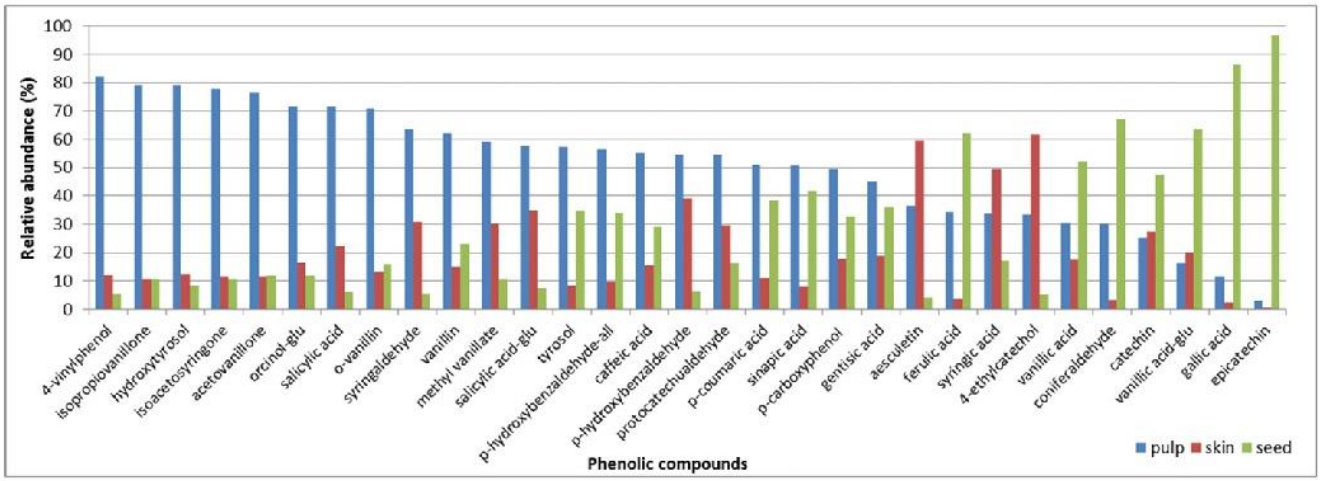

Fig. 1. Targeted simple phenolic compounds relative abundance in the three parts of grapes (pulp, skin and seed). Only compounds detected in 90% of samples of at least one grape fraction (pulp, skin or seed) of one variety were considered.

Table 2
Phenolic content (mean ± standard deviation; mg kg^{-1}) in seed, skin and pulp for each variety. Significant differences (Tukey's HSD test, $p < 0.05$) in the phenolic content of seed, skin and pulp for each variety are shown in brackets. Only compounds detected in 90% of samples for at least one grape fraction (pulp, skin or seed) of one variety were considered.

Compound	Hybrid varieties						*Vitis vinifera*		
	Muscaris (N = 6)			Solaris (N = 6)			Chardonnay (N = 3)		
	Seed	Skin	Pulp	Seed	Skin	Pulp	Seed	Skin	Pulp
4-Ethylcatechol	0.0012 ± 0.0002 (b)	0.0281 ± 0.0141 (a)	0.0104 ± 0.0037 (b)	0.0012 ± 0.0004 (c)	0.0295 ± 0.0103 (a)	0.0114 ± 0.0013 (b)	0.0017 ± 0.0002 (b)	0.0233 ± 0.0017 (a)	0.0208 ± 0.0059 (a)
4-Vinylphenol	<0.0123	<0.0276	<0.1839	<0.0123 (b)	0.0350 ± 0.0162 (b)	0.1993 ± 0.096 (a)	<0.0123	<0.0276	<0.1839
Acetovanillone	<0.0001	<0.0002	<0.0016	<0.0001	0.0001 ± 0.0001	<0.0016	<0.0001	<0.0002	0.0048 ± 0.0064
Aesculetin	0.0002 ± 0.0003	0.0144 ± 0.0066	0.0266 ± 0.0379	0.0001 ± 0.0002 (b)	0.0067 ± 0.0014 (a)	0.0050 ± 0.0041 (a)	0.0006 ± 0.0002	0.0065 ± 0.0005	0.0130 ± 0.0117
Caffeic acid	0.0031 ± 0.0044	0.1238 ± 0.2243	0.5375 ± 0.9092	0.0121 ± 0.0196	0.0012 ± 0.0016	0.0908 ± 0.1291	0.0086 ± 0.0052	<0.0002	0.0939 ± 0.1543
Catechin	44.7 ± 18.3 (a)	2.34 ± 1.03 (b)	2.85 ± 1.41 (b)	31.76 ± 13.61 (a)	0.641 ± 0.159 (b)	12.39 ± 13.20 (b)	32.57 ± 5.25 (a)	0.370 ± 0.071 (b)	1.730 ± 0.758 (b)
Coniferaldehyde	0.011 ± 0.0085 (a)	<0.0002 (b)	<0.0016 (b)	0.0095 ± 0.0063 (a)	<0.0002 (b)	<0.0016 (b)	0.0015 ± 0.0022	<0.0002	<0.0016
Epicatechin	286.4 ± 88.3 (a)	0.50 ± 0.31 (b)	3.90 ± 2.02 (b)	239.2 ± 83.7 (a)	0.240 ± 0.112 (b)	11.16 ± 8.62 (b)	246.8 ± 39.9 (a)	0.0924 ± 0.0152 (b)	2.054 ± 1.623 (b)
Ferulic acid	0.0074 ± 0.0062	0.0002 ± 0.00008	0.0123 ± 0.0236	0.0121 ± 0.0077	<0.0002	0.014 ± 0.0215	0.0131 ± 0.0044	<0.0002	0.0171 ± 0.0283
Gallic acid	8.374 ± 5.281 (a)	0.084 ± 0.0436 (b)	0.247 ± 0.201 (b)	4.430 ± 1.324 (a)	0.0569 ± 0.0094 (b)	0.6488 ± 0.6162 (b)	4.764 ± 0.671 (a)	0.043 ± 0.0099 (b)	0.6758 ± 0.4168 (b)
Gentisic acid	0.0199 ± 0.0093	0.0096 ± 0.0104	0.0277 ± 0.0479	0.014 ± 0.0061	0.0036 ± 0.0013	0.0188 ± 0.0348	0.024 ± 0.0048	0.0028 ± 0.0007	0.0457 ± 0.0326
Hydroxytyrosol	0.2366 ± 0.0427 (c)	0.3573 ± 0.0496 (b)	1.990 ± 0.073 (a)	0.224 ± 0.1023 (b)	0.370 ± 0.106 (b)	2.245 ± 0.645 (a)	0.217 ± 0.0515 (b)	0.2325 ± 0.0241 (b)	2.8029 ± 0.35 (a)
Isoacetosiringone	<0.0012	<0.0027	<0.0180	0.0015 ± 0.0011	<0.0027	<0.0180	<0.0012	<0.0027	<0.0180
Isopropiovanillone	<0.0059	<0.0133	<0.0887	0.0162 ± 0.0193	<0.0133	0.1501 ± 0.23	0.0223 ± 0.0189 (b)	<0.0133 (b)	0.2983 ± 0.0765 (a)
Methyl vanillate	0.0007 ± 0.0004	<0.0012	<0.0082	<0.0005	<0.0012	<0.0082	0.0008 ± 0.0002	<0.0012	<0.0082
o-Vanillin	<0.0011	<0.0024	<0.0164	<0.0011	<0.0024	<0.0164	<0.0011	<0.0024	0.0099 ± 0.0072
p-Carboxyphenol	0.021 ± 0.0111	0.0098 ± 0.0041	0.0175 ± 0.0242	0.0266 ± 0.0155	0.0056 ± 0.0017	0.0523 ± 0.0879	0.0505 ± 0.0212	0.0104 ± 0.0025	0.1268 ± 0.0978
p-Coumaric acid	0.0159 ± 0.0111	0.0088 ± 0.0137	0.0434 ± 0.0574	0.0152 ± 0.0103	0.0371 ± 0.0834	0.0292 ± 0.0377	0.0369 ± 0.0019	<0.0002	0.0261 ± 0.0322
p-Hydroxybenzaldehyde	<0.0110	0.0316 ± 0.0048	<0.1651	<0.0110	<0.0248	<0.1651	0.0147 ± 0.0033	<0.0248	<0.1651
Protocatechualdeihyde	0.002 ± 0.0013	0.0039 ± 0.0014	0.0032 ± 0.0041	0.0017 ± 0.0016	0.0039 ± 0.0019	0.015 ± 0.0289	0.0025 ± 0.0028	0.0022 ± 0.0005	0.0134 ± 0.0157
Salicylic acid	0.016 ± 0.0068 (b)	0.0634 ± 0.0431	0.2118 ± 0.1657 (a)	0.0184 ± 0.0098 (b)	0.0332 ± 0.0074 (b)	0.154 ± 0.0963 (a)	0.0101 ± 0.0026 (b)	0.0245 ± 0.0031 (b)	0.1692 ± 0.0248 (a)
Sinapic acid	0.007 ± 0.0075	<0.0012	<0.0082	0.0086 ± 0.0084 (a)	<0.0012 (b)	<0.0082	0.0105 ± 0.0126	<0.0012	<0.0082
Syringaldehyde	<0.0008	0.0036 ± 0.0035	<0.0123	<0.0008	0.0021 ± 0.001	<0.0123	<0.0008	0.0021 ± 0.0008	<0.0123
Syringic acid	0.0041 ± 0.0065	0.0008 ± 0.0016	<0.0016	0.0002 ± 0.0004	<0.0002	<0.0016	0.001 ± 0.0003 (a)	<0.0002 (b)	<0.0016
Tyrosol	0.0008 ± 0.0004	<0.0002	<0.0016	0.0009 ± 0.001	0.0002 ± 0.0003	<0.0016	0.0043 ± 0.003	<0.0002	<0.0016
Vanillic acid	0.0196 ± 0.0112	0.0034 ± 0.006	0.0108 ± 0.0153	0.0243 ± 0.0095 (a)	0.0012 ± 0.0011 (b)	0.0111 ± 0.0231	0.0463 ± 0.015	0.001 ± 0.0009	0.0416 ± 0.0406
Vanillin	0.0282 ± 0.0136 (b)	0.0223 ± 0.0058 (b)	0.0604 ± 0.0221 (a)	0.0449 ± 0.0264	0.0144 ± 0.0076	0.0841 ± 0.0857	0.0247 ± 0.007 (b)	0.0144 ± 0.0047 (b)	0.0935 ± 0.0327 (a)
Orcinol-glu	0.008 ± 0.0074	<0.0123	<0.0821	<0.0055	<0.0123	<0.0821	<0.0055	<0.0123	<0.0821
p-Hydroxybenzaldehyde-all	0.0259 ± 0.0145	<0.0246	<0.1642	0.0216 ± 0.0089	<0.0246	<0.1642	0.0525 ± 0.0094	<0.0246	<0.1642
Salicylic acid-glu	<0.0011	<0.0024	<0.0164	0.0177 ± 0.006 (c)	0.0392 ± 0.0146 (b)	0.0757 ± 0.0135 (a)	0.0167 ± 0.0015 (c)	0.0398 ± 0.0052 (b)	0.0736 ± 0.0068 (a)
Vanillic acid-glu	2.000 ± 0.386 (a)	0.3286 ± 0.0663 (b)	0.5758 ± 0.2961 (b)	1.7033 ± 0.7156 (a)	0.2403 ± 0.0607 (b)	<0.3449 (b)	2.628 ± 0.4119 (a)	0.2472 ± 0.0245 (b)	<0.3449 (b)

(continued on next page)

Table 2 (*continued*)

Compound	Hybrid varieties						*Vitis vinifera*		
	Muscaris (N = 6)			Solaris (N = 6)			Chardonnay (N = 3)		
	Seed	Skin	Pulp	Seed	Skin	Pulp	Seed	Skin	Pulp
	Prior (N = 3)			Cabernet Cantor (N = 6)			Merlot (N = 3)		
	Seed	Skin	Pulp	Seed	Skin	Pulp	Seed	Skin	Pulp
4-Ethylcatechol	0.0014 ± 0.0004	0.0054 ± 0.0022	<0.0082	0.002 ± 0.0007 (b)	0.021 ± 0.0016 (a)	0.011 ± 0.0039 (c)	0.0020 ± 0.0004 (c)	0.0256 ± 0.0022 (a)	0.0176 ± 0.0009 (b)
4-Vinylphenol	<0.0123	<0.0276	<0.1839	<0.0123 (b)	0.0304 ± 0.0166 (b)	0.2234 ± 0.0749 (a)	<0.0123	<0.0276	<0.1839
Acetovanillone	<0.0001	0.0018 ± 0.003	<0.0016	0.0011 ± 0.0006 (b)	0.0002 ± 0.00007 (b)	0.0064 ± 0.00603 (a)	0.0004 ± 0.0005	<0.0002	<0.0016
Aesculetin	0.0003 ± 0.0003	0.0095 ± 0.0009	0.0084 ± 0.0138	0.0014 ± 0.0006 (b)	0.0163 ± 0.0032 (a)	0.0046 ± 0.002 (b)	0.0011 ± 0.0007 (b)	0.0024 ± 0.0006	0.0059 ± 0.0029 (a)
Caffeic acid	0.0035 ± 0.0026	0.0178 ± 0.0293	0.1407 ± 0.2353	0.0112 ± 0.0033	0.0159 ± 0.0092	0.0172 ± 0.0143	0.0052 ± 0.0011 (b)	0.0022 ± 0.0004 (b)	0.0122 ± 0.0041 (a)
Catechin	0.6337 ± 0.1006 (b)	11.57 ± 5.77 (a)	18.60 ± 4.53 (a)	2.848 ± 0.790 (b)	46.64 ± 16.69 (a)	36.00 ± 35.98	1.20 ± 0.09 (b)	16.46 ± 4.13 (a)	5.23 ± 5.59 (b)
Coniferaldehyde	<0.0001	<0.0002	<0.0016	0.0031 ± 0.0044	<0.0002	<0.0016	0.0017 ± 0.0021	<0.0002	<0.0016
Epicatechin	148.5 ± 26.5 (a)	0.1947 ± 0.1043 (b)	10.70 ± 4.193 (b)	468.1 ± 72.1 (a)	0.6 ± 0.12 (b)	13.00 ± 16.08 (b)	185.3 ± 10.9 (a)	0.364 ± 0.2186 (b)	1.13 ± 0.24 (b)
Ferulic acid	0.0091 ± 0.0036	0.004 ± 0.0018	0.0198 ± 0.0159	0.0116 ± 0.0029 (a)	<0.0002 (b)	0.0062 ± 0.0126	0.0047 ± 0.0004	0.0007 ± 0.0011	0.0112 ± 0.009
Gallic acid	1.658 ± 0.422 (a)	0.1787 ± 0.1335 (b)	0.5169 ± 0.5195 (b)	8.193 ± 1.155 (a)	0.251 ± 0.1146 (b)	1.092 ± 1.186 (b)	3.134 ± 0.316 (a)	0.1053 ± 0.0205 (b)	0.5443 ± 0.1486 (b)
Gentisic acid	0.014 ± 0.0017	0.0277 ± 0.018	0.0840 ± 0.0956	0.0239 ± 0.0024 (b)	0.0311 ± 0.0032 (b)	0.0619 ± 0.0157 (a)	0.014 ± 0.0019 (b)	0.014 ± 0.0026 (b)	0.0756 ± 0.0121 (a)
Hydroxytyrosol	0.2148 ± 0.0296 (b)	0.3923 ± 0.2189 (b)	3.347 ± 1.233 (a)	0.4188 ± 0.0393 (c)	0.646 ± 0.0736 (b)	2.797 ± 0.240 (a)	0.213 ± 0.0146 (b)	0.274 ± 0.0038 (b)	2.841 ± 0.1619 (a)
Isoacetosiringone	0.005 ± 0.0013	<0.0027	<0.0180	0.0013 ± 0.0008 (b)	<0.0027 (b)	0.0082 ± 0.006 (a)	<0.0012	<0.0027	<0.0180
Isopropiovanillone	0.0179 ± 0.008	0.0631 ± 0.1049	0.3499 ± 0.4959	0.0315 ± 0.0212 (b)	0.0514 ± 0.03 (b)	0.2922 ± 0.0629 (a)	0.0062 ± 0.0049 (b)	<0.0133 (b)	0.1763 ± 0.038 (a)
Methyl vanillate	0.0006 ± 0.0008 (b)	0.0239 ± 0.0044 (a)	0.0086 ± 0.0055 (b)	0.0007 ± 0.0003 (b)	0.0037 ± 0.0018 (a)	<0.0082	<0.0005	0.0055 ± 0.0016	<0.0082
o-Vanillin	0.0686 ± 0.019	0.1981 ± 0.2102	0.621 ± 0.5441	0.2812 ± 0.1219	0.0941 ± 0.0731 (b)	0.4717 ± 0.2895 (a)	0.0895 ± 0.027	0.0214 ± 0.0177	0.5655 ± 0.5036
p-Carboxyphenol	0.0221 ± 0.0101	0.1162 ± 0.0989	0.3007 ± 0.2661	0.0494 ± 0.0088 (b)	0.0367 ± 0.0147 (b)	0.1206 ± 0.0286 (a)	0.0269 ± 0.0044 (b)	0.0254 ± 0.0025 (b)	0.1328 ± 0.0282 (a)
p-Coumaric acid	0.0094 ± 0.0042	0.0238 ± 0.0237	0.0728 ± 0.05	0.0157 ± 0.0041	<0.0002 (b)	0.029 ± 0.0292 (a)	0.0085 ± 0.0015	0.0012 ± 0.002 (b)	0.0376 ± 0.0228 (a)
p-Hydroxybenzaldehyde	<0.0110 (b)	0.1377 ± 0.0162 (a)	<0.1651	0.0064 ± 0.0057 (c)	0.2287 ± 0.0295 (a)	0.0853 ± 0.0702 (b)	<0.0110	0.0301 ± 0.0099	<0.1651
Protocatechualdeihyde	0.0032 ± 0.0013 (b)	0.0343 ± 0.0066 (b)	0.0968 ± 0.0279 (a)	0.0068 ± 0.0022 (b)	0.0171 ± 0.0082 (b)	0.043 ± 0.0158 (a)	0.0038 ± 0.001 (b)	0.0079 ± 0.0009 (b)	0.0695 ± 0.0194 (a)
Salicylic acid	0.0108 ± 0.004	0.0743 ± 0.0179	0.2888 ± 0.1967	0.0209 ± 0.0018 (c)	0.1539 ± 0.0264 (b)	0.281 ± 0.0408 (a)	0.0136 ± 0.0046 (b)	0.0646 ± 0.0089 (b)	0.3542 ± 0.0597 (a)
Sinapic acid	0.0058 ± 0.0016	<0.0012	<0.0082	0.0005 ± 0.0003	<0.0012	<0.0082	0.0043 ± 0.0007	<0.0012	<0.0082
Syringaldehyde	<0.0008	0.004 ± 0.0021	<0.0123	<0.0008	0.0046 ± 0.0023	<0.0123	<0.0008	0.0031 ± 0.0008	<0.0123
Syringic acid	0.0015 ± 0.0013 (b)	0.1474 ± 0.0155 (a)	0.0044 ± 0.0023 (b)	0.0019 ± 0.0021 (b)	0.2544 ± 0.0719 (a)	0.0103 ± 0.0103 (b)	0.0018 ± 0.0005 (b)	0.0955 ± 0.0146 (a)	0.0351 ± 0.0312 (b)
Tyrosol	0.0008 ± 0.0013	<0.0002	0.0099 ± 0.0157	0.0004 ± 0.0010	<0.0002	<0.0016	<0.0001	<0.0002	0.0181 ± 0.0146
Vanillic acid	0.0175 ± 0.0017	0.0584 ± 0.0262	0.0791 ± 0.0562	0.0261 ± 0.0086	0.0196 ± 0.0128	0.0148 ± 0.0102	0.0138 ± 0.0019 (c)	0.0266 ± 0.0019 (b)	0.0629 ± 0.0054 (a)
Vanillin	0.0308 ± 0.0104	0.0196 ± 0.0172	0.1559 ± 0.1026	0.0298 ± 0.0076 (b)	0.0319 ± 0.0062 (b)	0.0961 ± 0.0267 (a)	0.0141 ± 0.0036 (b)	0.0123 ± 0.0036 (b)	0.1125 ± 0.0223 (a)
Orcinol-glu	0.0306 ± 0.027	<0.0123	<0.0821	<0.0055	<0.0123	<0.0821	<0.0055 (b)	0.0601 ± 0.034 (a)	<0.0821
p-Hydroxybenzaldehyde-all	0.0143 ± 0.0029	<0.0246	<0.1642	0.1052 ± 0.0332 (a)	<0.0246 (b)	<0.1642 (b)	0.0463 ± 0.0103	<0.0246	0.2991 ± 0.422
Salicylic acid-glu	0.0291 ± 0.0134	0.3949 ± 0.0258	0.5185 ± 0.4444	0.0182 ± 0.0033 (b)	0.0859 ± 0.0276 (a)	0.1253 ± 0.0428 (a)	0.0072 ± 0.0001 (c)	0.2044 ± 0.0421 (a)	0.1175 ± 0.0402 (b)
Vanillic acid-glu	1.145 ± 0.1339 (a)	0.882 ± 0.1136 (a)	<0.3449 (b)	2.380 ± 0.219 (a)	1.532 ± 0.200 (b)	0.8291 ± 0.1829 (c)	1.598 ± 0.034	0.7920 ± 0.1804	4.820 ± 7.329

Note: glu = glucoside; all = alloside.

Regarding the characteristic phenolic profile found for each variety (Table 2), in Cabernet Cantor, hydroxytyrosol, *o*-vanillin, isopropiovanillone, salicylic acid, 4-vinylphenol, salicylic acid-glu, *p*-carboxyphenol, vanillin, gentisic/protocatechuic acid, protocatechualdehyde, *p*-coumaric acid, isoacetosyringone and acetovanillone were mainly present in pulp. Catechin, syringic acid, *p*-hydroxybenzaldehyde, 4-ethylcatechol, aesculetin and methyl vanillate were mainly present in skin. Epicatechin, gallic acid, vanillic acid-glu, *p*-hydroxybenzaldehyde-all and ferulic acid were mostly present in seed.

In Prior, catechin, hydroxytyrosol and protocatechualdehyde were mainly present in pulp; syringic acid, *p*-hydroxybenzaldehyde and methyl vanillate in skin; while epicatechin, gallic acid and vanillic acid-glu were mainly present in seed.

Merlot showed hydroxytyrosol, salicylic acid, isopropiovanillone, *p*-carboxyphenol, vanillin, gentisic/protocatechuic acid, protocatechualdehyde, vanillic acid, *p*-coumaric acid, caffeic acid and aesculetin as the main components in pulp, and catechin, salicylic acid-glu, syringic acid, orcinol-glu and 4-ethylcatechol in skin, while epicatechin and gallic acid were relevant in seed.

For Muscaris, salicylic acid, hydroxytyrosol and vanillin were mainly present in pulp; 4-ethylcatechol in skin; epicatechin, catechin, gallic acid, vanillic acid-glu and coniferaldehyde were mainly present in seed.

In the case of Solaris, hydroxytyrosol, 4-vinylphenol, salicylic acid and salicylic acid-glu were mostly present in pulp; while 4-ethylcatechol and aesculetin in skin and epicatechin, catechin, gallic acid, vanillic acid-glu, vanillic acid, coniferaldehyde and sinapic acid were mainly present in seed.

Finally, in Chardonnay the phenolic profile was characterised by hydroxytyrosol, isopropiovanillone, salicylic acid, vanillin and salicylic acid-glu as the main components in pulp; 4-ethylcatechol in skin, and epicatechin, catechin, gallic acid, vanillic acid-glu and syringic acid in seeds.

When the phenolic profiles of each grape part from the selected varieties were compared, it emerged that hydroxytyrosol and salicylic acid were always present in high levels in pulp, 4-ethylcatechol in skin, and epicatechin, gallic acid and vanillic acid-glu were prevalent in seed, confirming data reported in literature for European grapes (Di Lecce et al., 2014). Furthermore, syringic acid was present in larger amounts in skins of all red varieties and *p*-hydroxybenzaldehyde and methyl vanillate in skins of only hybrid red varieties, while catechin content was higher in skin for white cultivars and in seeds for the red ones.

Furthermore, particular phenolic profiles were found in the three grape fractions of each variety, with significant differences in the content of single compounds (Tukey's HSD test, $p < 0.05$; Table 2). Table 2 summarises the mean content and the standard deviations of targeted compounds measured in the three berry fractions and detected in at least 90% of samples of each selected variety.

In order to evaluate the differences between the phenolic profiles for each variety, total grape simple phenol content was considered. Preliminary and exploratory Principal Component Analysis (PCA) was applied to the entire dataset of 27 samples (Fig. 2). In such a way, PCA was used to objectively interpret and compare multiple independent phenolic groups present in all the *Vitis* types studied, and so explaining the maximum amount of variability present in the data.

Sinapic acid and 4-ethylcatechol were the most significant components with positive eigenvalues (0.54 and 0.27 respectively), while *p*-carboxyphenol, gentisic/protocatechuic acid and protocatechualdehyde were those with negative eigenvalues (−0.89, −0.88 and −0.83 respectively) for the first function (Fatt. 1), explaining 41.25% of total variability. Sinapic acid, salicylic acid-glu and methyl vanillate were the most significant components with positive eigenvalues (0.60, 0.52 and 0.42 respectively), while gallic acid, catechin and 4-ethylcatechol were those with negative eigenvalues (−0.95, −0.76 and −0.58 respectively) for the second function (Fatt. 2), which explained 22.35% of total variability.

In the experimental conditions previously described and with the number of samples evaluated, from an analytical point of view, PCA applied to simple phenol content (Fig. 2) made it possible to properly characterise Cabernet Cantor and Merlot samples, while Prior and Chardonnay samples were described reasonably well. In contrast, it was not possible to identify single clusters for Muscaris and Solaris samples. Further evidence of the possibility of defining a characteristic simple phenolic profile for each variety derived from Tukey's HSD test ($p < 0.05$; Table 3).

However, it was not possible, from the simple phenolic profile, to properly distinguish *Vitis vinifera* from the hybrid varieties. The mean content and standard deviations of target compounds for which

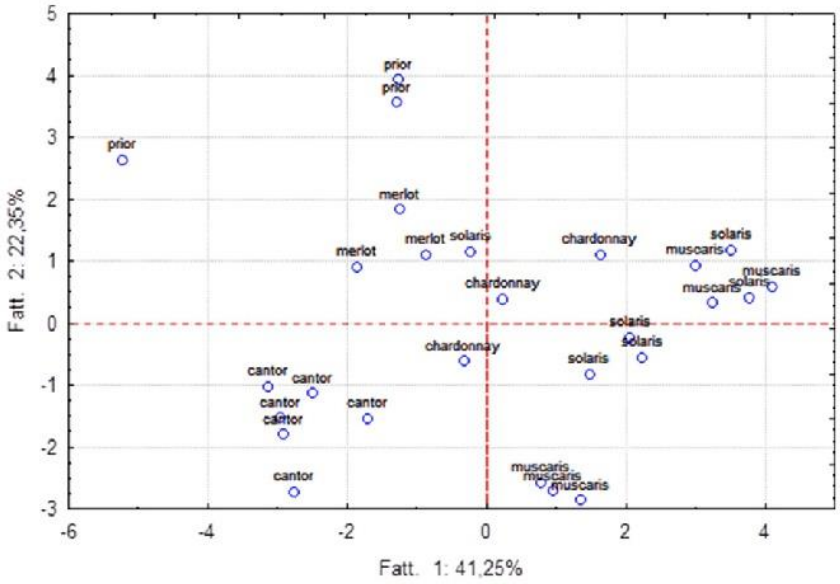

Fig. 2. Score plot of the first two principal components (Fatt. 1 and Fatt. 2) of total grape simple phenolic content for each selected variety.

Table 3
Total content (mean ± standard deviation; mg kg^{-1}) of the simple phenols in grapes found to be significantly different in varieties according to Tukey's HSD test ($p < 0.05$).

Compounds	Cabernet Cantor (N = 6)	Prior (N = 6)	Merlot (N = 6)	Solaris (N = 6)	Muscaris (N = 6)	Chardonnay (N = 6)
4-Ethylcatechol	0.034 ± 0.005 (a)	0.012 ± 0.006 (b)	0.045 ± 0.001 (a)	0.042 ± 0.01 (a)	0.039 ± 0.016	0.045 ± 0.005 (a)
Catechin	85.4 ± 32.0 (a)	30.8 ± 2.03	22.8 ± 4.64 (b)	44.7 ± 9.7	49.9 ± 17.3	34.7 ± 4.9
Epicatechin	481 ± 67.4 (a)	159 ± 30.4 (b)	186 ± 8.76 (b)	250 ± 76.6 (b)	290 ± 87.8	248 ± 33,7 (b)
Gallic acid	9.54 ± 1.16 (a)	2.35 ± 0.59 (bc)	3.78 ± 0.35 (b)	5.14 ± 1.08 (b)	8.71 ± 5.34 (b)	5.48 ± 0.81 (bd)
Methyl vanillate	<0.010 (b)	0.033 ± 0.005 (a)	<0.010 (b)	<0.010 (b)	<0.010 (b)	<0.010 (b)
p-Carboxyphenol	0.206 ± 0.047	0.439 ± 0.273 (a)	0.185 ± 0.017	0.084 ± 0.083 (b)	0.048 ± 0.032 (b)	0.187 ± 0.071
p-Hydroxybenzaldehyde	0.320 ± 0.064 (b)	0.232 ± 0.074	<0.201 (b)	<0.201 (b)	<0.201 (b)	<0.201 (b)
Protocatechualdeihyde	0.067 ± 0.017 (bd)	0.134 ± 0.028 (a)	0.081 ± 0.015 (ad)	0.020 ± 0.028 (bd)	0.009 ± 0.002 (bc)	0.018 ± 0.012 (bd)
Syringic acid	0.266 ± 0.079 (a)	0.153 ± 0.013 (ac)	0.132 ± 0.016 (bce)	<0.002 (bdf)	0.006 ± 0.006 (bdf)	<0.002 (bdf)
Vanillic acid	0.06 ± 0.004	0.155 ± 0.079 (a)	0.103 ± 0.004	0.036 ± 0.023 (b)	0.033 ± 0.025 (b)	0.089 ± 0.034
Salicylic acid-glu	0.229 ± 0.068 (b)	0.942 ± 0.427 (a)	0.329 ± 0.042 (b)	0.132 ± 0.021 (b)	<0.020 (b)	0.13 ± 0.01 (b)

Note: glu = glucoside.

significant differences between varieties were highlighted (according to Tukey's HSD Test) are summarised in Table 3.

3.2.2. Targeted approach: wine phenolic profile

The targeted approach allowed definition of the simple phenolic profile of wines produced using selected hybrid grape varieties. On comparing Cabernet Cantor's grape and wine profile, caffeic acid, tyrosol, gallic acid, tryptophol, syringic acid, *p*-coumaric acid, homovanillic acid, gentisic/protocatechuic acid, vanillic acid, methyl vanillate, *p*-carboxyphenol, protocatechualdehyde, ferulic acid, aesculetin, acetovanillone, salicylic acid, ethylvanillate, sinapic acid and 4 methylcatechol were mainly present in wine (list order reflects abundance in wine). In the same way, for Prior variety, caffeic acid, tyrosol, gallic acid, hydroxytyrosol, *p*-coumaric acid, syringic acid, vanillic acid, gentisic/protocatechuic acid, methyl vanillate, protocatechualdehyde, aesculetin, homovanillic acid, ethyl vanillate, pyrocatechol, homovanillic alcohol, acetovanillone, ferulic acid, syringaldehyde, sinapic acid and acetosyringone represent the main phenols present in wine. The Muscaris wine variety was mainly composed by tyrosol, caffeic acid, 4 vinylguaiacol, *p*-coumaric acid, ferulic

Table 4
Phenolic content (mean and standard deviation; mg kg^{-1} for grapes, mg L^{-1} for wine) in the grapes and wine of each variety. Only compounds detected in 90% of grape samples or in both wines of at least one variety were considered.

Compound	Cabernet Cantor		Prior		Muscaris		Solaris	
	Grape (N = 6)	Wine (N = 2)	Grape (N = 3)	Wine (N = 1)	Grape (*N* = 6)	Wine (N = 2)	Grape (N = 6)	Wine (N = 2)
4-Methylcatechol	<0.0100	0.008 ± 0.003	<0.010	0,005	<0.010	<0.005	<0.010	<0.005
4-Ethylcatechol	0.034 ± 0.005	0.007 ± 0.005	0.012 ± 0.006	0,007	0.039 ± 0.016	<0.005	0.042 ± 0.01	0.006 ± 0.004
4-Vinylguaiacol	0.109 ± 0.022	0.753 ± 1.02	<0.109	0,005	<0.109	1.12 ± 0.32	<0.109	1.04 ± 0.243
4-Vinylphenol	0.264 ± 0.068	<0.112	<0.224	<0.112	<0.224	0.124 ± 0.116	0.238 ± 0.098	<0.112
Acetosyringone	<0.002	<0.001	<0.002	0.005	0.002 ± 0.004	0.003 ± 0.003	<0.002	0.007 ± 0.001
Acetovanillone	0.007 ± 0.006	0.097 ± 0.007	0.006 ± 0.01	0.130	<0.002	0.004 ± 0.004	<0.002	0.005 ± 0.006
Aesculetin	0.022 ± 0.004	0.247 ± 0.08	0.018 ± 0.014	0.353	0.041 ± 0.038	<0.001	0.011 ± 0.004	<0.001
Caffeic acid	0.044 ± 0.014	661 ± 223	0.162 ± 0.265	229	0.664 ± 0.89	6.11 ± 1.74	0.104 ± 0.125	6.11 ± 2.63
Catechin	85.4 ± 32.0	24.5 ± 15.8	30.8 ± 2.03	6.01	49.9 ± 17.3	7.4 ± 4.6	44.7 ± 9.7	7.83 ± 1.00
Coniferaldehyde	0.004 ± 0.004	<0.001	0.008 ± 0.006	0,016	0.011 ± 0.008	<0.001	0.01 ± 0.006	<0.001
Epicatechin	481 ± 67.3	135 ± 82.3	159 ± 30.4	39,08	290.908 ± 87.808	2.558 ± 1.189	250 ± 76.6	4.22 ± 0.50
Ethyl vanillate	<0.011	0.059 ± 0.045	0.011 ± 0.004	0.292	<0.011	<0.006	<0.011	0.011 ± 0.001
Ethylvanillin	<0.200	0.102 ± 0.052	<0.200	0,283	<0.200	<0.100	<0.200	<0.100
Ferulic acid	0.018 ± 0.015	0.462 ± 0.328	0.033 ± 0.011	0.111	0.019 ± 0.027	0.489 ± 0.371	0.026 ± 0.021	0.48 ± 0.233
Gallic acid	9.54 ± 1.16	145 ± 64.6	2.35 ± 0.59	72.5	8.706 ± 5.34	2.957 ± 0.678	5.13 ± 1.08	3.21 ± 1.87
Gentisic/protocatechuic acid	0.117 ± 0.017	2.67 ± 1.38	0.125 ± 0.111	3.16	0.057 ± 0.053	0.181 ± 0.017	0.036 ± 0.034	0.308 ± 0.045
Homovanillic acid	<0.020	3.01 ± 0.57	<0.020	0.295	<0.020	0.109 ± 0.032	<0.020	0.199 ± 0.011
Homovanillyl alcohol	<0.102	0.180 ± 0.23	<0.102	0.289	<0.102	<0.051	<0.102	<0.051
Hydroxytyrosol	3.86 ± 0.32	3.92 ± 0.85	3.95 ± 1.44	15.3	2.584 ± 0.062	0.282 ± 0.112	2.84 ± 0.53	0.211 ± 0.029
Isoacetosiringone	<0.022	<0.011	0.022 ± 0.003	0,025	<0.022	<0.011	<0.022	<0.011
Isopropiovanillone	0.375 ± 0.099	0.183 ± 0.016	0.431 ± 0.595	0,726	<0.108	0.095 ± 0.02	0.173 ± 0.227	0.11 ± 0.012
Methyl vanillate	<0.010	2.29 ± 1.40	0.033 ± 0.005	2.56	<0.010	0.028 ± 0.006	<0.010	0.071 ± 0.062
Orcinol-glu	<0.100	<0.050	<0.100	<0.050	<0.100	0.094 ± 0.076	<0.100	0.063 ± 0.06
o-Vanillin	0.847 ± 0.371	0.488 ± 0.131	0.887 ± 0.752	2,13	<0.020	<0.010	<0.020	<0.010
p-Carboxyphenol	0.206 ± 0.047	1.68 ± 0.75	0.439 ± 0.273	1,74	0.048 ± 0.032	0.079 ± 0.02	0.084 ± 0.083	0.088 ± 0.016
p-Coumaric acid	0.044 ± 0.029	5.49 ± 3.47	0.106 ± 0.051	10.8	0.068 ± 0.066	0.715 ± 0.546	0.081 ± 0.089	0.864 ± 0.033
p-Hydroxybenzaldehyde	0.320 ± 0.064	0.253 ± 0.097	0.232 ± 0.074	0,182	<0.201	<0.101	<0.201	<0.101
p-Hydroxybenzaldehyde-All	<0.200	0.122 ± 0.083	<0.200	<0.100	<0.200	0.446 ± 0.579	<0.200	<0.100
Pyrocatechol	<0.010	<0.005	<0.010	0.292	<0.010	<0.005	<0.010	<0.005
Protocatechualdehyde	0.067 ± 0.017	0.983 ± 0.802	0.134 ± 0.028	2.32	0.009 ± 0.002	0.075 ± 0.0009	0.02 ± 0.028	0.107 ± 0.027
Salicylic acid	0.456 ± 0.051	0.069 ± 0.098	0.374 ± 0.210	0,296	0.291 ± 0.187	0.058 ± 0.007	0.205 ± 0.086	0.216 ± 0.099
Salicylic acid-glu	0.229 ± 0.068	0.123 ± 0.099	0.942 ± 0.427	0,107	<0.020	0.044 ± 0.0006	0.132 ± 0.021	0.076 ± 0.026
Sinapic acid	<0.010	0.023 ± 0.006	0.010 ± 0.003	0.036	0.011 ± 0.006	<0.005	0.014 ± 0.008	<0.005
Syringaldehyde	<0.015	0.017 ± 0.006	<0.015	0.108	<0.015	<0.008	<0.015	<0.008
Syringic acid	0.266 ± 0.079	5.89 ± 1.28	0.153 ± 0.013	9.55	0.006 ± 0.006	0.127 ± 0.012	<0.002	0.136 ± 0.01
Tryptophol	<2.20	6.94 ± 0.78	<2.20	<1.10	<2.20	3.164 ± 3.391	<2.20	<1.10
Tyrosol	<0.002	301 ± 9.72	0.010 ± 0.015	158	<0.002	165.04 ± 46.55	<0.002	147.24 ± 13.594
Vanillic acid	0.060 ± 0.004	2.46 ± 0.88	0.155 ± 0.079	4.95	0.033 ± 0.025	0.218 ± 0.073	0.036 ± 0.023	0.243 ± 0.058
Vanillic acid-glu	4.74 ± 0.46	1.51 ± 0.24	2.11 ± 0.03	0.292	2.905 ± 0.704	0.256 ± 0.159	2.19 ± 0.84	1.13 ± 1.09
Vanillin	0.157 ± 0.036	0.162 ± 0.002	0.206 ± 0.108	0,285	0.111 ± 0.036	0.065 ± 0.011	0.143 ± 0.082	0.043 ± 0.008

Note: glu = glucoside; all = alloside.

Table 5
UHPLC-MS parameters of glycosidically bound simple phenols tentatively identified in 90% of samples of at least one grape fraction (pulp, skin or seed) or in both wines of at least one variety.

Compounds	RT (min)	$[M-H]^-$ (m/z)	Δ m/z	MS/MS fragments
Hexose derivatives				
Pyrocatechol-hex	5.32	271.0823	0.8	109.0295; 108.0202
p-Carboxyphenol-hex	5.33	299.0772	1.2	137.0244; 93.0646
Gentisic/protocatechuic acid-hex	5.47	315.0721	1.1	153.0193; 109.0295
Gallic acid-hex	5.51	331.0670	1.4	169.0143; 125.0244
Syringic acid-hex	5.52	359.0983	1.5	197.0456; 182.0216
p-Coumaric acid-hex	5.74	325.0928	1.4	163.0401; 119.0502
Ferulic acid-hex	5.86	355.1034	1.5	193.0506; 178.0268
Caffeic acid-hex	5.94	341.0878	1.7	179.0350; 135.0452
protocatechualdehyde-hex	5.98	299.0772	1.2	137.0244; 108.0216
Hydroxytyrosol-hex	6.03	315.1085	1.2	153.0557; 123.0437
Homovanillic acid-hex	6.16	343.1034	2.1	181.0506; 137.0617
Catechin-hex	7.36	451.1245	1.1	289.0718; 245.0805
Ethylvanillin-hex	7.41	327.1085	0.7	165.0557; 136.0152
4-Methylcatechol-hex	7.43	285.0979	0.5	123.0452; 108.0214
Vanillin-hex	7.46	313.0928	0.8	151.0401; 136.0152
Epicatechin-hex	8.23	451.1245	1.0	289.0718; 245.0805
Isoacetosyringone-hex	8.63	357.1191	2.2	195.0663; 180.0426
Isopropiosyringone-hex	8.69	371.1347	1.6	209.0819; 194.0581
Isopropiovanillone-hex	9.66	341.1241	0.8	179.0714; 164.0478
Syringaldehyde-hex	10.33	343.1034	1.2	181.0506; 166.0268
Methyl vanillate-hex	10.71	343.1034	2.0	181.0506; 166.0268
Coniferaldehyde-hex	10.92	339.1085	1.9	177.0557; 162.0320
Ethyl vanillate-hex	11.26	357.1191	2.0	195.0663; 180.0415
Pentose derivatives				
p-Carboxyphenol-pent	5.25	269.0666	0.5	137.0244; 93.0646
Vanillic acid-pent	5.33	299.0772	1.2	167.0350; 152.0114
Syringic acid-pent	5.47	329.0878	1.5	197.0456; 182.0216
Salicylic acid-pent	5.53	269.0666	0.3	137.0244; 93.0344
p-Coumaric acid-pent	5.58	295.0823	0.7	163.0401; 119.0502
Hydroxytyrosol-pent	5.60	285.0979	0.7	153.0557; 123.0437
Homovanillic acid-pent	5.60	313.0928	1.1	181.0506; 137.0617
Caffeic acid-pent	5.61	311.0772	0.7	179.0350; 135.0452
Gentisic/protocatechuic acid-pent	5.63	285.0615	0.9	169.0143; 125.0244
Gallic acid-pent	5.63	301.0565	−0.3	153.0193; 109.0295
Pyrocatechol-pent	5.70	241.0717	0.1	109.0295; 108.0202
Ferulic acid-pent	5.74	325.0928	1.4	193.0506; 178.0268
Protocatechualdehyde-pent	5.79	269.0666	0.3	137.0244; 108.0216
p-Hydroxybenzaldehyde-pent	5.82	253.0717	0.4	121.0295; 108.0218
Guaiacol-pent	5.86	255.0874	1.3	123.0452; 108.0214
Vanillin-pent	5.93	283.0823	0.6	151.0401; 136.0152
Syringaldehyde-pent	5.97	445.1351	1.5	181.0506; 166.0268
Syringol-pent	7.41	285.0979	0.8	153.0557; 138.0321
Catechin-pent	7.52	421.1140	0.8	289.0718; 245.0805
Orcinol-pent	9.83	255.0874	6.0	123.0452; 108.0214
Ethylvanillin-pent	10.82	297.0979	0.6	165.0557; 136.0152
Hexose-hexose derivatives				
p-Carboxyphenol acid-hex-hex	5.26	461.1300	1.3	299.0772; 137.0244
Gallic acid-hex-hex	5.29	493.1198	0.7	331.0670; 169.0143
Pyrocatechol-hex-hex	5.31	433.1351	2.0	271.0823; 109.0295
Vanillic acid-hex-hex	5.37	491.1406	0.6	329.0878; 167.0350
Syringic acid-hex-hex	5.44	521.1511	0.4	359.0983; 197.0456
Caffeic acid-hex-hex	5.60	503.1406	−0.3	341.0878; 179.0350
Gentisic/protocatechuic acid-hex-hex	5.66	477.1249	0.9	315.0721; 153.0193
p-Coumaric acid-hex-hex	5.66	487.1457	0.5	325.0928; 163.0401
Salicylic acid-hex-hex	5.71	461.1300	0.9	299.0772; 137.0244
Homovanillic acid-hex-hex	5.74	505.1562	−0.4	343.1034; 181.0506
Hydroxytyrosol-hex-hex	5.85	477.1613	1.1	315.1085; 153.0557
Protocatechualdehyde-hex-hex	5.87	461.1300	1.2	299.0772; 137.0244
Ferulic acid-hex-hex	5.90	517.1562	0.6	355.1034; 193.0506
Catechin-hex-hex	7.01	613.1774	3.5	451.1245; 289.0718
Epicatechin-hex-hex	8.25	613.1774	3.8	451.1245; 289.0718
Isopropiosyringone-hex-hex	8.67	533.1875	0.1	371.1347; 209.0819
Syringaldehyde-hex-hex	10.44	505.1562	−0.4	343.1034; 181.0506
Sinapaldehyde-hex-hex	10.84	531.1719	2.3	369.1191; 207.0663
Hexose-pentose derivatives				
Vanillic acid-hex-pent	5.26	461.1300	1.1	329.0878; 167.0350
Gallic acid-hex-pent	5.56	463.1093	1.0	331.0670; 169.0143
Gentisic/protocatechuic acid-hex-pent	5.60	447.1144	0.9	315.0721; 153.0193
p-Coumaric acid-hex-pent	5.74	457.1351	1.0	325.0929; 163.0401
Pentose-hexose derivatives				

(continued on next page)

Table 5 (*continued*)

Compounds	RT (min)	$[M-H]^-$ (*m/z*)	Δ *m/z*	MS/MS fragments
Syringic acid-pent-hex	5.36	491.1406	1.3	329.0878; 197.0456
p-Carboxyphenol-pent-hex	5.46	431.1195	0.8	269.0666; 137.0244
Ferulic acid-pent-hex	5.66	487.1457	0.2	325.0928; 193.0506
Protocatechualdehyde/salicylic acid -pent-hex	5.96	431.1195	1.6	269.0666; 137.0244
Pentose-pentose derivatives				
Vanillic acid-pent-pent	5.53	431.1195	0.6	299.0772; 167.0350
Syringic acid-pent-pent	5.72	461.1300	0.4	329.0878; 197.0456

Note: hex = hexose; all = allose; pent = pentose.

acid, hydroxytyrosol, vanillic acid, gentisic acid, syringic acid, homovanillic acid, protocatechualdehyde, salicylic acid-glu and methyl vanillate. The Solaris variety wine was represented by tyrosol, caffeic acid, 4 vinylguaiacol, *p*-coumaric acid, ferulic acid, gentisic/protocatechuic acid, vanillic acid, homovanillic acid, syringic acid, protocatechualdehyde, methyl vanillate, ethyl vanillate and acetosyringone. Table 4 summarises the mean content and the standard deviations of targeted compounds detected in 90% of the grape samples of a single variety or in both the corresponding wines.

On comparing the total content of red hybrid grapes (N = 9) and the corresponding wines (N = 3), significant differences (Tukey's HSD test; $p < 0.05$) and compositional peculiarities in phenolic profiles were found for 4-methylcatechol, 4-vinylphenol, acetovanillone, aesculetin, hydroxybenzoic acids and their derivatives (gallic, gentisic/protocatechuic, homovanillic, *p*-carboxyphenol, salicylic, syringic, vanillic acid, protocatechualdehyde, syringaldehyde, and homovanillyl alcohol), hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric, and sinapic acid), ethyl/methyl vanillate, tryptophol and tyrosol, more abundant in wines, and for 4-ethylcatechol, epicatechin and vanillic acid-glucoside, more abundant in grapes. Since white wines from hybrids were produced without skin and seed contact, the phenolic content of pulp (N = 12) was compared with that of the corresponding wines (N = 4). Significant differences (Tukey's HSD test $p < 0.05$) were found for 4-vinylguaiacol, acetosyringone, hydroxybenzoic acids and their derivatives (gentisic, homovanillic, syringic, vanillic acid, and protocatechualdehyde), hydroxycinnamic acids (caffeic, ferulic, and *p*-coumaric acid), hydroxytyrosol, and tyrosol, detected in higher amounts in wines, and for 4-ethylcatechol and gallic acid, more abundant in pulp.

Those phenolic compounds always detected in greater amounts in wine were tyrosol, hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric), hydroxybenzoic acids (gentisic/protocatechuic, syringic, vanillic, homovanillic), and protocatechualdehyde. Indeed, tyrosol was produced by yeast during alcoholic fermentation (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), caffeic, ferulic and *p*-coumaric acids were released by hydrolysis from the corresponding hydroxycinnamic tartaric esters (García-Falcón, Pérez-Lamela, Matinez-Carballo, & Simal-Gándara, 2007), hydroxybenzoic acids increased (Tian et al., 2009), and vanillic acid can be produced from vanillin oxidation.

Finally, the mean content of simple phenols in the wines produced from hybrid grapes was in agreement with the data previously reported by the authors for European varieties (Barnaba et al., 2015; Barnaba et al., 2016).

3.2.3. Untargeted approach

The untargeted approach made it possible to tentatively identify 79 glycosilated simple phenols, 51 of which were in the form of monosaccharides (30 as –hexose and 21 as –pentose derivatives), and 28 in the form of disaccharides (17 as –hexose-hexose, 4 as hexose-pentose, 5 as pentose-hexose and 2 as pentose-pentose derivatives), previously described only for restricted classes of compounds (e.g. flavonols, Yang et al., 2014, or anthocyanidins, De Rosso et al., 2012).

Compounds that do not have any isobaric correspondence (Table 5) were univocally identified. Compounds that have in common the parent ion but not the ion corresponding to $[M-H-S]^-$ (*p*-hydroxybenzaldehyde-all/vanillin-pent, 4-methylcatechol-/orcinol-hex/hydroxytyrosol-/syringol-pent, *p*-carboxyphenol-/protocatechualdehyde-salicylic acid-hex/vanillic acid-pent, vanillin-hex/homovanillic acid-pent, *p*-coumaric acid-hex/ferulic acid-pent, vanillic acid-hex/syringic acid-pent, *p*-carboxyphenol-/protocatechualdehyde-/salicylic acid-hex-hex/vanillic acid-hex-pent/syringic acid-pent-pent, *p*-coumaric acid-hex-hex/ferulic acid-pent-hex, vanillic acid-hex-hex/syringic acid-pent-hex, *p*-carboxyphenol-/protocatechualdehyde-/salicylic acid-pent-hex/vanillic acid-pent-pent; Table 5) were distinguished on the basis of the accurate mass of the corresponding aglycon, partially hydrolysated of one or more sugar units in HESI. This approach to identification was applied both for coeluted compounds and in the case of detection of different peaks for the same *m/z* value, basing the attribution of peaks on the similar shift in the retention time with the corresponding aglycons. Isobaric compounds that have in common both the parent ion and the aglycon (glycosidic derivatives of aceto/isoacetovanillone and gentisic/protocatechuic acid and protocatechualdehyde-salicylic acid-pent-hex) could not be distinguished and were reported as both present. Only in the case of detection of different peaks for the same *m/z* value was possible to distinguish isobaric compounds on the basis of retention times, tracing the elution order of the corresponding free forms (4-methylcatechol-/orcinol-hex, *p*-carboxyphenol-/protocatechualdehyde-salicylic acid-hex, acetovanillone-/ethylvanillin-hex, homovanillic acid-/methyl vanillate-/syringaldehyde-hex, ethyl vanillate-/isoacetosyringone-hex, catechin-/epicatechin-hex, guaiacol-/orcinol-pent, *p*-carboxyphenol-/protocatechualdehyde-salicylic acid-pent, hydroxytyrosol-/syringol-pent, *p*-carboxyphenol-/protocatechualdehyde-salicylic acid-hex-hex, homovanillic acid-/syringaldehyde-hex-hex, catechin-/epicatechin-hex-hex, *p*-carboxyphenol-/protocatechualdehyde-salicylic acid-pent-hex; Table 5).

Furthermore, -hexose-pentose and -pentose-hexose derivatives could be distinguished on the basis of their fragmentation profile, considering in particular the different *m/z* value of the ion corresponding to the loss of one sugar unit (e.g. 325.0929 and 285.0823 *m/z*, for *p*-coumaric acid-hexose-pentose and *p*-coumaric acid-pentose-hexose respectively; Table 5). Table 5 shows the retention time, the accurate mass $[M-H]^-$, the difference between expected and experimental masses (Δ *m/z*) and fragments of the tentatively identified phenolic precursors.

Fourty 9% of putatively identified compounds were detected in at least 90% of pulp samples of at least one variety, 71% in skin samples, 79% in seed samples and 55% in wine. Specifically, caffeic acid-hex, ferulic acid-hex, gallic acid-hex, gentisic/protocatechuic acid-hex, hydroxytyrosol-hex, *p*-coumaric acid-hex and ferulic acid-pent were tentatively detected in all samples of the pulp, skin, seed and wine (Table 6). Syringol-pent was detected in all samples of the pulp, skin and seed; aceto-/isoacetovanillone-hex, gentisic/protocatechuic acid-hex-hex, *p*-carboxyphenol-hex, homovanillic acid-pent, gallic acid-hex-hex, and syringic acid-pent-pent were detected in all samples of the skin and seed; protocatechualdeihyde-hex was detected in all samples of the skin, seed and wine; isopropiosiringone-hex

Table 6
Presence of glycosidically bound simple phenols in the pulp, skin, seed and wine samples for each of the 6 varieties.

Compounds	Cabernet Cantor				Prior				Merlot			Muscaris				Solaris				Chardonnay		
	a	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*
Caffeic acid-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Ferulic acid-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Ferulic acid-pent	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Gallic acid-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Gentisic/protocatechuic acid-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Hydroxytyrosol-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
p-Coumaric acid-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Protocatechualdehyde-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	2	4	6	6	2	2	3	3
Salicylic acid-hex-hex	6	6	6	2	3	3	3	2	3	3	3	5	5	6	2	6	6	6	1	3	3	3
Gallic acid-hex-hex	6	6	6	2	3	3	3	2	3	3	3	4	6	6	1	6	6	6	2	2	3	3
p-Carboxyphenol-hex	6	6	6	2	2	3	3	–	3	3	3	6	6	6	2	6	6	6	2	1	3	3
Gentisic/protocatechuic acid-hex-hex	6	6	6	2	2	3	3	–	1	3	3	6	6	6	2	6	6	6	1	3	3	3
Syringic acid-pent-pent	6	6	6	2	3	3	3	2	3	3	3	5	5	6	–	6	6	6	–	3	3	3
Acetovanillone-hex	6	6	6	2	3	3	3	2	3	3	3	6	6	6	–	4	6	6	–	2	3	3
Homovanillic acid-pent	5	6	6	2	–	3	3	1	3	3	3	5	6	6	–	6	6	6	2	3	3	3
Salicylic acid-hex	1	1	6	1	–	2	3	1	1	–	3	6	6	6	2	6	6	6	2	3	3	3
Protocatechualdehyde-hex-hex	6	6	6	2	–	1	3	2	3	3	3	4	3	5	1	3	1	5	1	1	3	3
p-Coumaric acid-hex-hex	5	6	2	–	1	3	–	–	–	3	1	5	6	3	2	5	6	5	2	2	3	3
p-Hydroxybenzaldehyde-all	2	3	6	–	1	2	3	–	–	3	3	4	4	6	1	2	5	6	1	1	3	3
Vanillic acid-pent	–	–	5	–	–	–	3	–	–	–	3	6	6	6	2	6	6	6	2	1	3	3
Salicylic acid-pent-hex	6	6	6	–	–	2	3	–	–	2	3	1	5	6	–	1	5	5	1	–	3	3
Syringic acid-hex-hex	6	6	6	2	3	3	3	2	3	3	3	–	–	6	–	–	–	6	–	–	–	3
Gentisic/protecatechuic acid-pent	3	1	5	–	–	3	3	–	3	3	3	3	4	6	1	–	2	6	–	2	3	3
Vanillic acid-pent-pent	5	6	5	2	3	3	3	–	3	3	3	–	–	6	–	–	–	6	–	–	–	3
Syringic acid-pent	–	–	1	–	–	–	–	–	–	–	2	6	6	6	2	5	6	6	2	3	3	3
Vanillic acid-hex-hex	6	6	6	2	3	3	3	2	3	3	3	–	–	5	–	–	–	3	–	–	–	3
Gallic acid-hex-pent	–	4	6	–	3	3	3	2	–	3	3	–	2	6	–	1	2	6	1	–	2	3
Syringol-pent	6	6	6	2	3	3	3	1	3	3	3	6	6	6	2	6	6	6	2	3	3	3
Vanillic acid-hex	–	–	–	–	–	–	–	–	–	–	–	6	6	6	2	6	6	6	2	3	3	3
p-Hydroxybenzaldehyde-pent	2	2	6	–	2	–	3	–	1	2	3	3	3	6	–	2	3	6	–	1	–	3
Syringic acid-hex	–	–	–	–	–	–	–	–	–	–	–	6	6	6	1	6	6	6	2	3	3	3
Gallic acid-pent	1	4	6	–	1	3	3	–	2	3	2	1	4	5	–	2	2	5	–	–	2	1
Salicylic acid-pent	1	1	6	2	–	2	3	2	–	1	3	–	3	6	–	–	3	6	–	1	2	3
p-Carboxyphenol-pent	3	–	5	2	–	–	3	2	2	–	3	2	2	6	–	–	–	6	1	3	1	3
Salicylic acid/protocatechualdehyde-pent-hex	–	1	3	–	–	1	3	–	–	1	3	1	5	5	–	1	4	6	1	–	3	3
Ferulic acid-pent-hex	–	–	–	–	–	–	–	–	–	–	–	5	6	3	1	5	6	5	1	2	3	3
Gentisic/protocatechuic acid-hex-pent	–	4	–	1	3	3	3	2	–	2	–	–	4	3	–	3	4	4	–	–	3	1
Orcinol-hex	–	–	–	–	–	–	2	–	–	3	1	3	4	6	2	3	1	4	2	1	3	3
protocatechualdehyde-pent	1	1	6	2	–	2	3	1	–	3	3	–	–	6	–	–	–	6	–	–	1	3
Vanillin-hex	–	5	6	1	–	2	3	–	–	3	2	4	6	6	–	2	6	6	–	1	3	3
Ethylvanillin-hex	–	–	–	–	–	–		–	–	–	–	5	6	6	1	3	3	6	1	–	3	3
Isopropiosyringone-hex	–	6	–	2	–	3	–	2	–	3	–	1	6	–	2	–	6	–	2	–	3	–
Scopoletin-hex	–	–	6	1	1	1	3	1	–	2	3	4	5	–	–	2	3	2	–	–	2	–
Epicatechin-hex	1	1	6	2	–	–	3	2	–	1	3	–	–	6	–	–	–	6	–	–	–	3
Vanillin-pent	1	3	6	–	1	2	3	–	–	3	3	4	3	6	–	2	5	6	1	–	3	3
Isopropiovanillone-hex	–	–	–	–	–	–	–	–	–	–	–	6	6	2	–	4	5	1	–	3	3	2
p-Coumaric acid-hex-pent	1	–	3	–	–	3	3	–	–	–	–	1	2	3	–	–	6	6	–	–	1	3
Syringaldehyde-pent	3	5	6	2	–	2	2	2	2	3	3	–	–	–	–	–	–	–	–	–	–	–
Catechin-hex	–	–	6	2	–	–	3	–	–	–	3	–	–	6	–	–	–	6	–	–	–	3
Catechin-pent	–	–	6	–	–	–	3	–	–	–	3	–	–	6	–	–	–	6	–	–	–	3
p-Coumaric acid-pent	–	–	6	–	–	–	3	–	–	–	3	–	–	6	–	–	–	6	–	–	–	3
Catechin-hex-hex	–	–	6	–	–	–	2	–	–	–	3	–	–	6	–	–	–	6	–	–	–	3
Guaiacol-pent	–	1	–	1	–	1	1	–	–	–	1	1	2	6	–	2	1	6	–	–	–	3
Hydroxytyrosol-pent	–	–	5	–	–	–	3	1	–	–	2	–	–	6	–	–	–	6	–	–	–	3
Syringaldehyde-hex	–	–	–	–	–	–	–	–	–	–	–	1	2	6	–	–	4	6	1	–	3	3
p-Carboxyphenol-hex-hex	1	–	5	–	–	–	3	–	–	1	3	–	–	5	–	–	–	4	–	–	–	3
p-Carboxyphenol-pent-hex	–	–	6	–	–	1	3	–	–	–	3	–	–	6	–	–	–	3	–	–	–	3
Caffeic acid-hex-hex	–	–	–	–	–	–	–	–	–	–	–	1	5	–	–	4	6	2	2	1	3	–
Pyrocatechol-pent	–	–	5	–	–	–	3	–	–	–	3	–	–	6	–	–	–	5	–	–	–	2
Epicatechin-hex-hex	–	–	6	–	–	–	3	–	–	–	2	–	–	3	–	–	–	6	–	–	–	–
Pyrocatechol-hex	1	4	4	–	–	1	3	–	2	3	3	–	–	–	–	–	–	–	–	–	–	–
Vanillic acid-hex-pent	1	1	2	–	–	–	1	–	–	2	2	–	–	5	–	–	–	4	–	–	–	3
Syringaldehyde-hex-hex	–	1	6	–	1	3	3	–	–	–	2	–	–	–	–	–	4	–	–	–	–	–
Ethylvanillin-pent	2	1	–	–	–	2	–	1	–	–	–	2	5	–	2	–	1	–	2	–	–	–
Homovanillic acid-hex-hex	–	–	–	–	–	–	–	–	–	–	–	–	5	1	–	–	5	2	–	1	3	1
Syringic acid-pent-hex	–	–	3	–	–	–	3	–	–	–	3	–	–	2	–	–	–	3	–	–	–	3
Ethyl vanillate-hex	–	–	–	2	1	3	3	2	–	1	3	–	–	–	–	–	–	–	–	–	–	–
Caffeic acid-pent	–	–	–	–	–	–	–	–	–	–	–	–	–	6	–	–	–	6	–	–	–	3
Coniferaldehyde-hex	–	6	–	–	–	3	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–
Ferulic acid-hex-hex	–	–	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–	–	3
Hydroxytyrosol-hex-hex	1	–	–	2	–	3	–	1	–	3	–	–	1	–	–	–	–	–	–	–	1	–
Isoacetosyringone-hex	–	1	5	1	–	–	1	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–
Orcinol-pent	–	–	–	2	1	–	–	2	3	1	–	–	–	–	–	–	–	–	–	–	–	–
Homovanillic acid-hex	–	–	–	2	–	–	–	2	–	–	–	–	–	1	–	–	–	–	2	–	–	–

(continued on next page)

Table 6 (*continued*)

Compounds	Cabernet Cantor				Prior				Merlot			Muscaris				Solaris				Chardonnay		
	a	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*a*	*b*	*c*	*d*	*a*	*b*	*c*	*d*	*a*	*b*	*c*
Methyl vanillate-hex	–	–	–	–	–	1	3	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–
Pyrocatechol-hex-hex	–	–	1	–	–	–	1	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–
4-Methylcatechol-hex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	2	–	–	–
Sinapaldehyde-hex-hex	–	–	–	2	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isopropiosyringone-hex-hex	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–

Note: hex = hexose; pent = pentose; all = allose; *a* = pulp; *b* = skin; *c* = seed; *d* = wine. Cabernet Cantor grape (N = 6), wine (N = 2); Prior grape (3), wine (1); Merlot grape (3); Muscaris grape (6), wine (2); Solaris grape (6), wine (2); Chardonnay grape (3); – = not detected.

was detected in all samples of the skin and wine; *p*-coumaric acid-hex-hex was detected in all samples of the skin; catechin-hex, epicatechin-hex, salicylic acid-hex, *p*-hydroxybenzaldehyde-all, catechin-pent, *p*-hydroxybenzaldehyde-pent, *p*-coumaric acid-pent, protocatechualdehyde-pent, salicylic acid-pent, vanillin-pent, syringic acid-hex-hex and gallic acid-hex-pent were detected in all samples of the seed. Coniferaldehyde-hex, homovanillic acid-hex-hex and isopropiosiringone-hex-hex were detected only in the skin samples; isoacetosiringone-hex, methyl vanillate-hex, caffeic acid-pent, hydroxytyrosol-pent, pyrocatechol-pent, catechin-hex-hex, epicatechin-hex-hex, ferulic acid-hex-hex, pyrocatechol-hex-hex, vanillic acid-hex-pent and syringic acid-pent-hex were detected only in the seed samples; 4-methylcatechol-hex, catechin-hex, homovanillic acid-hex and sinapaldehyde-hex-hex were detected only in the wine samples. Isopropiovanillone-hex, gallic acid-pent, *p*-hydroxybenzaldehyde-pent, vanillin-pent, ferulic acid-pent-hex and protocatechualdehyde/salicylic acid-pent-hex were detected in the grape samples but never in the wines, probably since they were hydrolysed during winemaking.

Vanillic acid-hex, syringic acid-hex, ferulic acid-pent-hex, ethylvanillin-hex and isopropiovanillone-hex were detected in almost all samples of the pulp, skin and seed of white berry grapes, while they were never detected in samples of red berry grapes. Syringic acid-hex-hex, vanillic acid-pent-pent and vanillic acid-hex-hex were detected in almost all samples of the pulp, skin, seed and wine of red berry varieties, while for white berry varieties they were detected only in the seed samples. Syringaldehyde-pent was detected in many samples of the pulp, skin, seed and wine of red berry varieties but never detected in white berries, and coniferaldehyde-hex, never detected in white berry varieties, was detected only in red berry skins. Finally, no differences were highlighted in glycosidically bound simple phenol distribution in the 6 selected varieties, nor between hybrid and European ones.

Due to lack of information about glycosidically bound simple phenols in grapes, it was only possible to confirm the data reported for European grapes in hybrids (Perestrelo et al., 2012; Di Lecce et al., 2014) for gallic acid-hex, gentisic/protocatechuic acid-hex, *p*-coumaric acid-hex and *p*-carboxyphenol-hex. The glycosylated phenolic profiles of wines from hybrids corresponded with the 70% of those reported by Barnaba et al. (2016) for European wines, although in the former the number of detected glycosylated phenolic compounds was more than twice as high, possibly due to hydrolysis phenomena occurring during the ageing of the latter (Primitivo and Negroamaro wines).

Table 6 summarises the glycosidically bound simple phenols tentatively identified in the pulp, skin, seed and wines of the 6 selected varieties.

4. Conclusion

The high resolution mass approach furnished a detailed description of the distribution of free and glycosidically bound phenols in the pulp, skin and seed of 4 hybrid and 2 European grape varieties and the phenolic profiles of wines produced from hybrid varieties.

Through the high selectivity of high resolution mass spectrometry and the efficiency of SPE pre-treatment in reducing matrix interference, it was possible to quantify 60 simple phenols, 7 of which were glycosidic precursors. Besides, and up to our knowledge, 79 glycosidically bound simple phenols were tentatively identified for the first time in the selected matrices. In particular, using accurate mass, isotopic pattern matching and the presence of specific fragmentation profiles, it was also possible to distinguish between hexose, pentose, hexose-hexose, hexose-pentose and pentose-hexose derivatives.

In our experimental conditions, the content of targeted simple phenols provided individual descriptions for different hybrid grape varieties when compared to European ones, allowing to investigate the accumulation of these compounds in the different parts of berry. As regards the untargeted approach, characterization of the berry fractions and wine was based on the ionisation profile due to the reduced presence of commercially available standards. In brief, the present study opens up new opportunities for detailed description of phenolic profiles and investigation of grape phenolic composition.

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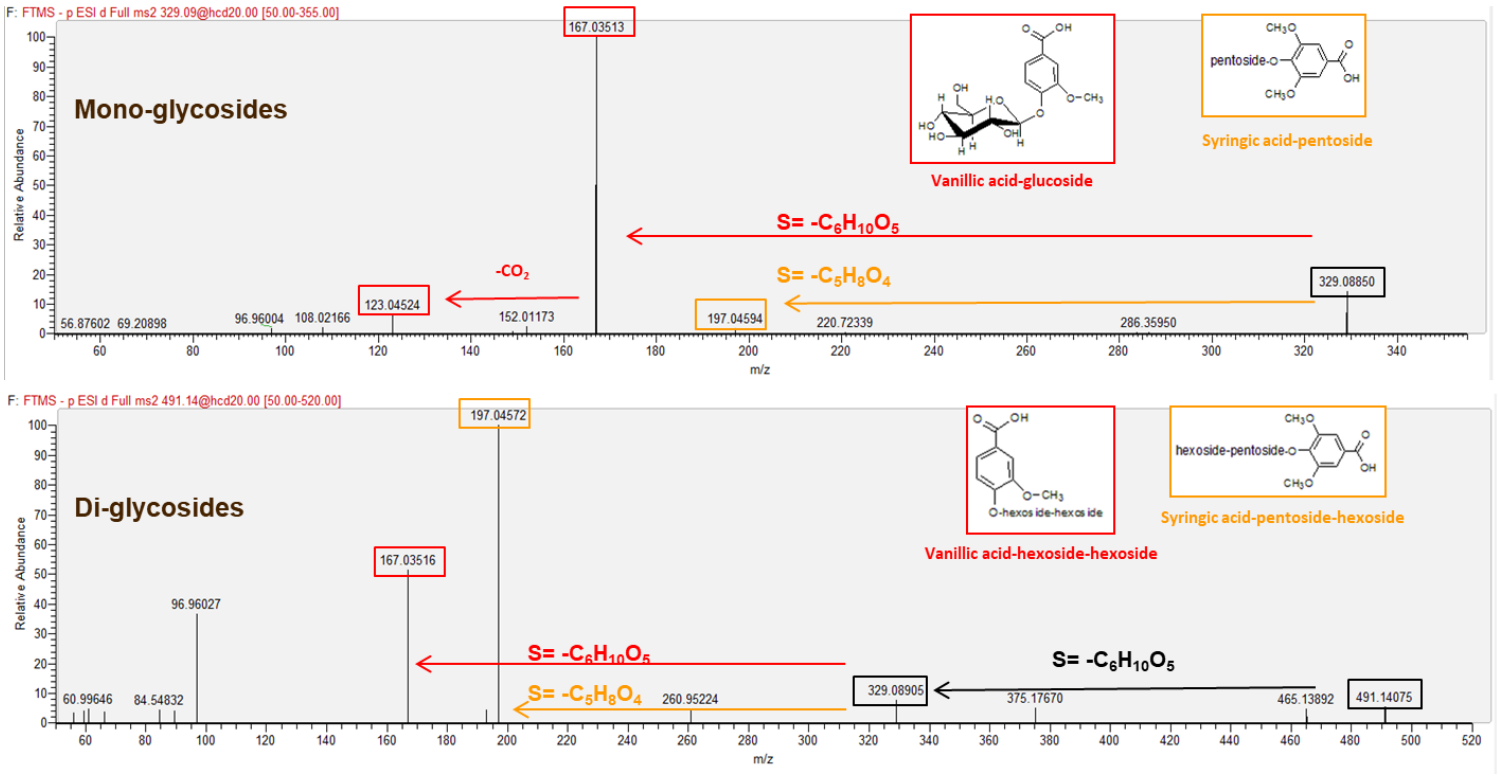
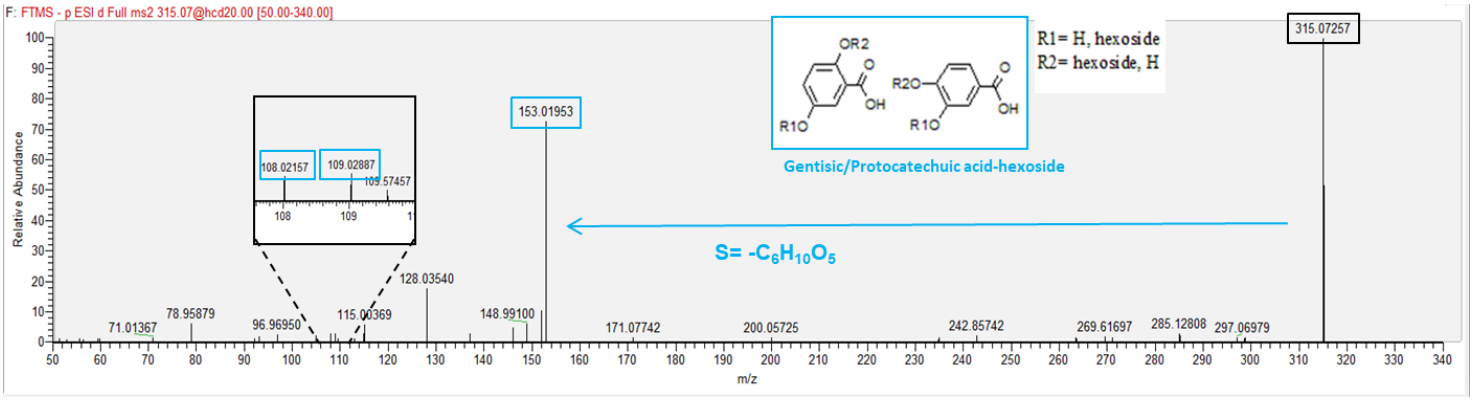
Isobaric parent ions different for fragments

(example of coeluted peak)



Supplementary Fig. 1: Example of distinction of isobaric compounds through their different typical fragmentation spectrum.

Isobaric parent ions indistinguishable



Supplementary Fig. 2: Example of the impossibility to distinguish isobaric compounds through their fragmentation spectrum, if parent and product ion are all isobaric.

Conclusion

In this work, the suspect screening approach developed proved to be an efficient tool for tentative identification of low-molecular-weight phenolic glycosides. Indeed, evaluation of the mass behavior of commercially available standards confirmed the possibility of basing tentative identification of suspect phenolic glycosides on the matching of accurate masses, experimental fragmentation and experimental isotope patterns with data reported in the literature or theoretically surmised. However, the greatest limit of this approach was the impossibility of tentatively identifying low-molecular-weight phenolic glycosides, which completely lost the sugar moieties in the source, since it was impossible to distinguish the source-released aglycone from that already occurring in the free form in the matrix in the absence of an analytical standard.

As regards sample pretreatment, solid samples (skin, pulp and seeds) underwent solvent extraction, paying attention to prevent sample fermentation and analyte oxidation or hydrolysis. The use of an online SPE clean-up procedure ensured the elimination of matrix interference and allowed the analysis of different matrices, such as berry fractions and red and white wines.

On comparing the free and glycosylated low-molecular-weight phenolic profile of berry fractions (pulp, skin and seed) with those of wines produced with the same grapes of selected hybrid varieties, it emerged that the possibility of detecting compounds already detected in grapes as a consequence of plant biosynthesis in wine, depends closely on the vinification process. Indeed, several phenols detected only in the pulp and seed can also be detected in wines, but only in the event of skin contact maceration. In contrast, compounds detected in larger amounts in wine were related to yeast production during alcoholic fermentation or to oxidation reactions.

SECTION 2.2.4.

Glycosylated simple phenolic profiling of food tannins using high resolution mass spectrometry (Q-Orbitrap)

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Aim of work

Tannins are polyphenolic compounds extensively present in plants and used by the food industry as processing aids (Codex Alimentarius, 2014). They are extracted from different botanical sources and are authorized by the International Organization of Vine and Wine (OIV) as clarifiers of musts and wines due to their affinity for binding proteins. In the last few years, different approaches aiming at correctly identifying the botanical origin of tannins have been developed, in order to satisfy the industry's request to verify product labels. However, despite the wide botanical variability of tannins, there is little information about their glycosylated phenolic profile.

Starting from recent evidence about the role of free low-molecular-weight phenols in distinguishing the origin of tannins (Malacarne *et al.*, 2016), this work aimed to characterize the profile of low-molecular-weight phenolic glycosides in tannins of different botanical origin, in order to investigate the potential of glycosylated phenols as effective markers for tannin traceability.

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Glycosylated simple phenolic profiling of food tannins using high resolution mass spectrometry (Q-Orbitrap)

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ABSTRACT

Tannins are polyphenolic compounds extensively present in plants and used by food industry as processing aids. Due to the heterogeneity of plant sources, actions involved in food processing and tannin commercial costs can be different. In the last years different approaches aimed at correctly identifying the tannin botanical origin have been developed, in order to satisfy the industry's request to verify product labels. This work aimed to define the glycosidic simple phenolic profile of a large selection of monovarietal commercial tannins of different origin, using a high-resolution untargeted approach. Using accurate mass, isotopic pattern and MS/MS fragmentation, 167 precursors, 89 as monoglycosylated and 78 as diglycosylated derivatives were tentatively identified in tannins, validating the untargeted approach with 3 custom-synthesized glycosidic precursors. Almost all tannin botanical varieties were shown to be characterised by a specific glycosylated phenolic profile, providing possible tools for tannin classification in the case of glycosylphenol standard availability.

1. Introduction

Tannins are polyphenolic compounds widespread in the plant kingdom and characterised by extensive structural heterogeneity, as reflected by a molecular weight ranging between 500 and 20,000 Da (Bate-Smith & Swain, 1962). They are found in vegetables (Haslam, Lilley, Cai, Martin, & Magnolato, 1989), fruits (Foo & Porter, 1981), legumes (Salunkhe, Chavan, & Kadam, 1990) or plant-derived beverages (Haslam, 2007; Kennedy & Jones, 2001; Luque-Rodríguez, Luque de Castro, & Pérez-Juan, 2007), such as tea, coffee or wine.

Tannins, also employed in leather production and mining activities, are principally used as processing aids in the food industry (Codex Alimentarius, 2014). The European Union authorises the addiction of tannins as food flavourings (EC No 1334/2008, EU Regulation No. 872/12), while the International Organization of Vine and Wine (OIV) restricts their use as an adjuvant for protein binding and precipitation in must and wine. Considering the wide variability of tannin sources, their chemical structure and resulting properties, and in particular the cost of tannins, the industry needs tools able to distinguish between commercial tannins of different origin, in order to verify the declarations of suppliers. Nowadays, different approaches are available to characterise tannins from different botanical sources, based on specific UV–vis, FT-IR, NMR or MS/MS spectra (Obreque-Slíer, Peña-Neira, López-Solís, Ramírez-Escudero, & Zamora-Marín, 2009; Salagoity-Auguste, Tricard, Marsal, & Sudraud, 1986; Laghi et al., 2010), proanthocyanidin (Vivas et al., 2004), minor sugars and phenol content (digallic acid, scopoletin, eugenol, 2-phenylethanol, vanillin and syringaldehyde; Malacarne, Nardin, Bertoldi, Nicolini, & Larcher, 2016), the polyalcohol and monosaccharide profile (e.g. quercitol, pinitol, myo-inositol, arabitol, muco-inositol, chiro-inositol, bornesitol; Alañón, Díaz-Maroto, Díaz-Maroto, Vila-Lameiro, & Pérez-Coello, 2011; Sanz, Martínez-Castro, & Moreno-Arribas, 2008), the mineral profile and the $^{12}C/^{13}C$ isotope ratio (Bertoldi et al., 2014).

In the last few years, the field of food analysis has made continuous progress improving food safety and quality, implementing control of all stages of food production, processing and distribution. Furthermore, the increasing number of foodomics studies based on untargeted methods shows that this approach is considered by scientists to be efficient in evaluating food safety and quality (Hjelmeland, Zweigenbaum, & Ebeler, 2015; Ibáñez, Simó, García-Cañas, Acunha, & Cifuentes, 2015; Barnaba, Nardin, Pierotti, Malacarne, & Larcher, 2017; Gil-Solsona et al., 2016).

Considering the role played by simple phenols in distinguishing the origin of tannin (Malacarne et al., 2016), this work aimed to characterise the glycosylated simple phenolic profile of a large selection of tannins from 16 botanical sources, in order to investigate the potential of glycosylated phenols to act as effective markers for tannin traceability. This was investigated using an untargeted approach, performed with ultra-high-performance liquid chromatography (UHPLC) coupled to hybrid quadrupole/high-resolution mass spectrometry (Q-Orbitrap).

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Table 1
Range of retention times (minimum–maximum, in minutes) for the glycosylated simple phenols tentatively identified in 73 tannins of different botanical origin. Aglycon retention times (minimum–maximum, in minutes) refer to available standards. The exact masses of aglycon's and sugar residues are reported.

A			H precursors [*m/z* A + 162.05282]	HH precursors [*m/z* A + 324.10560]	HP precursors [*m/z* A + 294.09500]	P precursors [*m/z* A + 132.04225]	PP precursors [*m/z* A + 264.08450]
	m/z	RT min–max (min)	RT min–max (min)	RT min–max (min)	RT min–max (min)	RT min–max (min)	RT min–max (min)
gallic acid[(b)]	169.01425	5.4–5.5	5.3–5.5	5.2–5.4	5.1*–5.8*	5.2–5.7	5.2–5.5
gentisic/protocatechuic acid[(a)]	153.01933	5.5–5.7	5.3–5.6	5.1–5.5	5.3*–6.0*	5.4–6.0	5.2–56.9
p-carboxyphenol[(a)]	137.02442	5.7–5.9	5.2–5.4	5.2–5.8	5.5*–6.3*	5.2–5.5	5.5–5.8
vanillic acid[(a)]	167.03498	5.9–6.5	5.2–5.6	5.1–5.5	5.2*–6.0*	5.5–6.0	5.8–6.4
caffeic acid[(a)]	179.03498	6.0–6.5	5.6–6.0	5.5–5.8	5.8*–6.4*	5.5–6.0	–
homovanillic acid[(b)]	181.05063	6.0–6.9	5.9–6.3	5.9–6.9	5.6*–6.0*	5.8–6.3	–
hydroxytyrosol[(e)]	153.05572	5.9–6.3	5.6–6.1	5.7–5.9	5.6*–5.7*	5.6–6.0	–
protocatechualdehyde[(b)]	137.02442	6.0–6.9	5.6–6.3	–	–	5.6–6.1	–
syringic acid[(a)]	197.04555	6.1–6.6	5.3–5.8	5.1–5.4	5.2*–5.4*	5.2–5.7	5.5–5.8
p-coumaric acid[(b)]	163.04007	6.3–7.4	5.7–5.9	5.6–7.0	5.8*–6.0*	5.6–6.4	–
salicylic acid[(g)]	137.02442	6.7–7.8	5.5–5.9	–	–	5.5–5.8	–
pyrocatecol[(b)]	109.02950	6.9–7.5	7.2–7.5	6.1–6.9	–	–	–
tyrosol[(b)]	137.06080	7.2–7.5	6.8–7.2	6.5–6.8	6.1*–6.5*	6.2–6.5	–
ferulic acid[(a)]	193.05063	7.3–7.5	5.7–5.9	–	5.6*–5.8*	5.7–5.9	–
catechin[(a)]	289.07176	7.6–8.0	5.6–6.0	–	–	5.7–6.5	–
phenol[(b)]	93.03459	7.6–7.8	6.5–6.6	–	–	6.7–7.0	–
sinapinic acid[(a)]	223.06120	7.6–8.8	7.1–7.5	–	–	–	–
aesculetin[(b)]	177.01933	8.1–8.4	6.4–6.9	–	5.6*–6.0*	5.5–6.0	–
homovanillyl alcohol[(b)]	167.07137	8.5–8.9	7.8–8.1	7.7–7.9	–	6.8–7.1	6.8–7.5
4-hydroxybenzaldehyde[(b)]	283.08233	8.5–8.8	5.9–6.3	–	6.2*–6.4*	6.5–7.0	–
orcinol[(b)]	123.04515	8.5–8.9	6.6–6.8	–	–	5.7–6.0	–
epicatechin[(b)]	289.07176	9.5–9.7	7.9–8.5	–	–	8.7–9.7	–
vanillin[(b)]	151.04007	9.7–9.9	5.5–5.9	5.3–5.9	5.6*–6.0*	5.7–6.1	–
ellagic acid[(a)]	300.99890	9.7–10.2	8.5–8.8	–	–	9.0–9.5	–
coniferyl alcohol[(b)]	179.07137	9.9–10.4	–	–	–	8.6–9.6	–
4-methylcatechol[(a)]	123.04515	10.1–10.3	6.9–7.5	–	–	7.3–7.5	6.7–7.0
syringaldehyde[(d)]	181.05063	10.3–10.5	10.0–10.8	–	–	6.1–7.0	–
aceto-/isoacetovanillone[(b)]	165.05572	10.5–10.8	7.9–8.6	8.4–8.6	–	6.2–6.7	–
isopropiovanillone[(b)]	179.07137	10.5–10.9	8.5–9.2	9.1–10.0	9.0*–10.0*	9.5–10.3	9.6–10.3
scopoletin[(a)]	191.03498	10.6–10.8	8.4–8.9	–	–	8.9–9.9	–
isopropiosyringone[(f)]	209.08193	10.7–10.9	8.4–8.8	8.6–8.7	–	7.4–7.6	–
acetosyringone[(b)]	195.06628	10.8–11.1	8.2–8.7	–	–	5,91–6,31	–
isoacetosyringone[(f)]	195.06628	11.1–11.3	8.2–8.9	–	–	6.8–7.4	–
syringol[(b)]	153.05572	11.3–11.5	8.9–9.2	8,28–8,91	8.0*–8.8*	7.2–7.6	8.4–8.9
coniferylaldehyde[(b)]	177.05572	11.4–11.8	11.1–11.5	11.2–11.4	–	11.2–11.7	–
ethylvanillin[(a)]	165.05572	11.4–11.8	8.2–8.6	–	–	10.3–11.1	–
sinapinaldehyde[(b)]	207.06628	11.6–11.8	10.7–10.9	–	–	10.8–11.2	–
tryptophol[(a)]	160.07679	11.8–12.2	–	–	–	–	–
o-vanillin[(b)]	151.04007	12.0–12.2	9.8–10.3	–	9.1**–9.4**	10.2–10.5	–
methyl vanillate[(d)]	181.05063	12.0–12.6	11.2–11.3	–	–	8.2–8.5	–
4-ethylcatechol[(c)]	137.06080	12.1–12.3	–	–	–	12.0–12.3	11.8–12.5
vanillyl ethyl ether[(f)]	181.08702	12.4–12.6	10.3–10.7	–	9.8*–10.1*	9.7–10.1	–
(*m* + *p*)-cresol[(b)]	107.05024	12.5–12.9	–	–	11.9–11.9	–	–
guaiacol[(b)]	123.04515	12.6–12.8	–	9.5–10.1	9.6*–10.3*	9.4–10.2	–
4-methylsyringol[(d)]	167.07137	12.8–13.0	–	–	–	9.1–9.7	9.1–9.7
3.4-xylenol[(a)]	121.06580	13.6–13.8	–	–	–	13.0–13.2	–
4-vinylguaiacol[(d)]	149.06080	13.5–13.9	13.4–13.6	13.2–13.6	–	13.2–13.8	–
4-vinylphenol[(d)]	119.05024	13.5–13.7	–	–	–	–	13.2–13.3
ethyl vanillate[(b)]	195.06628	13.7–13.7	11.1–11.5	–	–	8,17–8,54	–
4-ethylphenol[(a)]	121.06589	14.0–14.9	13.6–14.3	13.8–14.4	13.5*–14.3*	13.6–14.1	13.7–13.7
4-methylguaiacol[(b)]	137.06080	14.2–14.4	–	–	–	–	–
4-ethylguaiacol[(d)]	151.07645	14.4–14.6	13.5–14.3	13.5–14.3	13.8*–14.4*	13.6–14.7	–
4-allyl syringol[(b)]	193.08702	14.8–15.0	–	–	–	–	–
eugenol[(a)]/isoeugenol[(b)]	163.07645	15.0–15.2	14.7–15.4	15.0–15.5	–	14.6–15.4	–

Note: (a) = Fluka (St. Louis, MO, USA); (b) = Sigma Aldrich (St. Louis, MO, USA); (c) = Alfa Aesar (Karlsruhe, Germany); (d) = SAFC (St. Louis, MO, USA); (e) = CHEMOS GmbH (Regenstauf, Germany); (f) = TransMIT (Gießen, Germany); (g) = Aldrich (St. Louis, MO, USA); (h) = PhytoLab GmbH & Co. KG Vestenbergsgreuth, Germany). A. = aglycon; H = hexose; P = pentose; – as not detected; * as compounds (coeluted) detected in the form of both hexose-pentose and pentose-hexose derivatives; ** as compound detected in the form of pentose-hexose derivatives.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade acetonitrile (ACN, 99.9%), LC-MS grade methanol (MeOH, 99.9%) and MS grade formic acid (98%) were purchased from Fluka (St. Louis, MO, USA), while acetic acid (99%) and *p*-nitrophenol (99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Aesculetin-glucoside (aesculetin-6-*O*-β-D-glucoside, 98%), acetovanillone-glucoside (acetovanillone-4-*O*-β-D-glucoside, 99%), scopoletin-glucoside (scopoletin-7-*O*-β-D-glucoside, 99%) and vanillic acid-glucoside (vanillic acid-4-*O*-β-D-glucoside, 99%) were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany), while salicylic acid-glucoside (salicylic acid-2-*O*-β-D-glucoside, 98%), orcinol-

glucoside (98%) and *p*-hydroxybenzaldehyde-allopyranoside (4-formylphenyl beta-D-allopyranoside, 98%) were custom synthesized and supplied by TransMIT (Giessen, Germany). The suppliers of phenols in aglyconic form are summarised in Table 1. Deionised water was produced using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Water-methanol stock solutions of each glycosylated phenol were prepared by adding L-glutathione reduced (99%; Sigma Aldrich St. Louis, MO, USA) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol, 99.5%; Fluka, St. Louis, MO, USA) as antioxidant agents (2.5 g kg^{-1} each). Stock solutions of aglycons were prepared as reported by Barnaba and colleagues (Barnaba et al., 2015). All stock solutions were stored at −4 °C.

Instrument mass calibration was performed using a standard mixture of sodium dodecyl sulfate and sodium taurocholate ($[M\text{-}H]^-$ *m/z* 265.14690 and *m/z* 514.28440 respectively; Pierce® ESI Negative Ion Calibration Solution, Rockford, IL, USA), with the addition of formic and acetic acids (formiate dimer [M_2 + Na-2H] *m/z* 112.98560, acetate $[M\text{-}H]^-$ *m/z* 59.01385).

2.2. Sample preparation

Seventy-three samples of monovarietal tannins, whose botanical origin was confirmed using official OIV methods (COEI-1-TANINS:2015), were collected from the Italian market from commercial retailers and wineries: oak (N = 11), whole grape (9), grape skin (9), grape seed (6), chestnut (6), quebracho (6), gallnut (6), green tea (4), fruit tree (3), acacia (3), grape marc (3), citrus (2), tara (2), tea (1), mimosa (1) and blueberry (1). Oak tannins were supplied by Enologica Vason (EV; Verona, Italy) (N = 5), Tecnofood Italia (TI; Pavia, Italy) (4), Collis–Veneto Wine Group (CVW; Verona, Italy) (1) and Corimpex Service (CS; Gorizia, Italy) (1). Whole grape tannins were supplied by EV (N = 3), Ever (Venice, Italy) (2), Laffort Italia (Alessandria, Italy) (1), Lamothe-Abiet (Bordeaux, France) (1), Institut Oenologique de champagne (Épernay, France) (1) and Erbsloeh (Geisenheim, Germany) (1). Grape skin tannins were supplied by CRC Biotek (Orvieto, Italy) (N = 2), Enartis (EN; Novara, Italy), (2), Perdomini-IOC (IOC; Verona, Italy) (1), Ferrari (Trento, Italy) (1), CVW (1), TI (1) and CS (1), while grape seed tannins were provided by EV (N = 3), CVW (2) and G&B Italiana (Pordenone, Italy) (1). Chestnut tannins were supplied by TI (N = 4), IOC (1) and Figli di Guido Lapi (FGL; Pisa, Italy) (1), while quebracho tannins were provided by FGL (N = 2), TI (1), CVW (1), EV (1) and Oenobiotech (Paris, France) (1). Gallnut tannins were supplied by TI (N = 3) and CVW (3); green tea tannins by TI (N = 2), EV (1) and IOC (1), while fruit tree tannins were supplied by TI (N = 1), CS (1) and EV (1). Acacia (N = 3) and grape marc (N = 3) tannins were supplied by TI, and citrus tannins by Vinoblesse (Baarn, Hollande) (N = 1) and IOC (1). Tara tannins were supplied by TI (N = 1) and FGL (1), tea (1) and mimosa (1) tannins by TI, and blueberry tannin by Laboratorio Basel (Lanùs Oeste, Argentina) (1).

Each sample (125 mg) was dissolved in 50 mL of hydro alcoholic solution (water–ethanol 10%, v/v), filtered with 0.45 µm PTFE filter cartridges (Sartorius AG, Goettingen, Germany) and added of the internal standard (*p*-nitrophenol, 500 µg kg^{-1}).

2.3. Analytical conditions

The glycosylated phenolic profile of tannins was achieved by adapting the method proposed by Barnaba and colleagues (Barnaba et al., 2016). A Thermo Ultimate R3000 ultra-high performance liquid chromatograph (Thermo Scientific, Sunnyvale, CA, USA), furnished with a Rheodyne 6-port automated switching valve, and a high-resolution tandem mass spectrometer (Q-Exactive™; Thermo Scientific, Bremen, Germany), equipped with a heated electrospray source (HESI-II) were used.

Matrix interference was reduced with on-line SPE (HyperSep™ Retain PEP SPE cartridge; 3.0 mm × 10 mm, 40–60 µm, Thermo Scientific, Sunnyvale, CA, USA) using deionised water for 4 min at a flow rate of 0.250 mL min^{-1}. Analytical separation (Acquity UPLC BEH C18 column; 2.1 mm × 100 mm, 1.7 µm particle size; Waters, Milford, MA, USA) was performed in water-acetonitrile at a flow rate of 0.300 mL min^{-1}. The gradient of the organic solvent was set as follows: 4.0–5.5 min, isocratic elution at 5% of ACN; 5.5–17 min, linear ramp to 60%; 17.0–17.5 min, linear ramp to 100%; 17.5–18.5, isocratic elution at 100%; 18.5–22.0 min, isocratic equilibration at 5%.

High-resolution mass analysis was performed by acquiring mass spectra in negative ion mode through a full MS-data dependent MS/MS experiment (full MS–dd MS/MS). The mass resolving power was set at 140,000 full width at half-maximum (FWHM) for full MS, and at 17,500 FWHM for dd MS/MS. The HESI source operated by setting the spray voltage to 2.80 kV and the capillary temperature at 310 °C.

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software timed and controlled the injection system, switching valve and chromatographic gradient, while Thermo Fisher Scientific TraceFinder™ software (Thermo Scientific, San Jose, CA, USA) was used for data processing and evaluation.

2.4. Untargeted putative identification of glycosylated simple phenols

Glycosylated precursors of the 54 simple phenol aglycons reported in Table 1, in the forms of monosaccharidic (hexoside and pentoside) and disaccharidic (hexoside-hexoside, pentoside-hexoside, hexoside-pentoside and pentoside-pentoside) derivatives, were investigated in tannins.

The tentative identification approach used for glycosylated compounds was inferred from the experimental mass fragmentation behaviour of the four glycosylated phenols available as standards. Specifically, the mass spectra of the extracted chromatogram peaks for aesculetin-glucoside and vanillic acid-glucoside were characterised by the two ions $[M\text{-}H]^-$ and $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ (*m/z* 339.0722 and 177.0193 for the former compound, and 329.0878 and 167.0350 for the latter, respectively). The two ions corresponded to the deprotonated molecule and the aglycon released after sugar loss in HESI, respectively, and were used for untargeted putative identification of other monosaccharidic derivatives. The ion $[M\text{-}H\text{-}C_5H_8O_4]^-$ was considered characteristic of aglycons released after sugar loss in the case of pentosidic precursors.

In the same way, for tentative identification of disaccharidic precursors, ions corresponding to $[M\text{-}H]^-$, to $[M\text{-}H\text{-}S]^-$ and to $[M\text{-}H\text{-}S\text{-}S]^-$ (S = sugar moiety) should be detected in the mass spectrum of the extracted chromatogram peaks. In particular, the transitions $[M\text{-}H\text{-}C_6H_{10}O_5]^- \rightarrow [M\text{-}H\text{-}C_{12}H_{20}O_{10}]^-$, $[M\text{-}H\text{-}C_6H_{10}O_5]^- \rightarrow [M\text{-}H\text{-}C_{11}H_{18}O_9]^-$, $[M\text{-}H\text{-}C_5H_8O_4]^- \rightarrow [M\text{-}H\text{-}C_{11}H_{18}O_9]^-$ and $[M\text{-}H\text{-}C_5H_8O_4]^- \rightarrow [M\text{-}H\text{-}C_{10}H_{16}O_8]^-$ were considered characteristic of hexoside-hexoside, hexoside-pentoside, pentoside-hexoside and pentoside-pentoside derivatives respectively.

As regards acetovanillone-glucoside and scopoletin-glucoside, the precursor ion $[M\text{-}H]^-$ was not detectable, probably due to complete sugar loss in HESI. Thus identification was based on the ions $[M\text{-}H\text{-}C_6H_{10}O_5]^-$, taking advantage of different RTs for the aglycons and the corresponding bound forms (Table 1). However, in the case of putative identification of glycosydic precursors characterised by the same fragmentation behaviour as acetovanillone-glucoside and scopoletin-glucoside, the proposed untargeted approach cannot be applied without standards.

2.5. Method validation

Identification of the glycosylated compounds was based on accurate mass ($\Delta m/z$ < 5 ppm), isotopic pattern and MS/MS experiments. Matching of peak retention time was further required for compounds whose standards were available.

The identification capability of the untargeted approach was tested

with 3 glycosylated simple phenols (salicylic acid-glucoside, orcinol-glucoside and *p*-hydroxybenzaldehyde-allopyranoside), custom-synthesised in order to confirm the supposed retention times and fragmentation.

2.6. Statistical analysis

Statistical analysis was performed with Statistica 9.1 Software (StatSoft, 2010), using ionisation intensity expressed as the peak area, as adopted in a similar metabolome approach (Cuadros-Inostroza et al., 2010). The peak areas were normalised as relative areas (%) in comparison to the sum of glycosidic precursor areas. Samples were randomly processed in a single analytical batch and a quality control sample was repeated every 10 tannin samples, confirming the narrow repeatability of the analytes' normalised area (R.S.D. always < 8%).

In order to select new markers able to discriminate tannins of different botanical origin, the Kruskal-Wallis ($p < .05$) nonparametric statistical test was performed on the entire variable dataset, while Classification Tree Analysis, requiring a limited number of variables, was performed using variables resulting significant according to the Kruskal-Wallis test.

Parametric statistical analysis was used to select performing origin markers, but limited to the most commercially relevant varieties of tannins (oak, grape skin, quebracho, chestnut and gallnut). Tukey's Honestly Significant Difference HSD test ($p < .05$) and Forward Stepwise Discriminant Analysis were performed on data normally distributed and normalised by applying Box-Cox transformation.

3. Results and discussion

The untargeted profiling approach described allowed investigation of over 320 glycosylated simple phenols, both as mono- and disaccharidic derivatives of 54 simple phenol aglycons (Table 1), never previously described in this matrix to the best of our knowledge. Table 2 summarises the accurate mass $[M\text{-}H]^-$, difference between expected and experimental masses ($\Delta\, m/z$) and the fragmentation profile of the 167 tentatively identified phenolic precursors.

3.1. Glycosylated simple phenols

Among the 167 glycosylated simple phenols tentatively identified in tannins, 89 were monoglycosilated (hexoside, N = 43; pentoside, 46) and 78 were diglycosilated derivatives (hexoside-hexoside, N = 23; hexoside-pentoside, 21; pentoside-hexoside, 21; pentoside-pentoside, 13). Compounds that had in common the precursor ion $[M\text{-}H]^-$ could be distinguished based on the accurate mass of the corresponding aglycon $[M\text{-}H\text{-}S]^-$, released after one or more sugar loss in HESI (Table 2). These compounds were 1/48–49–50, 3/57–58, 4/59, 5–6/61–62, 7–8–9/67, 10/68–69, 11/74–75, 12–13/76–77–78, 14/79, 18/81, 20–21/82–83, 22/84, 26/85, 28/86, 91/118–134, 92/119, 93/121–122, 94/125/147, 98/131, 102/132, 104/133, 113/139, 115/141, 116/142 and 117/143–144 (Table 2). This approach was applied to coeluted compounds and in the event of detection of different peaks for the same m/z value, in the latter case basing the attribution of peaks on a similar shift in the retention time of the corresponding aglycons (Table 1).

Compounds that had in common both the precursor ion $[M\text{-}H]^-$ and the aglycon $[M\text{-}H\text{-}S]^-$ (Table 2) could only be distinguished in the case of detection of different peaks for the same m/z value and if the corresponding aglycons were characterised by different retention times. In this case, isobaric precursors were distinguished by tracing the elution order of the corresponding aglycons (e.g. catechin-/epicatechin derivatives, Table 1). These compounds were 5/6, 7/8/9, 12/13, 16/17, 20/21, 29/30/31, 35/36, 41/42, 46/47, 48/49/50, 51/52/53, 54/55, 57/58, 61/62, 65/66, 68/69, 74/75, 76/77/78, 82/83, 87/88, 100/101, 121/122 and 143/144 (Table 2). When the aglycons were coeluted, as in the case of aceto/isoacetovanillone, eugenol/isoeugenol, gentisic acid/protocatechuic acid and *m*-/*o*-/*p*-cresol, the corresponding isobaric precursors could not be distinguished.

Glycosidic precursors in the form of hexose-pentose and pentose-hexose can be distinguished on the basis of their fragmentation profile, using the different m/z value of the ion $[M\text{-}H\text{-}S]^-$ (e.g. gentisic acid-/protocatechuic acid-hexose-pentose and gentisic acid-/protocatechuic acid-pentose-hexose with $[M\text{-}H\text{-}S]^-$ at m/z 315.0721 and 285.0615 respectively, Table 2). *m*-/*o*-/*p*-cresol were always putatively identified in the form of a hexose-pentose derivative, and *o*-vanillin always as a pentose-hexose derivative, while the others (Table 2) were tentatively identified both as hexose-pentose and pentose-hexose derivatives in all samples and were coeluted.

The match between supposed and effective retention times and fragmentation of *p*-hydroxybenzaldehyde-allopyranoside and salicylic acid-glucoside confirmed the identification ability of the proposed untargeted approach. As expected, the two characteristic ions $[M\text{-}H]^-$ and $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ (m/z 283.0823–121.0295 and 299.0772–137.0244 for *p*-hydroxybenzaldehyde-allopyranoside and salicylic acid-glucoside respectively) were detected in the mass spectrum of the extracted chromatogram peaks (Δ RT = 0.06 and 0.09 min respectively). In the case of orcinol-glucoside, the precursor ion $[M\text{-}H]^-$ was not isolated, probably due to complete sugar loss in HESI. The aglyconic form $[M\text{-}H\text{-}C_6H_{10}O_5]^-$ at m/z 123.0452 was used for identification thanks to different RTs for the aglycon and the corresponding bound form (8.7 and 6.7 min respectively, Table 1), confirming that precursors that completely lose the sugar moiety in HESI could not be detected with the current method, given the lack of available standards.

3.2. Tannin characterisation

According to the Kruskal-Wallis test ($p < .05$), gallnut tannins were significantly different from oak (for compounds 8, 12, 49, 51, 52, 53, 54, 57, 63, 68, 71, 87, 99, 101, 116 and 138), quebracho (for 7, 8, 16, 49, 51, 57, 84 and 87), skin (for 7, 12, 16, 23, 27, 29, 34, 37, 61, 68, 81, 83, 102 and 138), seed (for 41 and 106), whole grape (for 16, 29 and 41), grape marc (for 12, 34 and 138), green tea (for 8, 27, 34, 54 and 71), fruit tree (for 83) and chestnut tannins (for 54). Oak tannins were significantly different from quebracho (for compounds 68 and 101), skin (for 5, 35, 51, 52, 60, 61, 70, 71, 81, 143 and 144), seed (for 40, 45, 49, 57, 60, 70, 94, 99, 101, 120 and 147), whole grape (8, 40, 43, 44, 45, 51, 54, 57, 60, 70, 71, 90 and 126), grape marc (for 8), green tea (53, 99, 126 and 147) and chestnut tannins (for 89 and 99). Chestnut tannins were significantly different from quebracho (for compound 7), skin (for 7, 16, 24, 29, 34, 61, 70 and 81), seed (for 19, 40, 41, 70, 89, 94 and 147), whole grape (for 16, 19, 40, 41, 43, 70 and 89), grape marc (for 24 and 34), green tea (for 34, 81 and 147) and acacia tannins (for 24). Quebracho tannins were significantly different from skin (for compounds 5, 34, 36, 61, 83 and 102), seed (for 45, 49 and 70), whole grape (for 51, 57 and 90), grape marc (for 138) and green tea tannins (for 34). Green tea tannins were significantly different from skin (for compound 73), seed (41 and 73), whole grape (for 73) and grape marc tannins (for 8 and 73). Seed tannins were significantly different from skin (for compound 102) and grape marc tannins (for 106).

As regards Classification Tree Analysis, C&RT-*style exhaustive search for univariate splits* was used as the split rule and *FACT-style direct stopping* with fraction of object 0.05 as the stopping rule (Fig. 1). The classification tree obtained allowed us to entirely characterise 7 out of the 16 botanical tannin varieties by the appropriate response of selected compounds (Fig. 1). These varieties were chestnut (N = 6; node F), acacia (3; H), grape seed (6; L), and with the limitations of reduced sampling, fermented tea (1; R), tara (2; W), mimosa (1; X) and blueberry (1; Y). As regards other varieties, 10 out of 11 oak samples were characterised by the appropriate response of several compounds (node M), while the remaining one was grouped with one sample of grape skin (node J). As regards whole grape, two groups were identified (node Q, 5

Table 2
Exact mass [M-H]$^-$, difference between exact and accurate masses (Δ *m/z*) and characteristic fragmentation profile (all expressed as *m/z*) of the 167 glycosylated simple phenols tentatively identified in tannin samples.

i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments	i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments	i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments
hexose derivatives											
1	phenol-hex	255.0874 (1.1)	93.035	**16**	hydroxytyrosol-hex	315.1085 (−1.3)	153.056	**31**	syringaldehyde-hex	343.1034 (0.3)	181.051
2	pyrocatechol-hex	271.0823 (0.1)	109.030	**17**	syringol-hex	315.1085 (1.0)	153,056	**32**	vanillyl ethyl ether-hex	343.1398 (0.7)	181.087
3	*p*-hydroxybenzaldehyde-all	283.0823 (−1.1)	121.030	**18**	*p*-coumaric acid-hex	325.0928 (0.2)	163.040	**33**	scopoletin-hex	353.0878 (−1.0)	191.035
4	4-ethylphenol-hex	283.1187 (0.8)	121.065	**19**	eugenol-/isoeugenol-hex	325.1292 (−0.4)	163.076	**34**	ferulic acid -hex	355.1034 (0.1)	193.051
5	4-methylcatechol-hex	285.0979 (0.1)	123.045	**20**	ethylvanillin-hex	327.1085 (0.5)	165.056	**35**	ethyl vanillate-hex	357.1191 (−0.4)	195.066
6	orcinol-hex	285.0979 (−0.3)	123.045	**21**	aceto-/isoacetovanillone-glu	327.1085 (1.7)	165.056	**36**	aceto-/isoacetosyringone-hex	357.1191 (0.1)	195.066
7	protocatechualdehyde-hex	299.0772 (0.2)	137.024	**22**	vanillic acid-hex	329.0878 (0.5)	167.035	**37**	syringic acid-hex	359.0983 (0.5)	197.046
8	salicylic acid-hex	299.0772 (0.8)	137.024	**23**	homovanillyl alcohol-hex	329.1241 (−0.1)	167.071	**38**	sinapaldehyde-hex	369.1191 (−0.3)	207.066
9	*p*-carboxyphenol-hex	299.0772 (0.3)	137.024	**24**	gallic acid-hex	331.0670 (1.5)	169.014	**39**	isopropiosyringone-hex	371.1347 (−1.1)	209.081
10	tyrosol-hex	299.1136 (−0.5)	137.061	**25**	aesculetin-hex	339.0721 (0.4)	177.019	**40**	sinapic acid-hex	385.1140 (0.8)	223.061
11	4-vinylguaiacol-hex	311.1136 (1.2)	149.061	**26**	coniferaldehyde-hex	339.1085 (−0.4)	177.056	**41**	catechin-hex	451.1245 (1.0)	289.072
12	vanillin-hex	313.0928 (−0.5)	151.040	**27**	caffeic acid-hex	341.0878 (0.9)	179.035	**42**	epicatechin-hex	451.1245 (−0.3)	289.072
13	*o*-vanillin-hex	313.0928 (−0.3)	151.040	**28**	isopropiovanillone-hex	341.1241 (0.6)	179.071	**43**	ellagic acid-hex	463.0518 (0.1)	300.999
14	4-ethylguaiacol-hex	313.1292 (−0.8)	151.076	**29**	homovanillic acid-hex	343.1034 (−0.3)	181.051				
15	gentisic-/protocatechuic acid-hex	315.0721 (0.3)	153.019	**30**	methyl vanillate-hex	343.1034 (−0.1)	181.051				
pentose derivatives											
44	phenol-pent	225.0768 (1.1)	93.035	**60**	gentisic-/protocatechuic acid-pent	285.0615 (−0.7)	153.019	**76**	homovanillic acid-pent	313.0928 (0.8)	181.050
45	*p*-hydroxybenzaldehyde-pent	253.0717 (0.7)	121.029	**61**	syringol-pent	285.0979 (0.1)	153.055	**77**	methyl vanillate-pent	313.0928 (1.8)	181.050
46	4-ethylphenol-pent	253.1081 (0.6)	121.065	**62**	hydroxytyrosol-pent	285.0979 (−0.9)	153.055	**78**	syringaldehyde-pent	313.0928 (0.1)	181.050
47	3.4-xylenol-pent	253.1081 (0.2)	121.065	**63**	p-coumaric acid-pent	295.0823 (−0.1)	163.040	**79**	vanillyl ethyl ether-pent	313.1292 (0.3)	181.087
48	4-methylcatechol-pent	255.0874 (0.7)	123.045	**64**	eugenol-/isoeugenol-pent	295.1187 (−1.2)	163.076	**80**	scopoletin-pent	323.0772 (0.1)	191.034
49	guaiacol-pent	255.0874 (1.7)	123.045	**65**	ethylvanillin-pent	297.0979 (−1.0)	165.055	**81**	ferulic acid -pent	325.0928 (0.2)	193.051
50	orcinol-pent	255.0874 (0.8)	123.045	**66**	aceto-/isoacetovanillone-pent	297.0979 (1.3)	165.055	**82**	ethyl vanillate-pent	327.1085 (0.7)	195.066
51	protocatechualdehyde-pent	269.0666 (0.7)	137.024	**67**	vanillic acid-pent	299.0772 (1.0)	167.035	**83**	aceto-/isoacetosyringone-pent	327.1085 (0.1)	195.066
52	salicylic acid-pent	269.0666 (1.1)	137.024	**68**	homovanillyl alcohol-pent	299.1136 (−0.6)	167.071	**84**	syringic acid-pent	329.0878 (−0.9)	197.046
53	*p*-carboxyphenol-pent	269.0666 (−0.2)	137.024	**69**	4-methylsyringol-pent	299.1136 (−1.2)	167.071	**85**	sinapaldehyde-pent	339.1085 (1.5)	207.066
54	tyrosol-pent	269.1031 (0.2)	137.060	**70**	gallic acid-pent	301.0565 (0.1)	169.014	**86**	isopropiosyringone-pent	341.1241 (−1.1)	209.081
55	4-ethylcatechol-pent	269.1031 (−0.9)	137.061	**71**	aesculetin-pent	309.0615 (−0.6)	177.019	**87**	epicatechin-pent	421.114 (−0.3)	289.071
56	4-vinylguaiacol-pent	281.103 (0.8)	149.060	**72**	coniferaldehyde-pent	309.0979 (1.1)	177.055	**88**	catechin-pent	421.114 (0.5)	289.071

(*continued on next page*)

Table 2 (*continued*)

i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments	i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments	i.d.	Compounds	[M-H]$^-$ (Δ *m/z*)	MS/MS fragments
57	vanillin-pent	283.0823 (0.3)	151.040	**73**	caffeic acid-pent	311.0772 (−1.2)	179.034	**89**	ellagic acid-pent	433.0412 (1.6)	300.998
58	*o*-vanillin-pent	283.0823 (0.6)	151.040	**74**	coniferyl alcohol-pent	311.1136 (1.1)	179.071				
59	4-ethylguaiacol-pent	283.1187 (0.1)	151.076	**75**	isopropiovanillone-pent	311.1136 (0.3)	179.071				
hexose-hexose derivatives											
90	pyrocatechol-hex-hex	433.1351 (−0.8)	271.082, 109.029	**98**	4-ethylguaiacol-hex-hex	475.1821 (0.1)	313.129, 151.076	**106**	gallic acid-hex-hex	493.1198 (−0.5)	331.067, 169.014
91	*p*-hydroxybenzaldehyde-hex-hex	445.1351 (2.4)	283.082, 121.029	**99**	gentisic-/protocatechuic acid-hex-hex	477.1249 (0.1)	315.072, 153.019	**107**	coniferaldehyde-hex-hex	501.1613 (0.1)	339.109, 177.056
92	4-ethylphenol-hex-hex	445.1715 (−0.8)	283.119, 121.066	**100**	hydroxytyrosol-hex-hex	477.1613 (1.1)	315.109, 153.056	**108**	caffeic acid-hex-hex	503.1406 (1.3)	341.088, 179.035
93	guaiacol-hex-hex	447.1508 (1.3)	285.098, 123.045	**101**	syringol-hex-hex	477.1613 (−0.4)	315.108, 153.055	**109**	isopropiovanillone-hex-hex	503.1770 (−0.8)	341.124, 179.071
94	*p*-carboxyphenol-hex-hex	461.1301 (0.2)	299.077, 137.024	**102**	*p*-coumaric acid-hex-hex	487.1457 (1.0)	325.093, 163.040	**110**	homovanillic acid-hex-hex	505.1562 (−0.5)	343.103, 181.051
95	tyrosol-hex-hex	461.1664 (0.6)	299.114, 137.061	**103**	eugenol-/isoeugenol-hex-hex	487.1821 (0.3)	325.129, 163.076	**111**	syringic acid-hex-hex	521.1511 (1.3)	359.098, 197.046
96	4-vinylguaiacol-hex-hex	473.1665 (−0.6)	311.114, 149.061	**104**	vanillic acid-hex-hex	491.1406 (0.3)	329.088, 167.035	**112**	isopropiosyringone-hex-hex	533.1875 (0.9)	371.135, 209.082
97	vanillin-hex-hex	475.1457 (0.6)	313.093, 151.040	**105**	homovanillyl alcohol-hex-hex	491.1770 (−0.9)	329.124, 167.071				
hexose-pentose derivatives											
113	*m-/o-/p*-cresol-hex-pent	401.1453 (1.7)	269.103, 107.050								
hexose-pentose/pentose-hexose derivatives											
114	4-ethylphenol-hex-pent/-pent-hex	415.1610 (−0.4)	283.119/ 253.108, 121.066	**121**	hydroxytyrosol-hex-pent/-pent-hex	447.1508 (1.8)	315.108/ 285.098, 153.056	**128**	caffeic acid-hex-pent/-pent-hex	473.1300 (0.1)	341.088/311.077, 179.035
115	guaiacol-hex-pent/-pent-hex	417.1402 (1.4)	285.098/ 255.087, 123.045	**122**	syringol-hex-pent/-pent-hex	447.1508 (−0.7)	315.109/ 285.098, 153.056	**129**	isopropiovanillone-hex-pent/-pent-hex	473.1665 (−0.5)	341.124/311.114, 179.071
116	*p*-carboxyphenol-hex-pent/-pent-hex	431.1195 (0.1)	269.067, 137.024	**123**	*p*-coumaric acid-hex-pent/-pent-hex	457.1351 (−0.9)	325.093/ 295.082, 163.040	**130**	homovanillic acid-hex-pent/-pent-hex	475.1457 (0.3)	343.104/313.093, 181.051
117	tyrosol-hex-pent/-pent-hex	431.1558 (−0.5)	299.114/ 269.103, 137.061	**124**	aceto-/isoacetovanillone-hex-pent/-pent-hex	459.1508 (0.9)	327.108/ 297.098, 165.056	**131**	vanillyl ethyl ether-hex-pent/-pent-hex	475.1821 (−1.3)	343.139/313.129, 181.087
118	vanillin-hex-pent/-pent-hex	445.1351 (−0.4)	313.093/ 283.082, 151.040	**125**	vanillic acid-hex-pent/-pent-hex	461.1301 (0.2)	329.088/ 299.077, 167.035	**132**	ferulic acid-hex-pent/-pent-hex	487.1457 (1.0)	355.104/325.093, 193.051
119	4-ethylguaiacol-hex-pent/-pent-hex	445.1715 (−1.0)	313.129/ 283.118, 151.077	**126**	gallic acid-hex-pent/-pent-hex	463.1093 (−0.1)	331.067/ 301.057, 169.014	**133**	syringic acid-hex-pent/-pent-hex	491.1406 (−0.9)	359.098/329.088, 197.046
120	gentisic-/protocatechuic acid-hex-pent/-pent-hex	447.1144 (0.5)	315.072/ 285.062, 153.019	**127**	aesculetin-hex-pent/-pent-hex	471.1144 (1.1)	339.072/ 309.062, 177.019				
pentose-hexose derivatives											
134	*o*-vanillin-pent-hex	445.1351 (0.5)	283.082, 151.040								
pentose-pentose derivatives											
135	4-vinylphenol-pent-pent	383.1347 (0.2)	251.093, 119.050	**140**	gentisic-/protocatechuic acid-pent-pent	417.1038 (0.5)	285.062, 153.019	**145**	gallic acid-pent-pent	433.0987 (0.1)	301.057, 169.014
136	4-ethylphenol-pent-pent	385.1504 (0.9)	253.108, 121.066	**141**	syringol-pent-pent	417.1402 (0.2)	285.098, 153.056	**146**	isopropiovanillone-pent-pent	443.1559 (0.6)	311.114, 179.071
137	4-methylcatechol-pent-pent	387.1296 (2.3)	255.087, 123.045	**142**	vanillic acid-pent-pent	431.1195 (−0.9)	299.077, 167.035	**147**	syringic acid-pent-pent	461.1301 (−0.6)	329.088, 197.046
138	*p*-carboxyphenol-pent-pent	401.1089 (0.3)	269.067, 137.024	**143**	4-methylsyringol-pent-pent	431.1558 (0.8)	299.114, 167.071				
139	4-ethylcatechol-pent-pent	401.1453 (0.8)	269.103, 137.061	**144**	homovanillyl alcohol-pent-pent	431.1558 (1.0)	299.114, 167.071				

Note: hex = hexose; pent = pentose; all = allose; glu = glucose.

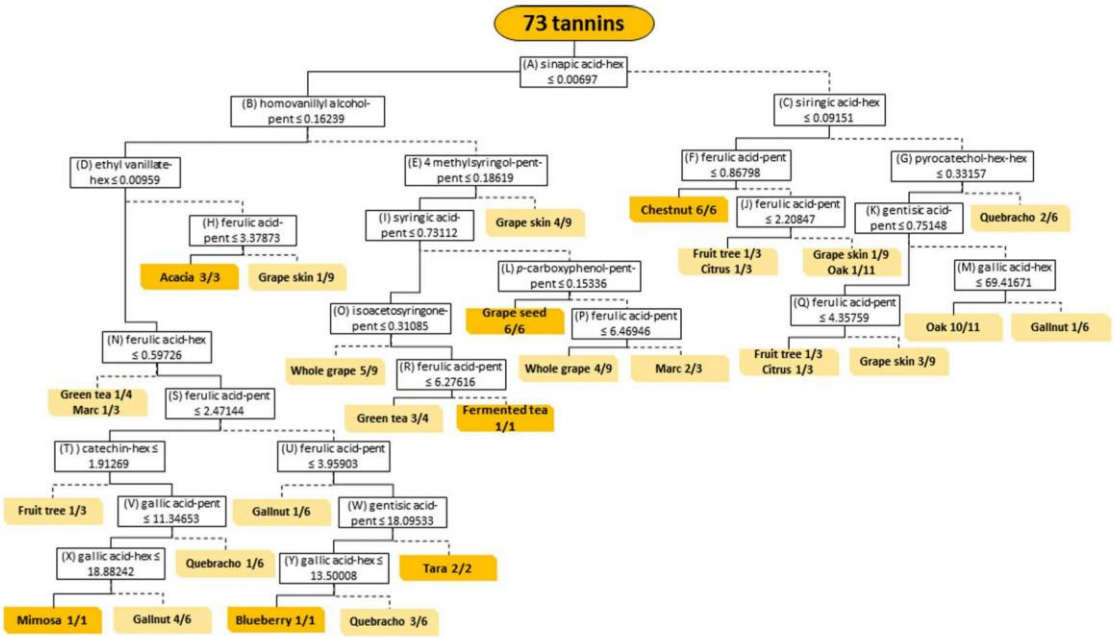

Fig. 1. Classification tree (C&RT-style exhaustive search for univariate splits; FACT-style direct stopping, fraction of object 0.05) for the 73 tannins of different botanical origin. The continuous line shows a positive answer to the split condition, the dashed line a negative one. Darker coloured terminal nodes show tannins of the same declared origin characterised by the same glycosidic simple phenolic profile.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples; node P, 4 samples). In the latter one, the response of compound 81 discriminated between whole grape (4 samples) and marc (2 samples). Three out of 4 samples of green tea were grouped at node R and distinguished from fermented tea (1 sample) by the appropriate response of compound 81. Finally, one sample of fruit tree was grouped twice with one of citrus, probably due to similarity in terms of botanical origin (nodes J and Q).

In order to obtain better characterisation of more commercially relevant tannin varieties, the same Classification Tree Analysis described above was performed considering oak, grape skin, quebracho, chestnut and gallnut tannins (Fig. 2). Chestnut and gallnut varieties were entirely characterised by the appropriate response of several compounds: 19 and 24 (node C), and 19, 90, 68 and 7 (node N) respectively. Five out of 6 quebracho tannins were characterised by the appropriate response of compounds 19 and 90 (node B) and the remaining 1 also by the appropriate response of compounds 68 and 7 (node F). Finally, 10 out of 11 samples of oak and 8 out of 9 samples of grape skin were grouped at node G, distinguished from each other by the appropriate response of compound 81. The remaining samples of both varieties were characterised by the appropriate response of compounds 19 and 24, and distinguished from each other by the appropriate response of compound 81 (node E).

As regards parametric statistical analysis, according to Tukey's Honestly Significant Difference HSD test ($p < .05$) oak tannins were significantly different from chestnut (for compounds 15, 24 and 81), skin (for compound 81) and gallnut tannins (for compounds 15 and 24).

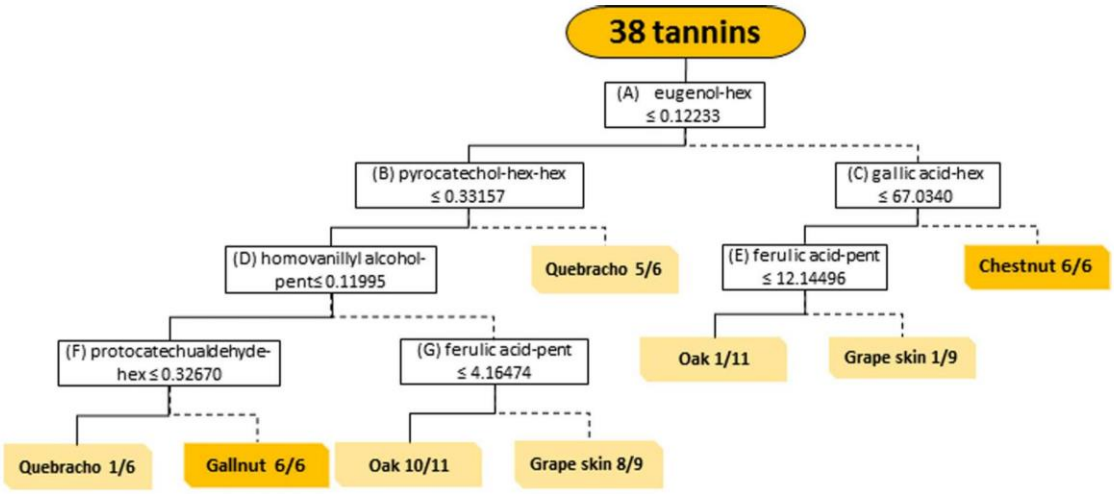

Fig. 2. Classification tree (Discriminant-based linear combination split; Prune on deviance, Minimum n = 5) of 38 tannins of the 5 most commercially relevant botanical varieties. The continuous line shows a positive answer to the split condition, the dashed line a negative one. Darker coloured terminal nodes show tannins of the same declared origin characterised by the same glycosidic simple phenolic profile.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Skin tannins were significantly different from chestnut and gallnut (for compounds 15, 24 and 81), and from quebracho tannins (for compounds 24 and 81). Quebracho tannins were significantly different from chestnut (for compound 81) and gallnut tannins (for compound 15). Compounds 24 and 81 recurred in tannin discrimination, as had already emerged in Classification Tree Analysis.

Finally, as regards Forward Stepwise Discriminant Analysis, the compounds used in the model and consequently with the greatest discriminatory power were gentisic-/protocatechuic acid-hex, gallic acid-hex and ferulic acid-pent (Wilks' Lambda was 0.17, 0.13 and 0.22 respectively). The Partial Wilks' Lambda indicated that ferulic acid-pent contributed most, gentisic-/protocatechuic acid-hex second most and gallic acid-hex contributed least to the overall discrimination (0.41, 0.52 and 0.72 respectively). Three discriminant functions were statistically significant for the model, even if the first function accounted for 93% of the explained variance. 6% of all discriminatory power was explained by function 2 and the left 1% by function 3. The first two discriminant functions (Roots 1 and 2) were weighted most heavily by the ionization response of ferulic acid-pent and gentisic-/protocatechuic acid-hex, and to a lesser extent by that of gallic acid-hex (standardized coefficients −0.77, −0.68 and 0.49 respectively for Root 1, and −0.65, 0.60 and −0.39 for Root 2); the third discriminant function was mostly marked by the ionization response of gallic acid-hex and gentisic-/protocatechuic acid-hex, and to a lesser extent by that of ferulic acid-pent (standardized coefficients 0.80, 0.52 and 0.28 respectively). Ferulic acid-pent was more highly correlated in order with the second and the first discriminant functions than with the third one (factor structure coefficients −0.76, −0.65 and 0.02 respectively), gentisic-/protocatechuic acid-hex with the second and the third than with the first one (0.71, 0.59 and −0.39 respectively), and gallic acid-hex with the third and the first than with the second one (0.86, 0.47 and -0.20 respectively).

The first discriminant function discriminated mostly between chestnut and gallnut tannins (mean of canonical variables 3.39 and 2.66 respectively) and the other tannin varieties (grape skin, quebracho and oak; −3.32, −0.30 and −0.41 respectively). The second discriminant function discriminated mostly between oak tannins (0.80) and the other tannin varieties (chestnut, grape skin, gallnut and quebracho; 0.16, −0.44, −0.82 and −0.15 respectively), and between chestnut and gallnut tannins (0.16 and −0.82 respectively). The third discriminant function discriminated mostly between quebracho and chestnut tannins (0.40 and 0.22 respectively) and the other tannin varieties (grape skin, gallnut and oak; −0.02, −0.30 and −0.16 respectively).

Finally, according to the classification model, 100% of grape skin tannins and 83% of chestnut tannins were correctly classified, with only one sample of the latter being incorrectly classified as gallnut. Oak and gallnut tannins were characterised reasonably well, correctly reattributing 63% and 50% of samples respectively. Three oak tannins were incorrectly classified as quebracho and three of gallnut as chestnut. Finally, quebracho tannins were not well characterised, since only two samples were correctly classified (33%), while three samples were incorrectly classified as oak and one as chestnut.

In conclusion, several tannin varieties were shown to be well characterised by specific glycosylated simple phenolic profiles. The commercial availability of pure standards of the most characteristic glycosylated phenols could permit the development of an effective new analytical approach to tannin classification.

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Conclusion

In this work, the suspect screening approach proved to be an efficient and well-performing analytical method for investigation of the nature and occurrence of low-molecular-weight phenolic glycosides in tannins of different botanical origin. Several tannin varieties were characterized by a specific profile for the selected glycosides. Consequently, in the event of availability of analytical standards, the proposed approach could become a useful tool for tannin classification and routine investigation and checking of tannin origin.

Furthermore, solid samples underwent solvent extraction in a wine-like solution, therefore the glycosides tentatively identified were those that could be transferred from tannins to wine during winemaking procedures or fining, contributing to defining its taste and aroma. This could be the case during the transfer of glycosides whose aglycone is directly related to wine defects, such as 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol and 4-vinylguaiacol, or there is consideration of their precursors, such as hydroxycinnamic acids, which can generate the previously mentioned volatile phenols if microbiological contamination of wine occurs.

SECTION 2.2.5.

The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine

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Aim of work

Wine ageing can occur in wood barrels, in particular if high quality wines are produced. Considering that many free low-molecular-weight phenolic compounds can be generated from the degradation of lignin (Bennett, & Wallsgrove, 1994), during wood heat-treatments they can be produced and then transferred to wines during fining. For this reason, one objective of this work was to investigate the nature and occurrence of free phenols in wine during the first three months of wood barrel ageing, in order to study the kinetics of transfer. Furthermore, considering that wood barrels undergo sanitation treatments in order to control spoilage microflora, this work also aimed to define the best sanitation procedures and evaluate their corresponding effects on the transfer of free phenolic compounds from wood to wine.

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ORIGINAL ARTICLE

The impact of different barrel sanitation approaches on the spoilage microflora and phenols composition of wine

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Abstract Careful control of spoilage microflora inside wine containers is a key issue during winemaking. To date, attention has been paid to the development of an effective protocol for the eradication of spoilage agents, especially *Brettanomyces*, from barrels. Few studies have taken into account the modifications caused by sanitation treatments in wine and wood barrels. In the present study the effects of two sanitation treatments (ozone and sodium hydroxide) on barrel spoilage microflora and the composition of the wine stored inside them were evaluated. The phenols of wine (38 compounds) were characterised using a UHPLC–MS during the first 3 months of wine ageing, to see possible alterations in composition due to the chemical exchange from wood to wine in presence of sanitising agents. With the same scope, a panel of 13 judges carried out sensorial analysis of wines. The results showed that the tested treatments had little effect on the organoleptic characteristics of wines, but underline the different performance of the sanitation treatments in terms of eradicating microorganisms.

Keywords Wine · Spoilage microflora · Ozone · Barrel · Simple phenols

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Introduction

Wood has always been used in the past, in oenology to make instruments and wine containers. Today the use of wood is limited to barrels, irreplaceable tools for the production of high quality wines. Wood provides a unique environment for wine ageing, because it acts as a semi-permeable barrier between wine and the environment, allowing a tailored exchange of gases, such as oxygen (Schmidtke et al. 2011), and the release of valuable compounds in wine (Chira and Teissedre 2015; Garde Cerdan and Ancin-Azpilicueta 2006; Singleton 1995). The combination of these two factors sparks off essential processes in wine ageing involving phenolic compounds. Wine colour stabilisation and the reduction of bitterness due to non-polymerized tannins are two of the most well-known examples of reactions occurring in wines during their permanence in barrels (Oberholster et al. 2015; Marginean et al. 2011).

Unfortunately, the use of barrels is accompanied by some problems. Wood is a material difficult to sanitise due to its porosity and chemical-physical inertness, which reduces the effectiveness of most of the sanitising agents (Guzzon et al. 2011; Stanga 2010). While the wine is present in barrels, the presence of organic matter due to fermentative microorganisms, and close contact between the wood and microbiota mediated by the liquid matrix allow the penetration of microorganisms into the wood. This phenomena is particular dangerous in the case of contamination of wine by spoilage microorganisms, such as *Brettanomyces* spp. The species, associated to the wine environment, belonging from this genera are two: *Dekkera/Brettanomyces bruxellensis* and *Brettanomyces anomalus*, with the majority of the strains being to the first one (Oelofse et al. 2008). These microorganisms are associated

at the organoleptic depreciation of wine, due to the accumulation of volatile phenols, acetic acid and other off-flavours. Also, some detrimental modifications in wine colour and taste are observed in the case of proliferation of Brettanomyces (Oelofse et al. 2008). It has been shown that *Brettanomyces* spp. is able to penetrate deeply into the wood, up to a depth of 8 mm below the surface of the staves (Suarez et al. 2007). Contact between wood and wine containing Brettanomyces for a period of two weeks is sufficient to cause significant contamination of barrels. If the contact between wine and wood is prolonged for 10 weeks, spoilage yeasts can reach a depth of more than 1 cm (Swaffield et al. 1997).

To reduce the risks of wine alteration during barrel ageing, preventive strategies are recommended but not always practicable. Immediately after alcoholic fermentation, there are technological limits in terms of the quantification of wine spoilage agents (OIV 2015; Guzzon et al. 2011) and it is not always possible to employ sanitization treatments, such as the addition of sulphur dioxide or filtration (Renouf et al. 2007; Ribereau-Gayon et al. 2006). Careful sanitation of barrels, therefore, plays a crucial role in avoiding cross-contamination between different wines and the settlement of an alterative microflora in the barrels. Traditionally, barrel sanitation was achieved using chemical agents such as organic acid or derivate of chlorine, sodium or potassium. There are some doubts about both the effectiveness and ecological sustainability of these approaches, due to the risk of residues remaining. Aqueous steam is widely used in wineries, but has not shown good efficacy because wood isolates microorganisms from high temperatures (Costantini et al. 2015; Guzzon et al. 2013). Other treatments, such as UV, high-power ultrasonics or ionizing radiation, have been proposed but have encountered difficulties in terms of widespread application due to operational limits or lack of efficacy (Guzzon et al. 2011; Schmid et al. 2011). Ozone has been proposed as a promising alternative for efficient and safe sanitation in the winery. The widespread application of this molecule in the agri-food industry (Erickson and Ortega 2006, Jin-Gab et al. 2003; Khadre et al. 2001; Foegeding 1985), and previous experience in winemaking (Guzzon et al. 2013; Guillen et al. 2010; Hester 2006; Coggan 2003), suggest that ozone could help to eliminate the problem of microbiological contamination of winemaking containers and equipment. However, to encourage the broad application of ozone to winemaking, doubts must be dispelled about residues in the winery environment that could lead to undesirable changes to wine.

In this work we compared ozone treatment with a traditional sanitation system based on chemical agents, for the purpose of eradicating spoilage microflora in wine barrels. The evolution of spoilage microflora was monitored by plate counts using a specifically tailored sampling approach and differential media, according to previous experiences of the authors (Guzzon et al. 2011). After sanitation, the barrels were filled with red wine and the evolution of a wide range of simple phenols was monitored during the first 3 months of wine ageing by an original UHPLC–MS method (Barnaba et al. 2015). This analytical dataset, accompanied by sensorial analysis of wines, provided information about the effectiveness of ozone as a sanitising agent of wine barrels, and about its interaction with the most valuable phenols characterising wood wine containers.

Materials and methods

Reagents and solutions

LC-MS grade acetonitrile (ACN, 99.9%), LC-MS grade methanol (MeOH, 99.9%), MS grade formic acid (98%) and DL-dithiothreitol (threo-1,4-dimercapto-2,3-butanediol; 99.5%) were purchased from Fluka (St. Louis, MO, USA), while L-glutathione reduced 99% and *p*-nitrophenol 99% were purchased from Sigma Aldrich (St. Louis, MO, USA). The target phenolic compound suppliers are summarised in Table 1. Water–methanol standard stock solutions were prepared with organic solvent content ranging from 15 to 55%, according to compound solubility.

Experimental plan

15 barrels (225 L each) were chosen in the cooperative winery of Girlan (Cornaiano, I), because contaminated by Brettanomyces at a concentration of over 3 log units. The barrels were made by the following suppliers: Fassbinderei Stockinger Gmbh (A), Tonnellerie Boutes (F), Tonnellerie Taransaud (F), Pauscha Fassbinderei Gmbh (A), and Tonnellerie Berthomieu (F). The barrels were washed with pressurised cold water (1×10^5 Pa, 5 min) and then filled with 50 litres of cold water, previously sterilised using a 0.45 µm filter. The water remained inside the barrels for 24 h, with periodic shaking, and was then sampled for microbiological analysis (1 L for each barrel). The barrels were emptied, dried for 24 h at 20 °C and randomly divided into 3 groups of 5 barrels. The 1st group (not treated, NT) was washed with cold water for 30 min. The 2nd group (chemical treatment, CT) was treated with the *TM Recond* system (Thonhauser GmbH, A), which provides for a barrel cleaning process in 4 steps: 4 min, 70 °C, 1% v/v of aqueous solution of *TM Recond AC* detergent (25% v/v aqueous solution of potassium hydroxide); 4 min, 70 °C, 1% of aqueous solution of *TM Recond pH*; 4 min, 70 °C, hot water; 2 min, cold water. The 3rd group of 5 barrels

Table 1 Phenolic compound characteristics and performance parameters for the chromatographic method

Compounds	Supplier	[M–H] (m/z)	MS/MS fragments	RT (min)	Linearity range (μg/L)	R^2	LOQ (μg/L)	Recovery (%)
Gallic acid	b	169.0142	125.0244	5.60	0.10–8802	0.999	0.10	83
Protocatechuic acid	a	153.0193	109.0294	5.76	0.10–5030	0.999	0.10	94
Gentisic acid	a	153.0193	109.0295, 108.0217	6.21	0.11–5300	0.999	0.11	94
Hydroxytyrosol	c	153.0557	123.0437, 95.0487	6.28	0.52–5150	0.990	0.52	83
Vanillic acid	a	167.0350	152.0114, 123.0452	6.42	0.10–3036	0.977	0.10	91
Syringic acid	a	197.0455	182.0216, 166.9984	6.57	0.11–4256	0.989	0.11	95
Caffeic acid	a	179.0350	135.0452	6.60	0.11–5330	0.996	0.11	76
Homovanillic acid	b	181.0506	137.0617, 122.0373	6.79	0.99–2970	0.988	0.99	105
Tyrosol	b	137.0608	119.0502, 106.0426	6.79	0.11–3150	0.984	0.11	95
Pyrocatechol	b	109.0295	108.0202, 91.0176	7.28	0.50–8946	0.992	0.50	95
p-coumaric acid	*b*	163.0401	119.0502, 93.1266	7.37	0.10–5200	0.993	0.10	88
Salicylic acid	d	137.0244	93.0346, 122.0374	7.72	0.10–8854	0.999	0.10	103
Catechin	a	289.0717	245.0805, 221.0812	7.89	5.1–5120	0.999	5.12	100
Ferulic acid	a	193.0506	178.0268, 149.0608	8.17	0.12–6210	0.997	0.12	94
Aesculetin	b	177.0193	133.0296, 105.0345	8.48	0.11–9720	0.998	0.11	91
Sinapinic acid	a	223.0611	208.0373, 179.0714	8.54	0.5–4990	0.999	0.50	89
Homovanillic alcohol	b	167.0714	152.0477, 122.0375	8.78	5.1–5100	0.995	5.10	92
Epicatechin	b	289.0718	245.0805, 221.0812	9.67	0.10–9018	0.992	0.10	94
Vanillin	b	151.0401	136.0152, 108.0202	9.86	0.11–5360	0.998	0.11	96
Coniferyl alcohol	b	179.0714	164.0478, 121.0296	10.11	10.7–5350	0.998	10.7	97
Syringaldehyde	e	181.0506	166.0269, 151.0035	10.42	0.75–13,500	0.999	0.75	89
Isopropiovanillone	b	179.0714	164.0477, 121.0295	10.55	5.4–5400	0.996	5.40	93
Scopoletin	a	191.0350	176.0112, 148.0166	10.66	1.0–9108	0.993	1.01	83
Acetovanillone	b	165.0557	150.0321, 122.0371	10.69	0.10–5120	0.998	0.10	89
Isoacetovanillone	b	165.0557	150.0321, 122.0371	10.79	0.10–5120	0.998	0.10	90
Isopropiosiringone	f	209.0819	194.0581, 179.0348	10.81	1.1–4392	0.997	1.10	94
Acetosyringone	b	195.0662	180.0426, 165.0190	11.00	0.10–5190	0.999	0.10	88
Isoacetosiringone	f	195.0662	180.0426, 165.0190	11.24	1.1–9720	0.998	1.08	91
Syringol	b	153.0557	138.0321, 123.0087	11.32	12.9–6460	0.996	12.9	90
Sinapinaldehyde	b	207.0663	192.0427, 177.0193	11.64	1.0–5040	0.997	1.01	77
Tryptophol	a	160.0767	142.0659, 130.0660	11.87	110–5510	0.997	110	95
O-vanillina	*b*	151.0401	136.0152, 123.0083	12.09	1.0–4980	0.999	1.00	89
Methyl vanillate	e	181.0506	166.0268, 151.0036	12.13	0.52–9270	0.995	0.52	93
4-ethylcatechol	c	137.0608	122.0374	12.30	0.0005–9.25	0.993	0.005	93
Guaiacol	b	123.0451	108.0215, 105.0346	12.85	11.0–9882	0.999	11.0	98
Ethyl vanillate	b	195.0662	180.0415, 130.9911	13.69	0.55–9900	0.995	0.55	100
4-ethylphenol	a	121.0658	106.0423, 83.9854	14.22	0.1022–5.11	0.999	0.1022	114
4-ethylguaiacol	d	151.0764	136.0529, 121.0293	14.58	0.0009–8.57	0.999	0.009	113

Supplier: a = Fluka (St. Louis, MO, USA); b = Sigma Aldrich (St. Louis, MO, USA); c = CHEMOS GmbH (Regenstauf, Germany); d = Aldrich (St. Louis, MO, USA); e = SAFC (St. Louis, MO, USA); f = TransMIT (Gießen, Germany); *RT* retention time; R^2 = coefficient of determination; *LOQ* limit of quantitation

(ozone treatment, OT) was treated with gaseous ozone generated by a cold plasma generator (Moving Fluid, I) with a nominal capacity of 32 g/h of ozone, equipped with an ozone detector B&C Electronics Srl (I); gas flow was set at 10 L/h with a treatment time of 30 min. After treatment, the barrels were again filled with sterile water, which was sampled as previously described. Finally, the barrels were filled with red wine (*Lagrein* cv., ethanol 13.1% vol., total acidity 5.70 g/L tartaric acid, acetic acid 0.45 g/L, malic acid 0.82 g/L, lactic acid 2.26 g/L, free SO_2 12.5 mg/L,

total SO_2 20.5 mg/L). The wine was aged in the barrels for 97 days, carrying out periodic sampling for microbiological and chemical analysis. Sensorial analysis was performed on the wine after barrel ageing.

Microbiological analysis

All microbiological analysis was performed as reported by the International Vine and Wine Organization (OIV 2015), using the following synthetic media: WL Agar (Oxoid, UK) for the enumeration of yeasts and acetic bacteria; Lysine Agar (Oxoid) for the quantification of non-*Saccharomyces* yeasts; DBDM (Morneau et al. 2011.) and WL Agar + 0.1% v/v of Cicloeximide solution (1% v/v aqueous solution, Oxoid) for the enumeration of Brettanomyces; MRS Agar for the quantification of lactic acid bacteria. All media were incubated at 25 °C for a time of between 4 and 10 days; MRS Agar was incubated under anaerobic conditions (Anaerogen KIT, Oxoid).

Simple phenolic characterisation of wine

Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, CA, USA) equipped with an on-line purification SPE system, adapting the method proposed by Barnaba et al. (2015). Mass analysis was performed with a Q-Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (HQ-OMS, Thermo Scientific) equipped with heated electrospray ionization (HESI-II) and operating in negative ion mode. A HyperSep™ Retain PEP spe cartridge (3.0 mm × 10 mm, 40–60 um, Thermo Scientific) was used for on-line sample purification and Acquity UPLC BEH C18 (2.1 mm × 100 mm, 1.7 µm particle size; Waters, MA, USA) as the analytical column. Chromatographic separation was performed with H_2O-ACN, by managing the ACN concentration as follows: from 4.0 to 5.5 min eluent B at 5%, from 5.5 to 17 min a linear increase to 60%, from 17.0 to 18.5 min a linear increase to 100%, then column equilibration from 18.5 to 22.0 min at 5%. During the first 4 min online SPE purification took place. During sample purification the flow rate was set to 0.250 mL/min, while during chromatographic separation the flow rate was set at 0.400 mL/min. The sample inject volume was 2 µL. Mass spectra were acquired in profile mode through full MS-data dependent MS/MS analysis (full MS–dd MS/MS). Full mass spectra were recorded at a mass resolving power of 140,000 full width at half-maximum (FWHM), while data-dependent mass spectra were recorded at a mass resolving power of 17,500 FWHM. The mass spectrometer operated with the following parameters: spray voltage, 2.80 kV; sheath gas flow rate at 30 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; capillary temperature at 310 °C; capillary gas heater temperature, 280 °C. Full mass spectral data were used for identification and quantification of analytes, while data-dependent mass spectral results were used to confirm the presence of analytes in real matrices. Table 1 summarises the exact masses and characteristic fragments used respectively for quantification and confirmation of the target compounds. Limits of detection (LODs) and of quantitation (LOQs) were established according to Mol et al. (2011) and Eurachem (1993) respectively. Method accuracy was estimated in terms of mean relative recovery by spiking 8 natural samples with 1000 µg/L of each phenol. Before analysis the sample was filtered using 0.45 µm PTFE filter cartridges (Sartorius AG, D), diluted 10 times, and added to the internal standard (*p*-nitrophenol, final concentration 500 µg/L) and formic acid (aqueous solution, 0.1% v/v).

Sensorial analysis of the wine

Wine from each individual barrel (15 samples) was tasted blindly following 97 days of ageing in the treated barriques. The panel consisted of 13 judges, previously trained wine sensory analysis at the laboratories of Laimburg Research Centre for Agriculture and Forestry (BZ, Italy). Judges were divided into two groups to which the wine were served in a different order to minimize systematic errors. Sensory evaluation was carried out using unstructured rating scales (Stone et al., 2008) that express each parameter on a linear scale from a minimum and a maximum. The evaluation scheme contained 12 parameters for both flavour (fruitiness, ink and leather, *Brett* smell/horse sweat, cleanness of the aroma, complexity, varietal typicality, reduced or oxidative character) and the taste (quantity of tannins, quality of tannins, balance, astringency and overall quality). 3 wine samples were replicated twice to check the sensitivity and reliability of each judge, using the method proposed by Kobler (1996).

Statistical analysis

All statistical analysis was performed using Statistica 9.1 (StatSoft, CA, USA). No detectable data were fixed at a value equal to half the LOQ determined for each parameter. Analytical compounds detectable in less than 10% of samples were not considered during statistical elaboration. Data not normally distributed (gentisic acid, syringaldehyde, sinapaldehyde, vanillin; Kolmogorov–Smirnov test, $p < 0.05$) were normalised by applying Box-Cox transformation. Honestly significant difference (HSD) Tukey test ($p < 0.05$) was applied in order to identify differences between treatments or sampling times both from microbiological and chemical determination. Principal Component Analysis was applied to phenols found to be significantly different due to ageing and treatment.

Results and discussion

Survey of the microbiological state of barrels before sanitation

Preliminary analysis of the wine stored in the barrels (data not shown) showed presence of *Brettanomyces* spp. at a concentration of up to 2 log units, an exceptionally higher value because it can lead to immediate alterations in wine (Renouf et al. 2007). Despite the diverse features of barrels (type of wood, degree of roasting, age) the profile of the microbiota found inside them, before sanitization treatments, was similar (Table 2) and related to the microbiological contamination of the wines previously stored in the barrels. This evidence led to consider the presence of spoilage microorganisms in winery equipment as a stable and widespread phenomenon that must be properly counteracted by a microbiological surveillance and an effective sanitization plan. The recovery onto petri plate containing a non-selective media (WL) was 1 log unit below the results of plate counts performed by DBDM and WLd, two media specifically tailored for *Brettanomyces* genera (Table 2). Comparison between the results of plate counts on WL Agar and Lysine Agar (Table 2) indicated that the yeast population was mainly made up of non-Saccharomyces yeasts. This observation agreed with previous works on the ecology of wine after alcoholic fermentation, revealing the presence of a complex yeast consortium during wine ageing (Perez-Martin et al. 2014; Sangorrin et al. 2008; Shinohara et al. 2000). The concentration of *Brettanomyces* genera, and of other yeasts grown onto the same selective media, was similar to that observed in wines earlier stored in barrels, about the 2–3 log units. Acetic bacteria were widely present in barrels, indeed lactic acid bacteria were not found in the samples, probably due to the standard practice of this winery of performing malolactic and alcoholic fermentation simultaneously, in the stainless-steel fermentation tanks. The differences in the recovery of analysis performed onto diverse synthetic media suggested that the microbiological monitoring of spoilage agents have consider different approaches, tailored for the microbial groups of interest in relation to the wine features (Zuehlke et al. 2011; Rodrigues et al. 2001).

Effectiveness of sanitation treatments

Barrels were treated using the 3 different protocols described in "Experimental plan" section. The first treatment was performed without a specific sanitising agent, using water to remove residues of wine from the internal surface of barrels. In the other cases (CT and OT) an aqueous solution of potassium hydroxide and gaseous ozone respectively were used as sanitising agents. Table 2 gives the results of the treatments, expressed as the microbial contamination found inside barrels after sanitisation. The first conclusion was that the sanitising agents did not result in complete removal of the microbes from the internal surface of barrels; this agreed with previous experience and confirmed the complexity of microbial control inside wood containers (Oelofse et al. 2008; Hester 2006; Du Toit and Pretorius 2000). In the case of washing with water (NT barrels), the decrease in cell concentration observed for certain groups of microorganism (Table 2) was probably related to mechanical removal of cells from the wood surface. In this context, one example was represented by acetic bacteria, whose aerobic nature and tendency to grow on the surface of the substrate (in this case wood) facilitated mechanical removal during washing with water. On the other hand, in the event of a more vigorous microorganism, *i.e.* yeasts, an increase in contamination was observed. A reasonable explanation for this behaviour would involve taking into account the increase in water activity inside the wood due to the water treatment, which is thus counterproductive.

Table 2 Results of microbiological tests in barrels before and after sanitation treatments

Sanitation treatment	Sampling	Total yeasts ($\times 10^1$) WL Agar (4 days)	Non-Saccharomyces yeasts ($\times 10^1$) LYS Agar (4 days)	Acetic bacteria ($\times 10^2$) WL Agar (7 days)	*Brettanomyces* spp. ($\times 10^2$)	
					DBDM (10 days)	WLd (10 days)
Chemical sanitizer (Sodium hydroxide)	BT	6.2 ± 3.7^{A}	7.3 ± 5.4^{A}	3.4 ± 2.7^{A}	4.9 ± 2.5^{A}	8.0 ± 4.1^{A}
	AT	2.2 ± 1.1^{A}	2.3 ± 0.6^{A}	2.2 ± 1.6^{A}	1.3 ± 0.7^{A}	1.1 ± 1.6^{B}
Cold water washing	BT	3.2 ± 1.6^{A}	3.2 ± 1.4^{A}	7.1 ± 1.8^{A}	7.1 ± 3.7^{A}	9.6 ± 0.8^{A}
	AT	3.6 ± 2.4^{A}	1.9 ± 1.0^{A}	1.6 ± 0.4^{B}	6.0 ± 2.2^{A}	8.1 ± 0.8^{A}
Gaseous ozone	BT	4.6 ± 1.6^{A}	4.8 ± 3.1^{A}	4.0 ± 2.3^{A}	6.4 ± 0.7A	6.8 ± 0.9^{A}
	AT	0.3 ± 0.4^{B}	0.8 ± 0.1^{A}	0.6 ± 0.1^{B}	nd.^{B}	nd.^{B}

The concentration is expressed in CFU/mL, referring to the microbial contamination of 50 litres of sterile water kept inside the barrels for 24 h with shaking. *BT* samples before sanitization treatment, *AT* samples after sanitization treatment; *nd* not detectable (<5 ufc/mL). Number of barrels with the same treatment (n) = 5, statistical analysis was performed comparing the microbial load before and after each treatment

In chemically treated barrels (CT) the residual microbial population was almost 30% of the initial level, for both yeasts and acetic bacteria, while it decreased to 15% in the case of Brettanomyces. More effective results were achieved in barrels subjected to the OT treatment, which reduced yeast contamination below 10% of the initial concentration and eliminated Brettanomyces: its concentration was negligible in sampling, after sanitations. The differences observed between CT and OT treatments were due to diverse mechanisms of action of the sanitising agents. CT was based on massive production of carbon dioxide due to the reaction of the active ingredients (potassium hydroxide, potassium carbonate and tetrapotassium pyrophosphate) with water, therefore mainly caused mechanical removal of the dirt. In contrast, OT acts by triggering radical reactions that target the double bonds typical of some molecules essential for cell life. It is therefore referred that the action of ozone was more strongly directed against living microorganisms, in comparison to CT. The various classes of microorganisms showed different sensitivity to OT. This behaviour could be explained by considering the different aerobic characteristics of oenological microorganisms and, consequently, different resistance to the oxidative stress exerted by ozone (Guzzon et al. 2013). *Brettanomyces* spp., residual concentration was $1.3 \pm 7 \times 10^2$ and $6.0 \pm 2 \times 10^2$ CFU/mL (Table 2) respectively in NT and CT barrels exposed wines to a significant risk of wine spoilage, considering that volatile phenols are generally produced when Brettanomyces exceeds 2 logarithmic units (Oelofse et al. 2008; Chatonnet et al. 1995). Conversely, in OT barrels the Brettanomyces concentration was close to the plate count detection limits and of no technological relevance.

Evolution of the phenolic content of wine stored in barrels subjected to different sanitation treatments over time

Almost all the target phenolic compounds were found in wines stored in barrels previously subjected to sanitation treatments at a concentration higher than their relative limits of quantitation. Two compounds differed from the general rule: sinapaldehyde and 4-ethylguaiacol. As regards phenols of microbiological origin, responsible for the "Brett character" in wine (Shinohara et al. 2000; Chatonnet et al. 1995), at the end of the 3rd month (97 days) of ageing 4-ethylphenol content ranged between 8.17 and 62.5 µg/L, with a median of 42.8 µg/L in the OT samples, between 4.9 and 45.2 µg/L, with a median of 31.9 µg/L in the CT samples, and between 29.4 and 46.7 µg/L, with a median of 34.9 µg/L in the NT samples. A similar situation was observed in the case of 4-ethylcatechol, which showed a median value of 30.2 µg/L in the OT samples (range 28.2 ÷ 31.9 µg/L), 28.6 µg/L in the CT samples (range 26.7 ÷ 30.4 µg/L), and 29.4 µg/L in the NT samples (range 27.5 ÷ 31.5 µg/L). Considering the sensorial threshold, generally above 420 µg/L as the sum of 4-ethylphenol and 4-ethylguaiacol (Knapp et al. 2010), all the wines were unaffected by "Brett character".

The proposed sanitation treatments did not directly involve the wine, but rather the wood of the barrels, so chemical characterisation therefore focused on the phenol profile (35 compounds), supposing that the variations of their concentrations should depend mainly on the interaction between wood and wine. As regards the content of wines subjected to the different sanitation treatments, significant differences (Tukey Test, $p < 0.05$) were observed between the wines sampled after 37 and 97 days of ageing, as reported in Fig. 1 for five compounds having different, and characteristics, trends. Minimum, median and maximum phenol content, and significant differences between the median concentration measured following 1, 2 and 3 months' ageing are summarised in Table 3. Acetovanillone increased by about 12% in wines aged in ozone-treated barrels (OT) over time, and also increased in wine from chemically treated barrels (+8%), albeit not significantly, but decreased in wine from untreated barrels (−3%). Gentisic acid increased in OT (+44%) and CT wines (+66%), and also in NT wines (+56%), albeit not significantly. Interestingly for wine aroma perception, vanillin and homovanillic acid increased significantly with ozone treatment (+72% and +12% respectively), but not significantly with the other treatments (+30% and +13% for CT; +13% and +10% for NT respectively). Isoacetovanillone and o-vanillin increased in OT (+20% and +26% respectively) and CT wines (+13 and +29% respectively), but did not significantly change in NT wines (−8 and +10% respectively). Catechin, which did not change in NT (+4%) and CT (+2%) wines, decreased in OT samples (−4%). Isoacetosyringone decreased over time both in NT (−27%) and OT (−36%) wines, but did not change in CT wine (−1%). Isopropiovanillone decreased significantly in OT wines (−36%), but not in NT (−15%) and CT wines (−18%). Epicatechin decreased in NT (−6%) and CT wines (−6%), albeit not significantly, but did not change in OT wines (+1%). Caffeic and salicylic acids decreased significantly in NT (−5 and −23% respectively), but not in CT (−3 and −15% respectively) and OT wines (−5 and −12% respectively). Gallic acid decreased significantly in NT (−35%) and CT samples (−28%), and also noticeably in OT (−28%) wine. Guaiacol increased significantly in NT (+27%) and noticeably in CT (+25%), but not in OT samples (−3%). Hydroxytyrosol increased in NT (+3%) and OT (+5%), but only significantly in CT wines (+4%). Methyl vanillate and protocatechuic acid decreased significantly in NT (−28%

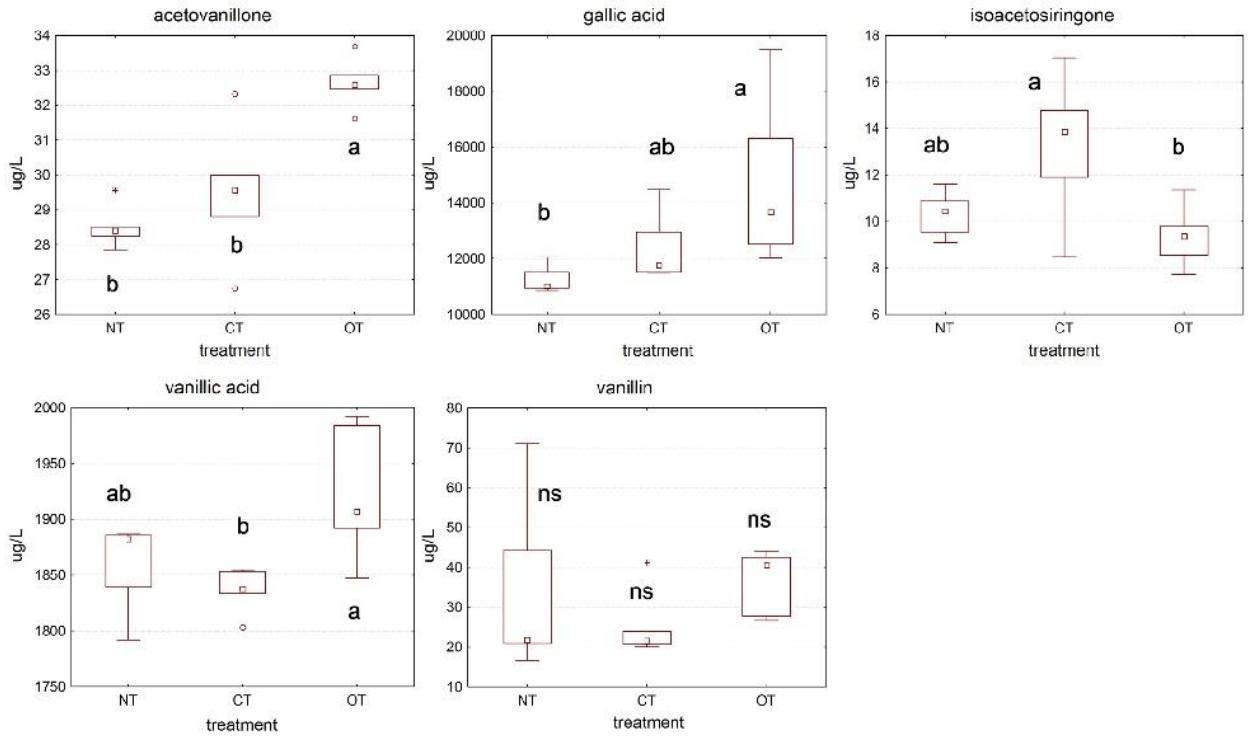

Fig. 1 Box plot of simple phenolic content after 3 months of ageing (97 days) in differently treated barrels (NT: non- treated barrels; CT: barrels treated with a chemical sanitising agent; OT: barrels treated with ozone). A single compound, of those found to be significantly different (Tukey Test, $p < 0.05$) for the treatments, is reported for each trend described. Vanillin, the only not significantly different compound, is given as an example of wood's impact on barrel-aged products

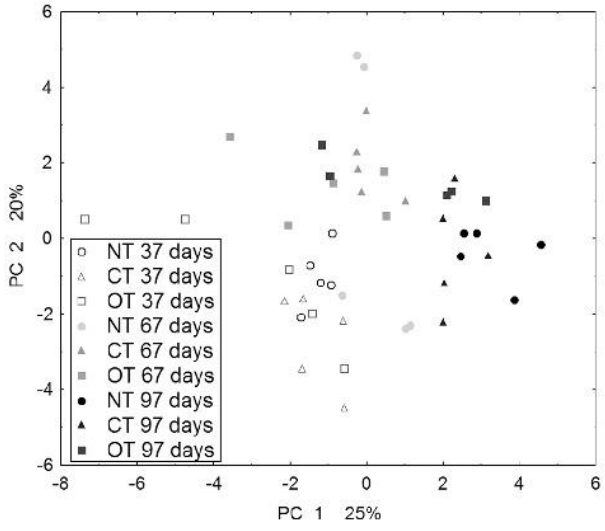

Fig. 2 Score plot (two first principal components: PC1 = 25%, PC2 = 20%) for wines aged for 37, 67 and 97 days in barrels distinguished on the basis of the three tested treatments (NT: non-treated barrels; CT: barrels treated with a chemical sanitising agent; OT: barrels treated with ozone)

and 29% respectively) and CT samples (−25 and −20% respectively), and also noticeably in OT (−19 and −13% respectively).

Impact of sanitation treatments on the wine phenolic profile following 97 days ageing

Statistical analysis of phenols for 97-day-aged samples had eight compounds with significant difference (Tukey Test, $p < 0.05$) between treatments were acetovanillone, isoacetovanillone, gallic acid, methyl vanillate, salicylic acid, protocatechuic acid, isoacetosyringone and vanillic acid. Aceto- and isoacetovanillone content was statistically higher for OT wines. Gallic acid, methyl vanillate, salicylic and protocatechuic acid were higher for OT than for NT. Isoacetosyringone content was lower for OT than for CT. Finally, vanillic acid was higher for OT than for CT. Figure 1 shows the distribution of these phenols after 3-month ageing for the different treatments. The vanillin content was very important due to the possible impact on wine aroma and although not significantly different for the barrel

Table 3 Original phenolic content of the test wine (not treated)

Compounds	Not treated mean (N = 3)	Treatment	After 37 days			After 67 days			After 97 days		
			Min	Median	Max	Min	Median	Max	Min	Median	Max
Acetovanillone	23.8	NT	28.7	29.3	30.4	27.3	28.3	35.3	27.8	28.4	29.6
		CT	26.2	27.4^{B}	28.7	31.0	31.7^{A}	34.2	26.7	29.6^{AB}	32.3
		OT	27.7	29.0^{B}	30.1	28.6	30.7^{B}	31.5	31.6	32.6^{A}	33.7
Caffeic acid	28,380	NT	31,752	$32,521^{A}$	33,771	30,662	$32,450^{AB}$	33,180	29,614	$31,032^{B}$	31,525
		CT	30,509	31,910	32,320	30,508	31,580	32,261	29,682	30,820	31,879
		OT	29,854	32,250	34,521	30,524	30,855	31,513	30,278	30,665	31,464
Catechin	26,800	NT	25,049	25,825	26,857	26,333	27,128	31,763	24,905	26,834	27,553
		CT	23,776	$25,484^{B}$	27,068	27,541	$28,643^{A}$	29,199	23,226	$26,081^{B}$	26,632
		OT	24,821	$26,042^{A}$	28,875	25,679	$26,532^{A}$	27,443	22,332	$25,009^{B}$	25,216
Coniferyl alcohol	799	NT	634	748	804	563	674	712	627	665	677
		CT	576	690^{A}	818	508	539^{B}	638	595	617^{AB}	728
		OT	679	729	762	457	492	630	549	644	811
Epicatechin	13,100	NT	12,051	$12,631^{A}$	13,233	11,477	$12,016^{B}$	12,301	11,534	$11,858^{B}$	12,062
		CT	12,054	12,480	13,157	11,465	12,085	12,431	11,294	11,737	13,042
		OT	11,583	$12,182^{A}$	13,080	10,962	$11,200^{B}$	11,497	11,912	$12,299^{A}$	12,932
Gallic acid	13,200	NT	16,258	$16,879^{A}$	18,831	12,662	$14,265^{B}$	15,601	10,852	$11,022^{C}$	12,053
		CT	15,059	$16,322^{A}$	19,064	14,285	$14,589^{A}$	15,398	11,485	$11,770^{B}$	14,473
		OT	17,161	19,067	21,756	14,677	18,279	22,265	12,014	13,661	19,477
Gentisic acid	54.8	NT	60.0	71.0	92.0	51.0	76.0	146	91.0	111	141
		CT	53.3	70.6^{B}	81.6	101	109^{A}	129	79.0	117^{A}	146
		OT	68.1	76.5^{B}	78.1	104	119^{A}	137	94.0	110^{A}	134
Guaiacol	198	NT	214	229^{B}	244	224	243^{B}	270	248	291^{A}	296
		CT	212	217	256	191	265	275	190	271	293
		OT	210	239	246	195	228	289	206	231	269
Homovanillic acid	173	NT	188	199	246	219	224	263	191	219	236
		CT	170	202	234	210	258	270	208	229	237
		OT	178	210^{B}	225	212	216^{AB}	238	217	236^{A}	257
Hydroxytyrosol	1260	NT	1283	1361	1388	1256	1404	1444	1277	1403	1420
		CT	1302	1330^{B}	1335	1313	1351^{B}	1367	1389	1401^{A}	1441
		OT	1261	1324	1401	1245	1324	1346	1269	1382	1415
Isoacetosyringone	5.9	NT	13.1	14.3^{A}	17.4	10.1	11.9^{AB}	14.9	9.07	10.4^{B}	11.6
		CT	11.4	14.1	16.2	11.9	13.4	15.9	8.5	13.9	17.0
		OT	12.4	14.6^{A}	21.2	8.65	10.1^{AB}	15.5	7.71	9.37^{B}	11.4

Table 3 continued

Compounds	Not treated mean (N = 3)	Treatment	After 37 days			After 67 days			After 97 days		
			Min	Median	Max	Min	Median	Max	Min	Median	Max
Isoacetovanillone	12.1	NT	13.9	14.6	15.5	12.5	12.9	16.9	13.3	13.5	14.1
		CT	12.4	12.5^{B}	13.9	14.8	15.1^{A}	16.4	12.7	14.1^{AB}	15.7
		OT	12.2	13.2^{C}	13.7	13.6	14.7^{B}	15.0	15.4	15.9^{A}	16.4
Isopropiovanillone	14.0	NT	12.0	13.3	16.0	10.5	17.6	22.6	10.7	11.3	13.3
		CT	11.8	14.7	23.0	12.6	14.2	15.5	9.35	12.0	15.4
		OT	14.7	19.0^{A}	19.7	10.6	14.4^{B}	17.1	11.0	12.1^{B}	15.0
Methyl vanillate	1080	NT	1548	1613^{A}	1730	1346	1468^{B}	1514	1094	1159^{C}	1193
		CT	1452	1691^{A}	1781	1385	1457^{A}	1592	1191	1275^{B}	1381
		OT	1729	1791	2571	1448	1747	2395	1220	1456	2113
o-Vanillin	116	NT	115	119	180	112	136	146	119	131	163
		CT	95.0	100^{B}	123	104	112^{AB}	142	125	129^{A}	155
		OT	93.3	124^{B}	127	102	119^{B}	139	142	156^{A}	166
Protocatechuic acid	1850	NT	2566	2587^{A}	2723	2007	2286^{B}	2503	1755	1827^{C}	1919
		CT	2370	2519^{A}	2748	2185	2336^{A}	2496	1785	2022^{B}	2119
		OT	2444	2555	3422	2228	2638	3305	1944	2224	3016
Salicylic acid	206	NT	280	298^{A}	305	251	282^{A}	322	220	229^{B}	256
		CT	261	295^{AB}	303	294	316^{A}	321	246	252^{B}	295
		OT	289	313	349	261	306	377	256	274	347
Scopoletin	5.6	NT	5.81	6.21	7.63	5.85	7.34	9.05	6.39	6.82	8.03
		CT	4.99	6.06^{B}	6.60	6.56	7.07^{A}	7.86	5.50	6.36^{AB}	7.03
		OT	6.45	7.32	8.34	6.45	6.94	7.58	5.98	7.33	9.58
Tryptophol	1490	NT	1519	1613^{B}	1659	1679	1696^{A}	1863	1596	1655^{AB}	1694
		CT	1570	1584	1782	1673	1715	1774	1609	1659	1705
		OT	1576	1650^{AB}	1789	1653	1722^{A}	1782	1526	1619^{B}	1645
Vanillin	15.6	NT	15.6	19.3	37.0	14.3	17.7	53.5	16.7	21.8	71.0
		CT	15.6	16.6	22.0	15.2	16.8	23.8	20.1	21.6	41.2
		OT	17.7	23.6^{B}	28.6	18.8	27.3^{AB}	31.4	26.6	40.6^{A}	44.1

Minimum, median and maximum content (μg/L) of phenolic compounds shown to be significantly different (Tukey Test, $p < 0.05$) for at least one barrel treatment in wines aged for one month (37 days), two months (67 days) and three months (97 days). Median superscript letters on the same row indicate significant differences between ageing times for the same treatment

treatments, it had a median content notably higher (almost double) for OT wines as observed in a previous work by Guzzon et al. (2013). All the variations described for hydroxybenzochetonic derivatives (methyl vanillate, isoacetosyringone, aceto- and isoacetovanillone), and hydroxybenzoic acids (gallic, salicylic, protocatechuic and vanillic acid) were consistent with earlier results for red wines (Barnaba et al. 2015). However, compounds with a possible impact on wine aroma, being vanillin derivatives and mostly related to wood transfer in barrel-aged products, such as aceto-, isoacetovanillone, methyl vanillate, vanillic acid and vanillin itself, were generally higher in wines aged in OT barrels as compared to those from differently treated barrels.

Figure 2 presents a graphic PCA description of data related to the phenols found to be significantly different in terms of ageing and treatment. *O*-vanillin, gentisic acid and guayacol were the most significant components with positive eigenvalues (0.41, 0.36 and 0.33 respectively), while protocatechuic acid, gallic acid and methyl vanillate had negative eigenvalues (−0.91, −0.91 and −0.90 respectively) for the first function (Fatt. 1), which explained 25% of total variability. Acetovanillone, isoacetovanillone and gentisic acid were the most significant components with positive eigenvalues (0.84, 0.80 and 0.76 respectively), while coniferyl alcohol, isoacetosyringone and isopropiovanillone had negative eigenvalues (−0.50, −0.41 and −0.35 respectively) for the second function (Fatt. 2), which explained 20% of total variability. Barrel treatment seems to have affected the wine phenolic profile in a more moderate way than the duration of ageing, and as it is reasonable to expect, the main compositional differences can be observed between 37-day and 97-day aged samples. These results provided further confirmation that OT, as well as being effective in eradicating spoilage microorganisms, did not effect the aroma profile of wines.

Sensorial analysis of wine

Sensorial analysis showed no significant differences between the different sanitation treatments for the descriptors considered (Table 4). According to the chemical assay, the flavour descriptors associated with aroma alterations due to the *Brettanomyces bruxellensis* metabolism (ink and leather, Brett smell/horse sweat, cleanness in the aroma) were detected at low levels. These results underlined that in the experimental conditions adopted, *Brettanomyces* was not able to cause alterations to the sensorial impression of wines. The main factor that limited *Brettanomyces* activity was probably the short ageing time (3 months), thus defined because it was specifically the standard procedure for the production of Lagrein wine. In future, additional experiments involving wines suitable for prolonged storage in barrels will be needed to better clarify this aspect. Other parameters in the sensorial tests (i.e. fruitiness, complexity, varietal typicality, reduced/oxidative character, quantity of tannins, quality of tannins and balance) were more related to wine features, deriving for example from the varietal contribution, rather than to winemaking variables such as the ageing time in barrels. Consequently, for the purpose of this work, these descriptors have the greatest relevance. The non-significant

Table 4 Sensory analysis (descriptive test) of Lagrein wine, 2014 vintage, after 97 days of barrel ageing

Parameter	Significance	NT	CT	OT
Fruitiness	n.s.	5.10 ± 0.66	5.23 ± 0.72	5.61 ± 1.00
Ink	n.s.	0.86 ± 0.31	0.90 ± 0.34	0.88 ± 0.19
Horse sweat	n.s.	0.92 ± 0.15	0.91 ± 0.31	0.81 ± 0.21
Cleanness in the aroma	n.s.	6.26 ± 0.50	6.34 ± 0.62	6.36 ± 0.39
Complexity	n.s.	5.81 ± .021	5.65 ± 0.42	5.86 ± 0.67
Varietal typicality	n.s.	5.98 ± 0.53	5.94 ± 0.35	5.96 ± 0.61
Reduced/oxidative character	n.s.	−1.73 ± 0.43	−1.78 ± 0.50	−1.48 ± 1.07
Quantity of tannins	n.s.	0.34 ± 0.72	0.22 ± 0.47	0.47 ± 0.24
Quality of tannins	n.s.	4.21 ± 0.36	4.52 ± 0.42	4.53 ± 0.17
Balance	n.s.	4.11 ± 0.65	4.43 ± 0.64	4.59 ± 0.63
Astringency	n.s.	3.83 ± 0.55	3.59 ± 0.30	3.43 ± 0.39
Overall quality	n.s.	4.97 ± 0.60	5.15 ± 0.48	5.03 ± 0.58

All data are expressed as the average of the five replicates ± standard deviation. *NT* non-treated barrels; *CT* barrels treated with a chemical sanitising agent; *OT* barrels treated with ozone. Statistical analysis: one-factor ANOVA and Tukey-B test. Different letters indicate statistically significant differences at $p < 0.05$. *n.s.* not significantly different. *Values are significantly different at $p < 0.05$. **Values are significantly different at $p < 0.01$. *** Values are significantly different at $p < 0.001$

n.s. not significantly different

differences detected for all of these descriptors proved that the treatments considered had no negative effects on the wine's organoleptic characteristics. While this was already known for the chemical treatment described, it is described for the first time, as far as we know, for sanitation treatment with ozone.

Conclusion

This work verified the applicability of ozone as a sanitising agent for barrels used to age wines in real winemaking conditions, maintaining accuracy and statistical robustness similar to laboratory tests in the experimental plan and analysis of results. Ozone proved to be an effective sanitising agent, against the main wine spoilage organisms, such as Brettanomyces. The extended range of chemical analysis performed on wines during the 3-months' ageing in barrels allowed the evolution of the phenolic profile of wines to be studied, both in terms of the kinetics of exchange between wine and wood, and the quantitative and qualitative composition of the finished wines. Finally, sensory analysis confirmed the results of chemical tests, excluding any depreciation of the wine due to treatment of the barrels with ozone. In conclusion, ozone proved to be an interesting alternative to traditional sanitising agents used in the winery, capable of guaranteeing effective and safe sanitation during winemaking.

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Conclusion

In this work, the targeted approach allowed study of the free phenolic composition of wood barrels, in order to evaluate phenolic enrichment during ageing. Many monitored compounds increased during ageing, showing that a relevant transfer from wood to wine occurred. Others, mainly representatives of flavan-3-ols, phenolic acids and benzoketones, decreased during ageing, probably because they underwent oxidation, condensation or polymerization reactions.

As regards sanitation treatments and their effect on the phenolic composition of wine, ozone proved to be an effective agent against spoilage microflora and did not affect either the phenolic or sensorial profile of wines. In particular, this sanitation procedure seemed to affect the phenolic profile of wine less than the duration of ageing, although compounds with a possible impact on wine aroma – such as vanillin derivatives –were generally higher in wines aged in barrels treated with ozone.

SECTION 2.2.6.

Free and glycosylated simple phenol profiling in Apulian Italian wines

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Aim of work

In food, phenols act as free radical scavengers (FRS), decreasing rancidity development and retarding the formation of toxic oxidation products (Shahidi, & Ambigaipalan, 2015). In wine, phenolic compounds have traditionally attracted attention due to their organoleptic properties, such as astringency and bitterness, their role in terms of wine colour and the different functions they have for plants and bacteria (Lesschaeve, & Noble, 2005). Furthermore, the taste and aroma of wines can also be partially influenced by the occurrence and levels of glycosidic precursors accumulated during grape maturation (Tamborra, & Esti, 2010), whose chemical hydrolysis can be significantly impacted by pH during ageing (Versini *et al.*, 2002) or treatment with β-glucosidase (Nicolini *et al.*, 1994).

The aim of this work was to characterize the nature and occurrence of free and glycosylated low-molecular-weight phenols in two wood-aged southern Italian wines – Primitivo di Manduria and Negroamaro – and evaluate the effect of wine ageing on selected compounds.

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Free and glycosylated simple phenol profiling in Apulian Italian wines

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ABSTRACT

Free simple phenols have a significant role in defining the sensory and nutritional characteristics of wines, affecting the organoleptic profile and having positive effects on health, but glycosidically bound phenols can also be hydrolysed during the winemaking process, releasing the corresponding volatile compounds and making a possible contribution to the final sensory profile.

In this work, application of on-line SPE liquid chromatography–high resolution mass spectrometry, operating in negative polarity with heated electrospray, allowed to detect over eighty free and glycosylated simple phenols in Primitivo di Manduria and Negroamaro wines. Sixty-one phenols, four of which phenolic glucosidic precursors, were quantified as having quantification limits ranging from 0.001 to 0.1 μg mL^{-1}, calibration R^2 of 0.99 for over 92% of compounds, and precision (R.S.D.%) always lower than 12%. Twenty-four simple phenolic precursors were tentatively identified as hexoside, pentoside and hexoside–hexoside derivatives, on the basis of accurate mass, isotopic pattern and MS/MS fragmentation.

1. Introduction

The wine aroma profile is affected by different factors, with grape variety, vinification practices and the ageing process playing the main role (Rapp & Mandery, 1986). Aroma compounds, present in grapes both as free and glycosidically bound forms, contribute to the final wine sensory profile, together with the volatiles originating during fermentation and with those extracted from wood barrels during ageing (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Consequently, wine flavour is the result of a complex equilibrium of volatile compounds with different characteristics and intensities. Flavourless glycoconjugates are considered aroma precursors, because they can be hydrolysed during the winemaking process or during wine ageing, releasing the corresponding volatile compounds (Williams, Strauss, Wilson, & Massy-Westropp, 1982a). In glycosidic precursors the aglycon can be linked both to mono- and disaccharides. In the first case they are usually identified as β-D-glucopyranoside, while in the second case the glycoside is further substituted with a pentose such as α-L-arabinofuranosyl-β-D-glucopyranosides, α-L-rhamnopyranosyl-β-D-glucopyranosides, β-D-apiofuranosyl-β-D-glucopyranosides and β-D-xylofuranosyl-β-D-glucopyranosides (Boido, Lloret, Medina, Carrau, & Dellacassa, 2002; Hayasaka et al., 2010; Williams, Strauss, Wilson, & Massy-Westropp, 1982b).

Among volatiles, phenolic compounds have traditionally attracted attention due to their organoleptic properties, such as astringency and bitterness, their role in terms of wine colour and the different functions they have for plants and bacteria (Bhattacharya, Sood, & Citovsky, 2010; Lesschaeve & Noble, 2005). Recently, interest has been shown in their antioxidant activity and their role in reducing the risk of cardiovascular diseases (Middleton, Kandaswami, & Theoharides, 2000). The aroma of wines produced with non-aromatic grapes can also be partially influenced by the occurrence and levels of glycosidic precursors accumulated during grape maturation (Tamborra & Esti, 2010), whose chemical hydrolysis can be significantly impacted by pH during ageing (Versini, Carlin, Dalla Serra, Nicolini, & Rapp, 2002) or treatment with β-glucosidase (Nicolini, Versini, Mattivi, & Dalla Serra, 1994).

Unfortunately, aroma precursor analysis generally involves complex and time-consuming procedures combining preliminary extraction of glycoconjugates, acid or enzymatic hydrolysis, aglyconic form separation and finally gas-chromatography detection of the latter (Boido et al., 2002; Williams et al., 1982a, 1982b).

The aim of this work was to further investigate the simple phenol composition of two of the most traditional red wines from southern Italy, providing quantitative information on over 50 aglycones and describing their possible glycosidic precursors. Despite their diffusion, little has been published regarding the free and bound simple phenol profiles of Primitivo (so named due to its early maturation, and characterised by its high-alcohol content,

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ruby-purple colour, and spicy and red-fruit aroma notes; Baiano, Terracone, Gambacorta, & La Notte, 2009), and Negroamaro (a mid-season maturing variety, characterised by its ruby-red colour, bitter taste, and earthy/fruity aroma notes; Tamborra & Esti, 2010; Toci et al., 2012) varieties. Our approach combined automatic on-line solid-phase extraction (SPE) clean-up with rapid chromatographic detection using ultra high-performance liquid chromatography (UHPLC) coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap).

2. Materials and methods

2.1. Reagents and solutions

LC–MS grade acetonitrile (ACN, 99.9%), LC–MS grade methanol (MeOH, 99.9%), MS grade formic acid (98%) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol, 99.5%) were purchased from Fluka (St. Louis, MO, USA), while L-glutathione reduced (99%) and *p*-nitrophenol (99%) were purchased from Sigma Aldrich (St. Louis, MO, USA). The target phenolic compound suppliers are summarised in Table 1. Deionized water was produced using an Arium®Pro Lab Water System (Sartorius AG, Goettingen, Germany).

A water–methanol (80:20 v/v) standard stock solution (10 mg L^{-1} of each phenol) was added of L-glutathione reduced and DL-dithiothreitol (2.5 g L^{-1}) as antioxidant agents, and used for external calibration in the range 0.0001–10 μg mL^{-1}. Stock solutions were stored at −4 °C.

2.2. Samples and preparation

Six samples of Primitivo di Manduria wine (DOP; 2006, 2010, 2012, 2013 and 2014 vintages; alcohol content between 13.5% and 14.4% vol; pH 3.4–3.7; total acidity 4.5–6.7 g L^{-1} tartaric acid; volatile acidity 0.5–0.9 g L^{-1} acetic acid) and six samples of Negroamaro wine (IGP; 2007, 2010, 2011, 2012, 2013 and 2014 vintages; alcohol content 13.3–14.8% vol; pH 3.4–3.6, total acidity 4.6–6.9 g L^{-1} tartaric acid; volatile acidity 0.6–0.9 g L^{-1} acetic acid) were collected at the Cantine San Marzano winery (southern Apulia, Taranto, Italy). All the wines were aged for 12 months in French and American oak barrels.

Before analysis, the samples were filtered using 0.45 μm PTFE filter cartridges (Sartorius AG, Goettingen, Germany), diluted 10 times and added of the internal standard (*p*-nitrophenol, 0.530 μg mL^{-1}).

2.3. Chromatographic separation

Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with a Rheodyne 6-port automated switching valve used for on-line clean-up, adapting the method recently proposed by Barnaba and colleagues (Barnaba et al., 2015).

On-line SPE clean-up was performed by loading 2 μl of sample on a HyperSep™ Retain PEP spe cartridge (3.0 mm × 10 mm, 40-60 μm, Thermo Scientific, Sunnyvale, CA, USA), with deionized water at a flow rate of 0.250 mL min^{-1}. After 4 min the analytical mobile phase (95% H_2O and 5% ACN, flow rate of 0.400 mL min^{-1}) flowed through the SPE cartridge, progressively removing the retained analytes and transferring them to the analytical column (Acquity UPLC BEH C18, 2.1 mm × 100 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Chromatographic separation was performed in H_2O-ACN, by managing ACN concentration as follows: from 4.0 to 5.5 min at 5%, from 5.5 to 17.0 min a linear increase to 60%, from 17.0 to 18.5 min a linear increase to 100%, then column equilibration from 18.5 to 22.0 min at 5%. During analytical column equilibration, the SPE cartridge was cleaned with formic acid (0.1%, v/v) aqueous solution/MeOH (50:50) for 1.5 min and activated with only formic acid aqueous solution for 2 min.

2.4. Mass conditions

Identification and quantification of phenolic compounds were performed using a Q-Exactive™ hybrid quadrupole-orbitrap mass spectrometer (HQ-OMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionisation (HESI-II). Mass spectra were acquired in negative ion mode through full MS-data dependent MS/MS analysis (full MS-dd MS/MS), recording full mass spectra at a mass resolving power of 140,000 full width at half-maximum (FWHM), and data-dependent mass spectra at 17,500 FWHM. The mass spectrometer operated as follows: spray voltage, 2.80 kV; sheath gas flow rate, 30 arbitrary units; auxiliary gas flow rate, 20 arbitrary units; capillary temperature, 310 °C; capillary gas heater temperature, 280 °C.

2.5. Identification of free and bound simple phenols (target analysis)

Full mass spectral data were used for identification and quantification of analytes, through comparison of high resolution *m/z* values (mass tolerance <5 ppm, SANCO/12571/2013), retention time (RT) and dd-MS/MS spectra, with data collected from experiments performed on commercially available standards (see Table 1). Specifically, quantification of aesculetin-glucoside (aesculetin-6-O-β-D-glucoside) was performed with the ion at *m/z* 339.0722 (expected mass), corresponding to the deprotonated molecules $[M-H]^-$, while ions at *m/z* 177.0193 and 133.0296, corresponding respectively to the loss of the glucosidic unit $[M-H-C_6H_{10}O_5]^-$ and to the further loss of CO_2 $[M-H-C_6H_{10}O_5\text{-}44]^-$, were used for confirmation. In the same way, quantification of vanillic acid-glucoside (vanillic acid-4-O-β-D-glucoside) was performed with the ion at *m/z* 329.0878 $[M-H]^-$, while ions at *m/z* 167.0350 $[M-H-C_6H_{10}O_5]^-$ and 152.0114 $[M-H-C_6H_{10}O_5\text{-}CH_3]^-$ were used for identification. For acetovanillone-glucoside (acetovanillone-4-O-β-D-glucoside) and scopoletin-glucoside (scopoletin-7-O-β-D-glucoside), ions corresponding to deprotonated molecules $[M-H]^-$ were not isolated, probably due to the sugar loss in HESI. Their quantification was thus performed by considering the ions corresponding to the aglyconic forms $[M-H-C_6H_{10}O_5]^-$ (Fig. 1). Consequently, quantification was performed with ions at *m/z* 165.0557 and *m/z* 191.0350 respectively, while ions at *m/z* 150.0321 $[M-H-C_6H_{10}O_5\text{-}CH_3]^-$ and *m/z* 176.0112 $[M-H-C_6H_{10}O_5\text{-}CH_3]^-$ were used for identification.

The efficiency of the on-line clean-up method described in reducing the matrix effect that can occur when analysing real samples was tested by spiking a wine at 8 levels with standards (calibration range 0.1–2 μg mL^{-1}) and comparing the linearity (R^2) of its calibration curves with those obtained by direct injection (sample directly injected into the analytical column, without the SPE clean-up step).

The linearity range of the selected SPE method was defined including concentration levels allowing a coefficient of determination (R^2) of at least 0.99. Limits of quantification (LOQs) were determined according to EURACHEM (EURACHEM Secretariat, 1993), and limits of detection (LODs) according to Mol, Van Dam, Zomer and Mulder (2011). Method precision (R.S.D.%) was estimated through standard deviation of 7 repetitions carried out subsequently at 13 concentration levels inside the working range. Method accuracy was evaluated in terms of relative recovery by spiking 4 wines with 1 μg mL^{-1} of each phenol and analytical results were corrected with recoveries.

Table 1
UHPLC–MS parameters of phenolic compounds.

Compounds	Supplier*	$[M-H]^-$ (m/z)	$\Delta m/z$	RT (min)	NCE	MS/MS fragments	R^2	Linearity range** (μg mL^{-1})	LOQ** (μg mL^{-1})
3.4-Xylenol	a	121.0658	1.2	13.73	90	119.0503, 96.9445	0.999	0.0100–4.98	0.0100
4-Allyl syringol	b	193.0870	1.0	14.85	10	178.0632, 163.0399	0.999	0.0202–10.1	0.0202
4-Ethylcatechol	c	137.0608	1.5	12.30	35	122.0374	0.993	0.0005–9.25	0.0005
4-Ethylguaiacol	d	151.0764	1.7	14.58	10	136.0529, 121.0293	0.999	0.0009–8.57	0.0009
4-Ethylphenol	a	121.0658	0.9	14.22	90	106.0423, 83.9854	0.999	0.1022–5.11	0.1022
4-Methylcatechol	a	123.0451	1.2	10.18	100	108.0214, 90.0591	0.998	0.0005–9.36	0.0005
4-Methylguaiacol	b	137.0608	1.4	14.37	35	122.0374	0.999	0.0105–9.59	0.0105
4-Methylsyringol	d	167.0713	1.4	12.87	20	152.0478, 137.0243	0.991	0.0101–5.06	0.0101
4-Vinylguaiacol	d	149.0608	1.7	14.00	20	134.0375, 87.0088	0.996	0.0055–9.83	0.0055
4-Vinylphenol	d	119.0502	0.1	13.60	100	91.0550, 93.0346	0.996	0.0112–5.60	0.0112
Aceto-/isoacetovanillone	b	165.0557	1.0	10.69	40	150.0321, 122.0371	0.999	0.0001–5.12	0.0001
Acetosyringone	b	195.0662	1.3	11.00	30	180.0426, 165.0190	0.998	0.0001–5.19	0.0001
Aesculetin	b	177.0193	1.4	8.48	50	133.0296, 105.0345	0.998	0.0001–9.72	0.0001
Caffeic acid	a	179.0350	1.5	6.60	40	135.0452	0.996	0.0001–5.33	0.0001
Catechin	a	289.0717	1.3	7.89	35	245.0805, 221.0812	0.999	0.0051–5.12	0.0051
Coniferyl alcohol	b	179.0714	1.4	10.11	35	164.0478, 121.0296	0.998	0.0107–5.35	0.0107
Coniferylaldehyde	b	177.0556	1.3	11.51	35	162.0320	0.998	0.0001–5.07	0.0001
Ellagic acid	a	300.9989	1.0	14.00	60	229.0149, 185.0071	0.967	3.03–5.05	3.03
Epicatechin	b	289.0718	1.7	9.67	40	245.0805, 221.0812	0.992	0.0001–9.02	0.0001
Ethyl vanillate	b	195.0662	1.3	13.69	40	180.0415, 130.9911	0.995	0.0006–9.90	0.0006
Ethylvanillin	a	165.0557	1.3	11.59	30	136.0152, 108.0202	0.994	0.0100–5.00	0.0100
Eugenol	a	163.0764	1.7	15.11	30	148.0529	0.998	0.0087–15.6	0.0087
Ferulic acid	a	193.0506	1.4	8.17	40	178.0268, 149.0608	0.997	0.0001–6.21	0.0001
Gallic acid	b	169.0142	1.5	5.60	45	125.0244	0.999	0.0001–8.80	0.0001
(Gentisic + protocatechuic) acid	a	153.0193	1.5	6.21	45	109.0295, 108.0217	0.999	0.0001–5.30	0.0001
Guaiacol	b	123.0451	1.0	12.85	70	108.0215, 105.0346	0.999	0.0110–9.88	0.0110
Homovanillic acid	b	181.0506	1.4	6.79	45	137.0617, 122.0373	0.988	0.0010–2.97	0.0010
Homovanillic alcohol	b	167.0714	1.2	8.78	35	152.0477, 122.0375	0.995	0.0051–5.10	0.0051
Hydroxytyrosol	e	153.0557	0.3	6.28	50	123.0437, 95.0487	0.990	0.0005–5.15	0.0005
Isoacetosyringone	f	195.0662	1.2	11.24	30	180.0426, 165.0190	0.998	0.0011–9.72	0.0011
Isoeugenol	b	163.0764	1.8	15.47	30	148.0529, 118.9925	0.999	0.0102–9.14	0.0102
Isopropiosyringone	f	209.0819	1.3	10.81	35	194.0581, 179.0348	0.997	0.0011–4.39	0.0011
Isopropiovanillone	b	179.0714	1.3	10.55	40	164.0477, 121.0295	0.996	0.0054–5.40	0.0054
Methyl vanillate	d	181.0506	1.3	12.13	40	166.0268, 151.0036	0.995	0.0005–9.27	0.0005
(*m* + *p*)-Cresol	b	107.0502	1.5	12.27	60	79.0551, 65.7207	0.999	0.1010–5.05	0.1010
o-Cresol	*b*	107.0502	1.5	12.41	60	82.5568	0.999	0.1170–5.85	0.1170
o-Vanillin	*b*	151.0401	1.3	12.09	40	136.0152, 123.0083	0.999	0.0010–4.98	0.0010
p-Carboxyphenol acid	*a*	137.0244	1.5	6.14	40	93.0646	0.997	0.0001–5.28	0.0001
p-Coumaric acid	*b*	163.0401	1.3	7.37	35	119.0502, 93.1266	0.993	0.0001–5.20	0.0001
Phenol	b	93.0345	0.0	7.73	100	65.0382	0.996	0.1050–9.49	0.1050
Pyrocatecol	b	109.0295	0.9	7.28	80	108.0202, 91.0176	0.992	0.0005–8.95	0.0005
Protocatechuic aldehyde	b	137.0244	1.6	7.10	60	108.0216, 93.0344	0.999	0.0001–5.05	0.0001
Salicylic acid	g	137.0244	1.4	7.72	60	93.0346, 122.0374	0.999	0.0001–8.85	0.0001
Scopoletin	a	191.0350	1.4	10.66	40	176.0112, 148.0166	0.993	0.0010–9.11	0.0010
Sinapinaldehyde	b	207.0663	1.3	11.64	35	192.0427, 177.0193	0.997	0.0010–5.04	0.0010
Sinapinic acid	a	223.0611	1.4	8.54	30	208.0373, 179.0714	0.999	0.0005–4.99	0.0005
Syringaldehyde	d	181.0506	1.5	10.42	40	166.0269, 151.0035	0.999	0.0008–13.5	0.0008
Syringic acid	a	197.0455	1.3	6.57	35	182.0216, 166.9984	0.989	0.0001–4.26	0.0001
Syringol	b	153.0557	1.4	11.32	50	138.0321, 123.0087	0.996	0.0129–6.46	0.0129
Tryptophol	a	160.0767	1.5	11.87	70	142.0659, 130.0660	0.997	0.1102–5.51	0.1102
Tyrosol	b	137.0608	1.5	6.79	40	119.0502, 106.0426	0.984	0.0001–3.15	0.0001
Vanillic acid	a	167.0350	1.0	6.42	40	152.0114, 123.0452	0.977	0.0001–3.04	0.0001
Vanillin	b	151.0401	1.6	9.86	40	136.0152, 108.0202	0.998	0.0001–5.36	0.0001
Vanillyl ethyl ether	f	181.0870	1.3	12.67	30	166.0633, 153.0656	0.992	0.0010–9.16	0.0010
Acetovanillone-glucoside	h	327.1085	1.5	8.40	20	165.0557, 150.0321	0.982	0.0140–4.20	0.0140
Aesculetin-glucoside	b	339.0722	2.2	6.79	35	177.0193, 133.0296	0.998	0.0055–5.50	0.0055
Scopoletin-glucoside	h	353.0878	2.0	8.60	20	191.0350, 176.0112	0.982	0.0090–2.70	0.0090
Vanillic acid-glucoside	h	329.0878	2.0	5.47	20	167.0350, 152.0114	0.995	0.0105–5.25	0.0105

Note: RT = retention time; $\Delta m/z$ = difference between expected and experimental masses (ppm); NCE = normalised collision energy; R^2 = coefficient of determination; LOQ = limit of quantitation.

* a = Fluka (St. Louis, MO, USA); b = Sigma Aldrich (St. Louis, MO, USA); c = Alfa Aesar (Karlsruhe, Germany); d = SAFC (St. Louis, MO, USA); e = CHEMOS GmbH (Regenstauf, Germany); f = TransMIT (Gießen, Germany); g = Aldrich (St. Louis, MO, USA); h = PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

** Linearity range and LOQs are defined without considering sample dilution.

2.6. Tentative identification of glycoconjugated simple phenols

Untargeted analysis of bound compounds was directed at finding the precursors of all the free simple phenols considered, including both monosaccharidic (hexoside, pentoside) and disaccharidic (hexoside–hexoside, pentoside–hexoside) forms. Tentative identification was based on accurate mass ($\Delta m/z < 5$ ppm), fragmentation profile and isotopic pattern. With reference to

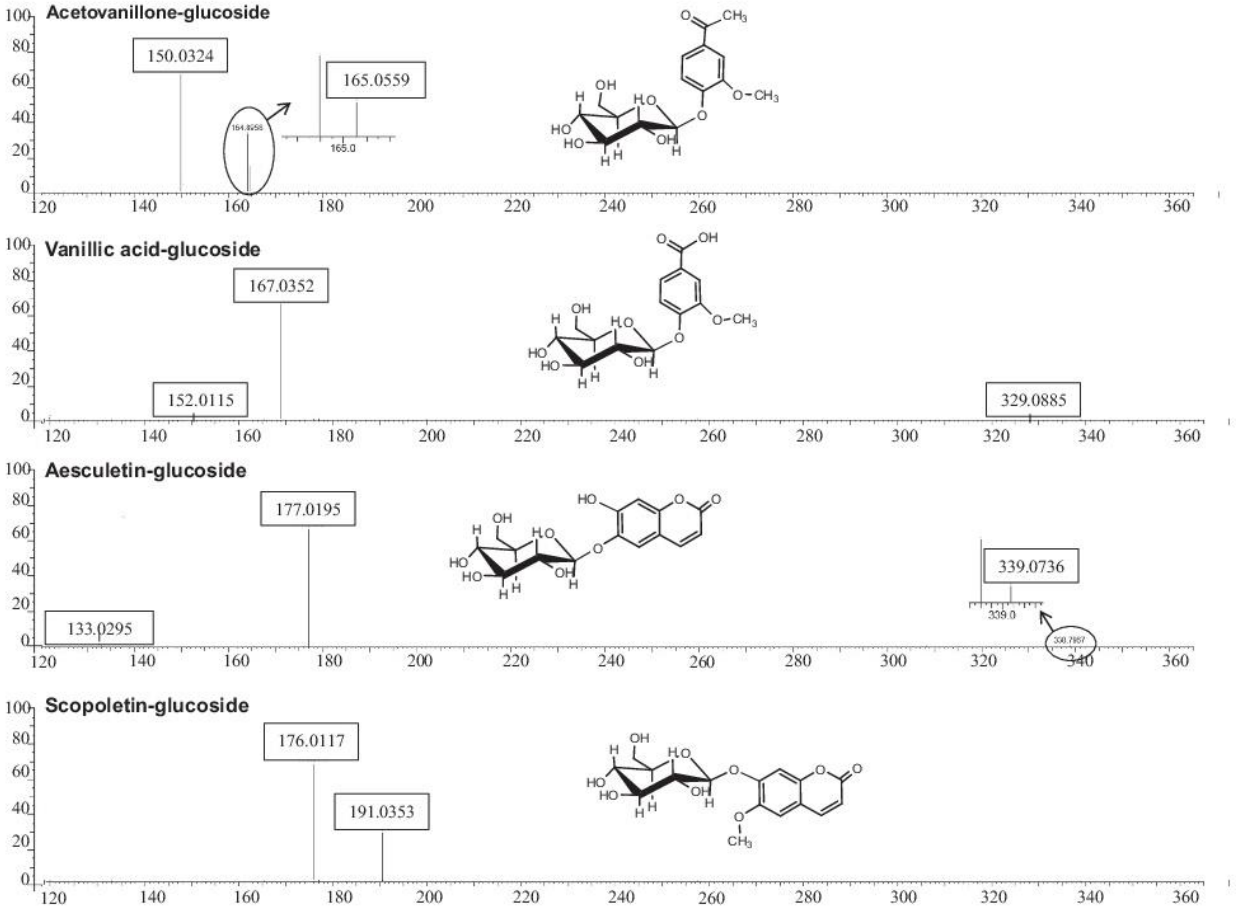


Fig. 1. Characteristic experimental fragmentation profile of four commercially available bound glucosylated simple phenols.

aesculetin-glucoside and vanillic acid-glucoside (Fig. 1), monosaccharidic precursor identification required the presence of the ions corresponding to $[M-H]^-$ and the loss of the sugar unit $[M-H-S]^-$ (S = -$C_6H_{10}O_5$, -hexoside; S = -$C_5H_8O_4$, -pentoside) in the mass spectrum of the peak detected in the extracted ion chromatograms. Disaccharidic precursors were tentatively identified, requiring the presence of the ions corresponding to $[M-H]^-$, the loss of one sugar unit $[M-H-S]^-$ and two sugar units $[M-H-S-S]^-$.

3. Results and discussion

3.1. Method optimisation

Due to the high selectivity of high resolution mass detection, perfect chromatographic separation was not required, except for target compounds with the same accurate mass. With the exception of isomer pairs of protocatechuic/gentisic acids, aceto-/isoacetovanillone and *m*- and *p*-cresol, determined as an isomeric sum, all the target compounds were individually quantified.

Mass spectra were acquired in negative ion mode to obtain greater target compound ionisation. Normalised collision energy (NCE, Table 1), used during dd-MS/MS experiments, was tuned for each target compound.

3.2. Method validation

Linearity range was defined from the LOQ to the highest standard concentration level allowing a R^2 value of at least 0.99. The only exceptions were tyrosol (0.984), vanillic acid (0.977), ellagic acid (0.967), acetovanillone-glucoside and scopoletin-glucoside (0.982). The linearity range varied from 2 orders of magnitude to 6 orders of magnitude. Linearity range, R^2 and LOQs are summarised in Table 1.

Acceptable recovery, ranging from 80% to 120%, was obtained for almost 90% of compounds. The remaining recovery ranged from 50% to 132% (vanillic acid-glucoside, 50%; scopoletin-glucoside, 68%; caffeic acid and ethylvanillin, 76%; sinapinaldehyde, 77%; acetovanillone-glucoside and coniferylaldehyde, 78%; ellagic acid, 132%). Method precision (R.S.D.%) was always lower than 12% for all phenols with concentrations over the LOQs.

3.3. Method application

The analytical UHPLC/HQ-OMS method, used in this work for the analysis of Primitivo di Manduria and Negroamaro wine samples, allowed the identification and quantification of 61 simple phenols, 4 of which were glucosidic precursors. Table 2 summarises the mean levels of target compounds in the two varieties and the standard deviations.

It also made it possible to tentatively identify 24 glycosylated simple phenols never previously described in these two varieties, as far as we know. Table 3 shows the chemical structure, expected mass $[M-H]^-$, $\Delta m/z$ and fragments of the phenolic precursors.

3.3.1. Target simple phenols

The mean content of simple phenols, both in the free and bound form, was generally similar in the two varieties. The only

Table 2
Phenolic compound content (mean, standard deviation; μg mL^{-1}) in selected monovarietal samples (Primitivo di Manduria wines, $N = 6$; Negroamaro, 6).

Compounds	Negroamaro		Primitivo di Manduria	
	Mean (μg mL^{-1})	SD	Mean (μg mL^{-1})	SD
3.4-Xylenol	<0.100	–	<0.100	–
4-Allyl syringol	<0.202	–	<0.202	–
4-Ethylcatechol	<0.005	–	<0.005	–
4-Ethylguaiacol	<0.009	–	<0.009	–
4-Ethylphenol	<1.02	–	<1.02	–
4-Methylcatechol	0.022	0.015	0.011	0.008
4-Methylguaiacol	<0.105	–	<0.105	–
4-Methylsyringol	<.0101	–	<.0101	–
4-Vinylguaiacol	<0.055	–	<0.055	–
4-Vinylphenol	<0.112	–	<0.112	–
Acetosyringone	0.049	0.034	0.053	0.051
Aceto-/isoacetovanillone	0.057	0.024	0.057	0.022
Aesculetin	0.160	0.123	0.096	0.027
Caffeic acid	6.54	1.60	6.64	1.16
Catechin	37.5	10.7	40.4	13.3
Coniferyl alcohol	0.146	0.291	<0.001	–
Coniferylaldehyde	<0.107	–	<0.107	–
Ellagic acid	<30.3	–	<30.3	–
Epicatechin	11.0	4.50	11.8	5.32
Ethyl vanillate	0.328	0.575	0.184	0.220
Ethylvanillin	<0.105	–	<0.105	–
Eugenol	<0.087	–	<0.087	–
Ferulic acid	0.310	0.047	0.344	0.050
Gallic acid	43.6	11.3	36.8	8.27
Gentisic + protocatechuic acids	1.12	0.196	1.09	0.380
Guaiacol	<0.110	–	<0.110	–
Homovanillic acid	0.789	0.162	1.21	0.549
Homovanillic alcohol	<0.051	–	<0.051	–
Hydroxytyrosol	2.05	0.440	1.73	0.569
Isoacetosiringone	0.006	0.003	<0.011	–
Isoeugenol	<0.102	–	<0.102	–
Isopropiosyringone	0.076	0.106	0.037	0.050
Isopropiovanillone	<0.054	–	0.065	0.085
(*m* + *p*)-Cresol	<1.01	–	<1.01	–
Methyl vanillate	1.18	0.530	0.845	0.296
o-Cresol	<1.17	–	<1.17	–
o-Vanillin	<0.010	–	<0.010	–
p-Carboxyphenol acid	0.578	0.276	0.464	0.205
p-Coumaric acid	5.03	4.57	4.71	2.87
Phenol	<1.05	–	<1.05	–
Pyrocatecol	<0.005	–	0.098	0.220
Protocatechuic aldehyde	0.002	–	0.002	–
Salicylic acid	0.116	0.136	0.139	0.061
Scopoletin	0.021	0.016	0.026	0.029
Sinapinaldehyde	<0.010	0.000	<0.010	0.000
Sinapinic acid	0.019	0.024	0.015	0.016
Syringaldehyde	0.214	0.421	0.255	0.530
Syringic acid	7.71	2.75	6.47	2.33
Syringol	<0.129	–	<0.129	–
Tryptophol	<1.102	0.600	1.10	0.874
Tyrosol	0.005	0.006	0.019	0.040
Vanillic acid	9.10	10.1	5.76	3.80
Vanillin	0.169	0.190	0.129	0.182
Vanillyl ethyl ether	<0.010	–	<0.010	–
Acetovanillone-glucoside	<0.140	–	<0.140	–
Aesculetin-glucoside	<0.055	–	<0.055	–
Scopoletin-glucoside	<0.090	–	<0.090	–
Vanillic acid-glucoside	0.147	0.111	0.161	0.072

Note: SD = standard deviation.

exceptions were aesculetin, coniferyl alcohol, ethyl vanillate, isopropiosyringone, vanillic acid and vanillin, which were present in slightly larger amounts in Negroamaro wines. Our results are confirmed by previous studies focusing on these varieties (Capone, Tufariello, & Siciliano, 2013a; Capone et al., 2013b; Tufariello, Capone, & Siciliano, 2012; Tufariello et al., 2014), from which it emerges that simple phenols cannot correctly differentiate Primitivo from Negroamaro.

The most abundant compounds were hydroxybenzoic acids (gallic, syringic and vanillic acid, respectively 37, 6.5 and 5.8 μg mL^{-1} in Primitivo, and 44, 7.7 and 9.1 μg mL^{-1} in Negroamaro) and hydroxycinnamic acids (caffeic and *p*-coumaric acids, respectively 6.6-6.5 and 4.7-5.0 μg mL^{-1} in both varieties).

Considering wines of different vintages, it was noted that catechin and epicatechin decreased during ageing in both the varieties: catechin by 60% in Negroamaro, comparing the most recent sample (2014 vintage) with the oldest one (2007), and by 66% in Primitivo, comparing the 2014 and 2006 vintages. Epicatechin decreased by 71% and 79% in Negroamaro and Primitivo respectively. This behaviour is consistent with the polymerisation reactions that take place during ageing.

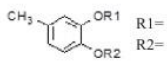
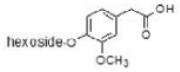
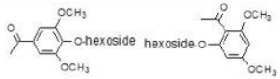
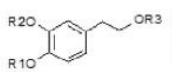
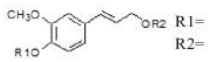
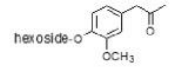
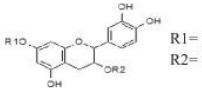
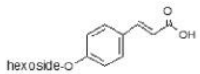
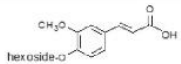
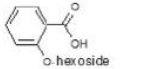
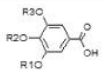
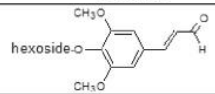
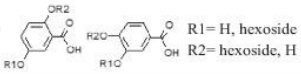
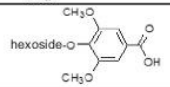
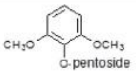
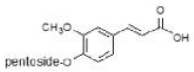
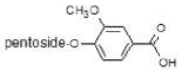
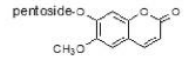
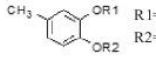
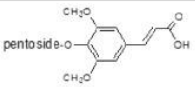
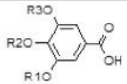
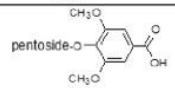
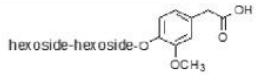
As regards glucosidic precursors, vanillic acid-glucoside, never previously found as far as we know, was present in both varieties, although it was slightly more abundant in Primitivo, while Negroamaro was definitely richer in free vanillic acid.

3.3.2. Untargeted simple phenols

To tentatively identify bound simple phenols, the accurate mass of deprotonated molecules and the ions corresponding to the loss of sugar units (one or two depending on whether they are mono- or disaccharides) should be present in the full mass spectrum. Our approach was defined on the basis of target bound phenol mass behaviour and compounds already tentatively identified in the literature in other matrices. *p*-coumaric acid-hexoside (*m*/*z* 325.0928, expected mass) has previously only been identified in Albariño grapes, tomatoes and beer (Di Lecce et al., 2014; Quifer-Rada et al., 2015; Vallverdú-Queralt, Jáuregui, Di Lecce, Andres-Lacueva, & Lamuela- Raventós, 2011; Vallverdú-Queralt, Jáuregui, Medina-Remón, Andres-Lacueva, & Lamuela- Raventós, 2010), homovanillic acid-hexoside (*m*/*z* 343.1034) in tomatoes (Vallverdú-Queralt et al., 2010, 2011), ferulic acid-hexoside (*m*/*z* 355.1034) in tomatoes and beer (Quifer-Rada et al., 2015; Vallverdú-Queralt et al., 2010, 2011), and gallic acid-hexoside (*m*/*z* 331.0670), gallic acid-dihexoside (*m*/*z* 493.1198) and epicatechin-hexoside (*m*/*z* 451.1245) in grapes (Di Lecce et al., 2014). Following the aforementioned criterion, the hexoside precursors of 4-methylcatechol (*m*/*z* 285.0979, expected mass), aceto-/isoacetosyringone (*m*/*z* 357.1191), coniferyl alcohol (*m*/*z* 341.1241), gentisic + protocatechuic acids (*m*/*z* 315.0721), hydroxytyrosol (*m*/*z* 315.1085), isopropiovanillone (*m*/*z* 341.1241), salicylic acid (*m*/*z* 299.0772), sinapinaldehyde (*m*/*z* 369.1191) and syringic acid (*m*/*z* 359.0983), and the dihexoside precursors of 4-methylcatechol (*m*/*z* 447.1508) and homovanillic acid (*m*/*z* 505.1562) were tentatively identified in wines for the first time, as far as we know, specifically in the Primitivo di Manduria and Negroamaro varieties (Table 3). Isomeric forms such as aceto-/isoacetosyringone-hexoside, gentisic/protocatechuic acid-hexoside and the coniferyl alcohol/isopropiovanillone-hexoside pair could not be distinguished, because they have the same accurate mass and fragments.

As regards pentoside phenolic precursors, the ions considered for tentative identification were $[M-H]^-$ and $[M-H-C_5H_8O_4]^-$. The pentoside precursors tentatively identified were aceto-/isoacetosyringone-pentoside (*m*/*z* 327.1085, expected mass), ferulic acid-pentoside (*m*/*z* 325.0928), scopoletin-pentoside (*m*/*z* 323.0772), sinapic acid-pentoside (*m*/*z* 355.1035), syring acid-pentoside (*m*/*z* 329.0878), syringol-pentoside (*m*/*z* 285.0979) and vanillic acid-pentoside (*m*/*z* 299.0772) (Table 3). The isomeric forms of acetosyringone- and isoacetosyringone-pentosides could not be distinguished because they have the same accurate mass and fragments. It was possible to distinguish the *p*-coumaric acid-hexoside/ferulic acid-pentoside, syringic acid-pentoside/vanillic acid-hexoside and salicylic acid-hexoside/vanillic acid-pentoside

Table 3
Chemical structure, expected mass $[M-H]^-$, difference between expected and experimental masses ($\Delta m/z$) and characteristic fragmentation profile of simple phenol glycosidic derivatives tentatively identified both in Primitivo di Manduria and Negroamaro wines.

Compounds (RT)	Chemical structure	MS fragments	Δ *m/z*	Compounds (RT)	Chemical structure	MS fragments	Δ *m/z*
4-methylcatechol-hexoside (7,47)	 R1= H, hexoside R2= hexoside, H	**285.0979**, 123.0451	0.3	homovanillic acid-hexoside (6,56)		**343.1035**, 181.0506	1.4
aceto-/isoacetosyringone-hexoside (8,74)		**357.1191**, 195.0662	0.7	hydroxytyrosol-hexoside (6,07)	 R_1= hexoside, H, H R_2= H, hexoside, H R_3= H, H, hexoside	**315.1085**, 153.0557	1.8
coniferyl alcohol-hexoside (9,68)	 R1= H, hexoside R2= hexoside, H	**341.1242**, 179.0714	0.9	isopropiovanillone-hexoside (9,68)		**341.1242**, 179.0714	0.9
epicatechin-hexoside (8,25)	 R1= H, hexoside R2= hexoside, H	**451.1246**, 289.0718	1.1	*p*-coumaric acid-hexoside (5,87)		**325.0929**, 163.0401	1.9
ferulic acid-hexoside (6,07)		**355.1035**, 193.0506, 149.0608	0.3	salicylic acid-hexoside (6,10)		**299.0772**, 137.0244	0.7
gallic acid-hexoside (5,55)	 R_1= hexoside, H, H R_2= H, hexoside, H R_3= H, H, hexoside	**331.0671**, 169.0142, 125.0244	0.9	sinapaldehyde-hexoside (10,85)		**369.1191**, 207.0663	0.7
gentisic/protocatechuic acid-hexoside (5,40)	 R1= H, hexoside R2= hexoside, H	**315.0722**, 153.0193, 109.0295	1.5	syringic acid-hexoside (5,50)		**359.0984**, 197.0455	1.8
aceto-/isoacetosyringone-pentoside (6,03)		**327.1085**, 195.0662	1.2	syringol-pentoside (7,50)		**285.0979**, 153.0557	0.8
ferulic acid-pentoside (5,87)		**325.0929**, 193.0506, 149.0608	1.8	vanillic acid-pentoside (6,11)		**299.0772**, 167.0350, 123.0452	0.7
scopoletin-pentoside (9,50)		**323.0772**, 191.0350	1.3	4-methylcatechol-dihexoside (9,60)	 R1= H, hexos.-hexos.; hexos. R2= hexos.-hexos., H; hexos.	**447.1508**, 285.0979, 123.0451	0.6
sinapic acid-pentoside (6,07)		**355.1035**, 223.0611	1.5	gallic acid-dihexoside (5,10)	 R1= S-S, H, H; H, S, S; R2= H, S-S, H; S, H, S; R3= H, H, S-S; S, S, H; S = hexoside	**493.1199**, 331.0670, 169.0142, 125.0244	0.4
syringic acid-pentoside (5,47)		**329.0878**, 197.0455	1.0	homovanillic acid-dihexoside (5,89)		**505.1563**, 343.1034, 181.0506	0.7

Note: RT = retention time in minute; MS fragments (m/z): in bold the expected mass corresponding to the deprotonated molecules $[M-H]^-$; $\Delta m/z$ = difference between expected and experimental masses (ppm). Bold lines separate hexoside, pentoside and hexoside–hexoside derivatives.

pairs, as they have the same accurate mass $[M^{-}H]^{-}$, but different fragments $[M-H-S]^{-}$, even if coeluted.

In relation to the identification of the glycosidic precursors of gallic acid, gentisic + protocatechuic acids, ferulic acid and vanillic acid, ions corresponding to the loss of CO_2 after the loss of the sugar units $[M-H-S-44]^{-}$, were used as further confirmation. Moreover, all the identification hypotheses were strengthened by limited differences between expected and experimental masses ($\Delta m/z < 3$ ppm, Table 3), and the matching of isotopic patterns, helped by SPE purification, which reduced the possibility of isobaric interference.

No disaccharidic precursors of the phenols studied were found in the pentoside–hexoside or hexoside–pentoside forms.

In conclusion, on the basis of data reported by Nonier, de Gaulejac, Vivas and Vitry (2005), the glycosidically bound simple phenols tentatively identified in the selected wines seemed to derive mainly from grapes. However, none of the phenolic glycosides that we identified in our study have been reported in previous studies specifically focusing on the free and bound aroma compounds of these two varieties, but which used indirect identification through GC-MS analysis of the hydrolysed bound forms (Fragasso et al., 2012; Tamborra & Esti, 2010; Toci et al., 2012)

4. Conclusions

The high selectivity of the mass spectrometer in determining the accurate mass of target compounds and the efficiency of SPE pretreatment in reducing matrix interference, made it possible to quantify sixty-one phenolic compounds in wines, four of which are phenolic glucosidic precursors, and for the first time to tentatively identify 24 new precursors of simple phenols as hexoside, pentoside and hexoside–hexoside derivatives. Profile fragmentation and the matching of isotopic patterns reinforced our identification hypotheses.

The adoption of new high resolution mass approach opens up new opportunities for the direct description of phenolic glycosidic profiles, offering an effective tool for interpreting nutraceutical and sensorial wine properties.

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Conclusion

In this work, the availability of an analytical method able to simultaneously quantify and tentatively identify free and glycosylated low-molecular-weight phenolic compounds made it possible to define the occurrence and profile of these compounds in red wines of different vintages. As regards the targeted approach, it was not possible to find phenolic compounds able to distinguish the two selected varieties, in agreement with what has been reported in the literature. However, it was possible to confirm that wine ageing can affect the phenolic profile through oxidation, condensation or polymerization reactions, since flavan-3-ols in particular decreased when comparing recent and older vintages. As regards the suspect screening approach, for the first time, as far as we know, this allowed structural characterization of low-molecular-weight phenolic glycosides occurring in selected wines. The profile did not seem to be affected by wine ageing and thus potential hydrolysis would seem to be general and widespread.

SECTION 2.2.7.

Targeted and untargeted characterisation of free and glycosylated simple phenols in cocoa beans using high resolution-tandem mass spectrometry (Q-Orbitrap)

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Aim of work

In order to extend the focus of my work to non-oenological food matrices, attention was paid to cocoa beans, since they are a worldwide commodity and the raw material of many cocoa-derived food products.

Different studies have revealed that consumption of cocoa-based products has positive effects on human health, because cocoa is considered a major dietary source of antioxidants due to its high content of phenolic compounds (Lamuela *et al.*, 2005; Tomás-Barberán *et al.*, 2007).

Considering that almost all the works about cocoa phenolic content reported in the literature concern flavonoids, the aim of this work was to investigate the nature and occurrence of free and glycosylated low-molecular-weight phenolic compounds in a wide selection of one of the most widespread global varieties of cocoa beans. Furthermore, the work aimed to evaluate the possibility of using selected compounds as markers for cocoa bean traceability.

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Targeted and untargeted characterisation of free and glycosylated simple phenols in cocoa beans using high resolution-tandem mass spectrometry (Q-Orbitrap)☆

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ABSTRACT

Free simple phenols have positive effects on health and influence the organoleptic profile of cocoa products, contributing towards defining their aroma and nutritional properties. Glycosidically bound simple phenols can be hydrolysed during the production phase to the corresponding free forms, and thus potentially contribute to the final sensory profile. In this work, 60 samples of *Forastero* cocoa beans from all over the world were analysed, combining on-line solid phase extraction (SPE) clean-up with ultra-high performance liquid chromatography (UHPLC), coupled with high resolution mass spectrometry. Operating in negative ion mode and with a heated electrospray, 62 simple phenols were measured, of which four were glucosidic precursors, with quantification limits ranging from 0.04 to 40 mg kg^{-1}, calibration R^2 of 0.99 for over 93% of compounds, and precision (R.S.D.%) always lower than 12%. On the basis of accurate mass, isotope pattern and MS/MS spectrum, 32 monoglycosylated simple phenols such as hexoside and pentoside precursors, and 14 diglycosylated simple phenols such as hexoside-hexoside, hexoside-pentoside and pentoside-hexoside precursors, were tentatively identified. The untargeted approach was validated using 3 glucosidic precursors synthesized by an external supplier. Honestly Significant Difference Tukey's test ($p < 0.05$) and Discriminant Analysis showed it was possible to distinguish the geographical origin of cocoa beans. In particular, the absolute free phenol profile made it possible to characterise 4 out of 5 production macro-areas well, while an untargeted approach based on the ionisation profiles of glycosylated forms allowed complete characterisation of all the 5 macro-areas.

1. Introduction

In the last few years increasing interest has been shown in naturally diffused compounds, previously generally considered to be non-nutritive substances. They are usually called "secondary plant products" [1], "phytochemicals" [2], or "chemo-preventers" [3], because they are products of plants' secondary metabolism [4] and they play a significant role in plant growth and protection [5].

Polyphenols make up a broad group of these compounds, being extensively present in plant food (fruit, vegetables, legumes, cereals, cocoa, etc.) and beverages (tea, cider, wine, beer, etc.) [5]. They are widely used in the food, cosmetics and pharmaceutical industries [5,6–9]. Polyphenols have aroused scientific interest thanks to their effect on health, due to anticarcinogenic, antiatherogenic, anti-ulcer, anti-thrombotic, anti-inflammatory, anti-allergenic, immune modulating, anti-microbial, vasodilatory and analgesic activities [10]. They achieve these biological effects by acting as antioxidants, chelators of divalent cations, enzyme inhibitors and modulators of enzymes [10].

In the class of polyphenols, simple phenols are low molecular weight compounds characterised by a simpler chemical structure and the same biological effects as more complex phenols [11–13]. They generally include hydroxybenzoic acids or their aldehydic derivatives, hydroxycinnamic acids and cinnamyl alcohols [14]. In nature, they can be found in both free and glycosidically bound forms, linked in the latter case to mono- or disaccharides. In monosaccharides, glucose is the most usual sugar residue but others include galactose, rhamnose and xylose [15]. In disaccharides, the hexose-hexose, hexose-pentose and pentose-hexose pairs can be identified, but there is little information about the specific sugar units making them up.

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Cocoa beans and products deriving from them, such as cocoa powder, dark chocolate and cocoa liqueurs, are particularly rich in polyphenols. The first evidence of cocoa's medicinal properties dates back to the Maya and Aztec era [16], to be better described in 16th century historical texts [17]. Medicinal use of cocoa and chocolate prevailed until the 19th century and only after 1930s did consumption shift towards confectionery [18]. Despite a few works documented in the 1930s [19], more detailed studies on cocoa polyphenolic content have only been carried out since 1995 [20].

Theobroma cacao, the cocoa tree belonging to the *Sterculiaceae* family, is an important crop in tropical regions of America, Africa and Asia [21]. Three cocoa varieties can be distinguished – Forastero, Criollo and Trinitario – but the former is the most widespread. Commercially, cocoa is produced from fermented, dried and roasted cocoa beans, and is consumed worldwide due to its almost unique flavour and aroma [22].

12–18% of the dry weight of unfermented cocoa beans is made up of phenolic compounds [23], 60% of which are flavanol monomers, epicatechin and catechin, and procyanidin oligomers [24]. Moreover, other polyphenolic compounds identified in cocoa include simple phenols, benzoquinones, phenolic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, phenylpropenes, coumarines, chromones, naphtoquinones, xanthones, stilbenes, anthraquinones, flavonoids, lignans and lignins [22].

The methods used for polyphenol determination include colourimetric methods [25], thin-layer chromatography [26], counter-current chromatography and gas chromatography [10]. However, HPLC methods are the most widely used, due to their high efficiency, high reproducibility and relatively short analysis time. Furthermore they do not require derivatisation and are easily coupled to different detectors. Compositional studies are also useful for fingerprinting and the geographical traceability of cocoa products [27]

The aim of this work was to further investigate the simple phenol composition of 60 samples of Forastero cocoa beans, providing quantitative information on over 50 aglycones and describing their possible glycosidic precursors. Our approach took advantage of tandem-high resolution mass spectrometry detection (Q-Orbitrap), combined with automatic on-line SPE clean up to reduce matrix interference, coupled with UHPLC.

2. Materials and methods

2.1. Reagents and solutions

LC–MS grade acetonitrile (ACN, 99.9%) and MS grade formic acid (98%) were purchased from Fluka (St. Louis, MO, USA), while *p*-nitrophenol (99%) was purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was produced using an Arium® Pro Lab Water System (Sartorius AG, Goettingen, Germany). Standards used for quantitative determination of free targeted phenolic compounds (Table 1) were prepared as reported by Barnaba and colleagues [28]. Aesculetin-glucoside (aesculetin-6-*O*-β-D-glucoside, 98%) was purchased from Sigma Aldrich (St. Louis, MO, USA), while vanillic acid-glucoside (vanillic acid-4-*O*-β-D-glucoside, 99%), acetovanillone-glucoside (acetovanillone-4-*O*-β-D-glucoside, 99%) and scopoletin-glucoside (scopoletin-7-*O*-β-D-glucoside, 99%) were supplied by PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Salicylic acid-glucoside (Salicylic acid-2-*O*-β-D-glucoside, 98%), orcinol-glucoside (98%) and *p*-hydroxybenzaldehyde-allopyranoside (4-formylphenyl beta-D-allopyranoside, 98%) were custom synthesized and supplied by TransMIT (Giessen, Germany). Three water-methanol stock solutions (20 mg kg^{-1} of each glycosylated phenol) were prepared by adding L-glutathione reduced and DL-dithiothreitol (2.5 g kg^{-1} each) as antioxidant agents. The organic solvent ranged from 10% to 40% according to the component's solubility. Stock solutions were used to prepare calibration solutions in the range 0.0001–1 mg kg^{-1}. Stock solutions were stored at −4 °C.

Instrument mass calibration was performed using a standard mixture of sodium dodecyl sulfate and sodium taurocholate (2.6 mg L^{-1} and 4.9 mg L^{-1} respectively; Pierce® ESI Negative Ion Calibration Solution, Rockford, IL, USA), with the addition of formic and acetic acids (5 mg L^{-1} each).

2.2. Samples and sample extraction

Sixty samples of fermented and dried *Forastero* cocoa beans were collected from Central America (CA; Cuba, Dominican Republic, Grenada, Mexico, Trinidad; N = 9), South America (SA; Brazil, Ecuador, Peru, Venezuela; N = 14), East Africa (EAF; Madagascar, Tanzania, Uganda; N = 8), West Africa (WAF; Congo, Ghana, Ivory Coast, Nigeria, São Tomé and Principe, Sierra Leone; N = 21) and Asia (AS; Indonesia, Java, Malaysia, Papua New Guinea; N = 8).

The cocoa beans were crumbled and sifted (0.5 mm) in order to obtain a uniform cocoa powder. The basic composition of selected compounds was evaluated considering residual moisture (105 °C, 24 h), carbon and nitrogen content (Dumas method), all expressed as a percentage of dried sample weight.

For analysis of free simple phenols, 50 mg of the obtained powder was extracted in 10 mL of water-methanol mixture (50:50, v/v, with the addition of formic acid 0.1%, v/v), dispersing the suspension with Ultra-Turrax (T 25 Basic, Ika® – Werke GmbH & Co. KG, Staufen, Germany) for 30 s and keeping the solution at 50 °C in an ultrasound bath (Labsonic LBS1–6L, FALC INSTRUMENTS, Treviglio, Italy) for 30 min. After centrifugation (30 min, 4000 rpm), the supernatant was filtered with 0.45 μm PTFE filter cartridges (Sartorius AG, Goettingen, Germany), diluted 2 times, and added of the internal standard (*p*-nitrophenol, 500 μg kg^{-1}) and formic acid (0.1%, v/v).

In order to detect glycosidically bound phenols, usually present at low levels as compared to corresponding free forms, 200 mg of sifted cocoa powder was used. Moreover, to avoid acidic hydrolysis of the glycosidic bond, formic acid was never used. All the other operational conditions were unchanged.

2.3. Chromatographic conditions

A Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), furnished with a Rheodyne 6-port automated switching valve, was used for chromatographic separation, slightly adapting the approach proposed by Barnaba and colleagues [28].

A HyperSep™ Retain PEP SPE cartridge (3.0 mm x 10 mm, 40–60 μm, Thermo Scientific, Sunnyvale, CA, USA), loaded with 2 μL of sample using deionised water at a flow rate of 0.250 mL min^{-1}, was used to perform the on-line SPE clean-up. After 4 min of matrix washing, the Rheodyne valve switched position and the analytical mobile phase (H_2O-ACN 95:5, flow rate of 0.400 mL min^{-1}) flowed through the SPE cartridge, progressively removing the retained analytes and transferring them to the analytical column (Acquity UPLC BEH C18, 2.1 mm x 100 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Chromatographic separation was performed in H_2O-ACN, setting the organic solvent concentration as follows: from 4.0 to 5.5 min, 5%; from 5.5 to 17 min it increased linearly to 60%; from 17.0 to 17.5 min it increased linearly to 100%; from 17.5 to 18.5, 100%. From 18.5 to 22.0 min, the analytical column was equilibrated with ACN at 5%, while the SPE cartridge was washed with formic acid (0.1%, v/v) aqueous solution/MeOH (50:50) for 1.5 min and equilibrated with formic acid

Table 1
UHPLC–MS parameters of targeted compounds (free and glycosidically bound phenols).

	RT (min)	$[M-H]^-$ (m/z)	Δ m/z	NCE	MS/MS fragments	R^2	LOQ (mg kg^{-1})	Linearity range (mg kg^{-1})	Recoveries (%)
gallic acid [b]	5.60	169.0142	0.6	45	125.0244	0.999	0.039	0.039–3520	83
protocatechuic acid [a]	5.90	153.0193	0.4	45	109.0295, 108.0217	0.999	0.042	0.042–2120	86
p-carboxyphenol [a]	6.14	137.0244	0.6	40	93.0646	0.997	0.042	0.042–2112	87
gentisic acid [a]	6.21	153.0193	0.4	45	109.0295, 108.0217	0.999	0.042	0.042–2120	98
hydroxytyrosol [e]	6.28	153.0557	−0.4	50	123.0437, 95.0487	0.990	0.206	0.206–2060	88
vanillic acid [a]	6.42	167.0350	0.1	40	152.0114, 123.0452	0.977	0.040	0.040–1216	95
syringic acid [a]	6.57	197.0455	0.7	35	182.0216, 166.9984	0.989	0.042	0.042–1704	91
caffeic acid [a]	6.60	179.0350	0.4	40	135.0452	0.996	0.043	0.043–2132	84
homovanillic acid [b]	6.79	181.0506	0.4	45	137.0617, 122.0373	0.988	0.396	0.396–1188	96
tyrosol [b]	6.79	137.0608	03	40	119.0502, 106.0426	0.984	0.042	0.042–1260	100
protocatechuic aldehyde [b]	7.10	137.0244	0.6	60	108.0216, 93.0344	0.999	0.040	0.040–2012	114
pyrocatecol [b]	7.28	109.0295	0.1	80	108.0202, 91.0176	0.992	0.199	0.199–3580	97
p-coumaric acid [b]	7.37	163.0401	0.4	35	119.0502, 93.1266	0.993	0.042	0.042–2080	90
salicylic acid [g]	7.72	137.0244	0.5	60	122.0374, 93.0346	0.999	0.039	0.039–3540	134
phenol [b]	7.73	93.0345	0.3	100	65.0382	0.996	42.0	42.0–3796	112
catechin [a]	7.89	289.0717	0.5	35	245.0805, 221.0812	0.999	2.05	2.05–2048	93
ferulic acid [a]	8.17	193.0506	0.2	40	178.0268, 149.0608	0.997	0.050	0.050–2484	91
aesculetin [b]	8.48	177.0193	0.4	50	133.0296, 105.0345	0.998	0.043	0.043–3888	96
sinapinic acid [a]	8.54	223.0611	0.5	30	208.0373, 179.0714	0.999	0.200	0.200–1996	89
4-hydroxybenzaldehyde [b]	8.67	121.0295	0.6	130	108.0218, 92.0267	0.993	4.02	4.02–2012	92
orcinol [b]	8.77	123.0451	0.4	60	81.0345, 79.0553	0.991	4.00	4.00–2000	97
homovanillic alcohol [b]	8.78	167.0714	−0.9	35	152.0477, 122.0375	0.995	2.04	2.04–2040	108
epicatechin [b]	9.67	289.0718	0.6	40	245.0805, 221.0812	0.992	0.040	0.040–3608	133
vanillin [b]	9.86	151.0401	0.4	40	136.0152, 108.0202	0.998	0.043	0.043–2144	86
coniferyl alcohol [b]	10.11	179.0714	0.1	35	164.0478, 121.0296	0.998	4.28	4.28–2140	88
4-methylcatechol [a]	10.18	123.0451	0.1	100	108.0214, 90.0591	0.998	0.208	0.208–3744	96
syringaldehyde [d]	10.42	181.0506	0.8	40	166.0269, 151.0035	0.999	0.300	0.300–5400	88
isopropiovanillone [b]	10.55	179.0714	0.4	40	164.0477, 121.0295	0.996	2.16	2.16–2160	102
scopoletin [a]	10.66	191.0350	0.7	40	176.0112, 148.0166	0.993	0.405	0.405–3644	83
aceto-/isoacetovanillone [b]	10.69	165.0557	0.4	40	150.0321, 122.0371	0.999	0.042	0.042–2076	97
isopropiosyringone [f]	10.81	209.0819	0.7	35	194.0581, 179.0348	0.997	0.439	0.439–1756	102
acetosyringone [b]	11.00	195.0662	0.7	30	180.0426, 165.0190	0.998	0.041	0.041–2048	92
isoacetosyringone [f]	11.24	195.0662	0.3	30	180.0426, 165.0190	0.998	0.432	0.432–3888	116
syringol [b]	11.32	153.0557	0.5	50	138.0321, 123.0087	0.996	5.17	5.17–2584	88
coniferylaldehyde [b]	11.51	177.0556	0.5	35	162.0320	0.998	0.040	0.040–2028	75
ethylvanillin [a]	11.59	165.0557	0.4	30	136.0152, 108.0202	0.994	4.00	4.00–2000	90
sinapinaldehyde [b]	11.64	207.0663	0.8	35	192.0427, 177.0193	0.997	0.403	0.403–2016	79
tryptophol [a]	11.87	160.0767	0.8	70	142.0659, 130.0660	0.997	44.1	44.1–2204	106
o-vanillin [b]	12.09	151.0401	0.6	40	136.0152, 123.0083	0.999	0.398	0.398–1992	110
methyl vanillate [d]	12.13	181.0506	0.8	40	166.0268, 151.0036	0.995	0.206	0.206–3708	96
(*m*+*p*)-cresol [b]	12.27	107.0502	0.4	60	79.0551, 65.7207	0.999	40.4	40.4–2020	123
4-ethylcatechol [c]	12.30	137.0608	0.5	35	122.0374	0.993	0.206	0.206–3700	102
o-cresol [b]	12.41	107.0502	0.4	60	82.5568	0.999	46.8	46.8–2340	123
vanillyl ethyl ether [f]	12.67	181.0870	0.5	30	166.0633, 153.0656	0.992	0.407	0.407–3664	110
guaiacol [b]	12.85	123.0451	−0.4	70	108.0215, 105.0346	0.999	4.39	4.39–3952	107
4-methylsyringol [d]	12.87	167.0713	0.4	20	152.0478, 137.0243	0.991	4.05	4.05–2024	95
4-vinylphenol [d]	13.60	119.0502	−1.2	100	91.0550, 93.0346	0.996	4.48	4.48–2240	117
ethyl vanillate [b]	13.69	195.0662	0.3	40	180.0415, 130.9911	0.995	0.220	0.220–3960	102
3.4-xylenol [a]	13.73	121.0658	−0.1	90	119.0503, 96.9445	0.999	3.98	3.98–1992	126
4-vinylguaiacol [d]	14.00	149.0608	0.5	20	134.0375, 87.0088	0.996	2.18	2.18–3932	130
4-ethylphenol [a]	14.22	121.0658	−0.1	90	106.0423, 83.9854	0.999	40.9	40.9–2044	127
4-methylguaiacol [b]	14.37	137.0608	0.4	35	122.0374	0.999	4.22	4.22–3836	122
4-ethylguaiacol [d]	14.58	151.0764	0.3	10	136.0529, 121.0293	0.999	0.343	0.343–3428	96
4-allyl syringol [b]	14.85	193.0870	0.4	10	178.0632, 163.0399	0.999	8.10	8.10–4040	103
eugenol [a]	15.11	163.0764	0.5	30	148.0529	0.998	3.48	3.48–6240	110
isoeugenol [b]	15.47	163.0764	0.5	30	148.0529, 118.9925	0.999	4.06	4.06–3656	113
vanillic acid-glucoside [h]	5.47	329.08781	0.5	20	167.0350, 152.0114	0.995	4.20	4.20–2011	85
aesculetin-glucoside [b]	6.79	339.07216	1.4	35	177.0193, 133.0296	0.998	2.20	2.20–2200	95
acetovanillone-glucoside [h]	8.40	327.10854	1.1	20	165.0557, 150.0321	0.982	5.60	5.60–1680	113
scopoletin-glucoside [h]	8.60	353.0878	0.9	20	191.0350, 176.0112	0.982	3.60	3.60–1080	97

Note: RT = retention time; Δ m/z = difference between expected and experimental masses (ppm); NCE = normalized collision energy; R^2 = coefficient of determination; LOQ = limit of quantitation. a = Fluka (St. Louis, MO, USA); b = Sigma Aldrich (St. Louis, MO, USA); c = Alfa Aesar (Karlsruhe, Germany); d = SAFC (St. Louis, MO, USA); e = CHEMOS GmbH (Regenstauf, Germany); f = TransMIT (Gießen, Germany); g = Aldrich (St. Louis, MO, USA); h = PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

aqueous solution (0.1%, v/v) for 2 min, in order to activate ureidic functions for the next analysis.

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software timed and controlled the injection system, switching valve and chromatographic gradient.

2.4. High resolution mass analysis

A Q-Exactive™ high-resolution tandem mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with a heated electrospray source (HESI-II), was used for mass analysis. Mass spectra were acquired in negative ion mode through a full MS-data dependent MS/MS experiment (full MS–dd MS/MS), adapting the method proposed by Barnaba and colleagues [29].

Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software was used for instrument control, Thermo Fisher Scientific TraceFinder™ software (Thermo Scientific, San Jose, CA, USA) was used for data processing and evaluation.

2.5. Targeted analysis: free and bound simple phenols

In order to identify and quantify all targeted compounds, full mass spectral data were selected. Matching of high-resolution m/z values (mass tolerance < 5 ppm [30]), retention times (RT) and isotope patterns was required when comparing sample analysis data and experiments performed on commercially available standards (56 free simple phenols and 4 glycosylated forms, Table 1). The conformity of sample dd-MS/MS spectra with those collected from available standards was used as further evidence of the presence of the targeted compounds in real matrices.

In the case of free simple phenols, the precursor ion detected in the extracted ion chromatograms (EICs) and corresponding to the deprotonated molecules [M-H] − was always used for quantification. Identification was performed considering the characteristic fragment ions of each compound chemical group, such as [M-H-44] − for hydroxybenzoic acids (loss of CO_2), [M-H-15] − for methoxyderivatives (loss of a methyl group) or [M-H-CH_2CHOH] − for catechin and epicatechin [28,30]. Table 1 summarises the exact mass, normalised collision energy (NCE) used for MS/MS experiments and fragment ions of targeted compounds.

As regards glycosidically bound phenols, aesculetin-glucoside and vanillic acid-glucoside were quantified respectively with ions at m/z 339.0721 and m/z 329.0878, corresponding to the deprotonated molecules [M-H] −. Ions corresponding to the loss of the glucosidic unit [M-H-$C_6H_{10}O_5$] −, respectively m/z 177.0193 and m/z 167.0350, and ions at m/z 133.0296, aesculetin-glucoside loss of CO_2 [M-H-$C_6H_{10}O_5$-44] −, and at m/z 152.0114, vanillic acid-glucoside loss of a methyl group [M-H-$C_6H_{10}O_5$-CH_3] −, were used for identification [29].

In the case of acetovanillone-glucoside and scopoletin-glucoside, precursor ions [M-H] − were not isolated, probably due to the sugar loss in HESI. Consequently, EICs ions corresponding to the aglyconic forms [M-H-$C_6H_{10}O_5$] −, m/z 165.0557 and m/z 191.0350 respectively, were used for quantification, taking advantage of different RTs in the free and bound forms (Fig. 1). Finally, ions at m/z 150.0321 [M-H-$C_6H_{10}O_5$-CH_3] − and m/z 176.0112 [M-H-$C_6H_{10}O_5$-CH_3] − were used for identification.

No ions characteristic of sugar residues were observed.

2.6. Tentative identification of glycoconjugated simple phenols

In order to investigate the presence of glycosidically bound precursors of all targeted free simple phenols, in the form of both monosaccharidic (hexoside, pentoside) and disaccharidic (hexoside-hexoside, pentoside-hexoside, hexoside-pentoside) derivatives, the accurate mass ($\Delta m/z$ < 5 ppm), isotope pattern and fragmentation profile needs to be verified.

As regards the fragmentation profile, the mass behaviour of aesculetin-glucoside and vanillic acid-glucoside was accepted as a reference. As in the case of these precursors the two characteristic ions [M-H] − and [M-H-$C_6H_{10}O_5$] − were also detected in the mass spectrum of the extracted chromatogram peaks, due to poor loss of sugar units in HESI. In the same way, their presence was required for identification of other monosaccharidic derivatives. Obviously, the loss of sugar in the case of pentosidic precursors should correspond to [M-H-$C_5H_8O_4$] −.

In order to identify disaccharidic precursors, the presence of ions corresponding to [M-H] −, to the loss of one sugar unit [M-H-S] − and the loss of two sugar units [M-H-S-S] − was required. The transitions [M-H-$C_6H_{10}O_5$] − → [M-H-$C_{12}H_{20}O_{10}$] −, [M-H-$C_6H_{10}O_5$] − → [M-H-$C_{11}H_{18}O_9$] − and [M-H-$C_5H_8O_4$] − → [M-H-$C_{11}H_{18}O_9$] − were considered characteristic of hexoside-hexoside, hexoside-pentoside and pentoside-hexoside derivatives respectively.

2.7. Method validation

External solvent calibration curves, obtained by drawing up graphs of the relative area ($A_{sample}/A_{internalstandard}$), were used for analyte quantification. Limits of quantification (LOQs) were established according to EURACHEM [31]. Standard levels allowing a regression coefficient (R^2) of at least 0.990 were included in the linearity range. The accuracy of the method was estimated in terms of relative recovery, determining the amount of the targeted compound as a percentage of the theoretical amount present in the matrix in four real samples spiked with a standard mix of all the 62 phenols at a concentration of 1 mg kg^{-1}, approximately doubling the original content. The final concentration of the internal standard was kept at 500 μg kg^{-1}. Method precision (R.S.D.%) was estimated through the standard deviation of 7 repeated analyses at 13 different concentration levels.

The capability identification of the untargeted approach, regarding the supposed retention times and fragmentations, was confirmed using three glycosylated phenols (salicylic acid-glucoside, orcinol-glucoside and *p*-hydroxybenzaldehyde-allopyranoside), expressly synthesised by an external laboratory for this purpose.

2.8. Statistical analysis

Statistical analysis was performed separately on free and glycosidically bound phenols, using Statistica 9.1 Software (StatSoft, 2010). As regards free phenols, statistical processing was carried out based on concentration values, fixing non-detectable data at half of the LOQ values, while for untargeted results, it was based on ionisation intensity, expressed as the peak area. In particular, the peak area was normalised to the sum of glycosidic precursors areas.

For both the targeted and untargeted approaches, a nonparametric statistical test (Kruskal-Wallis; $p < 0.05$) was performed with the entire dataset in order to characterise cocoa beans on the basis of the different geographical origins. Honestly Significant Difference HSD Tukey's test ($p < 0.05$) and Forward Stepwise Discriminant Analysis were also performed with data normally distributed or normalised by applying Box-Cox transformation, in order to obtain further evidence. The Neural Network approach was used to differentiate the geographical origin of samples, using the entire untargeted dataset.

3. Results and discussion

3.1. Basic composition of samples

The sixty selected samples of cocoa beans appeared to be almost homogenous in terms of their basic composition, independently of

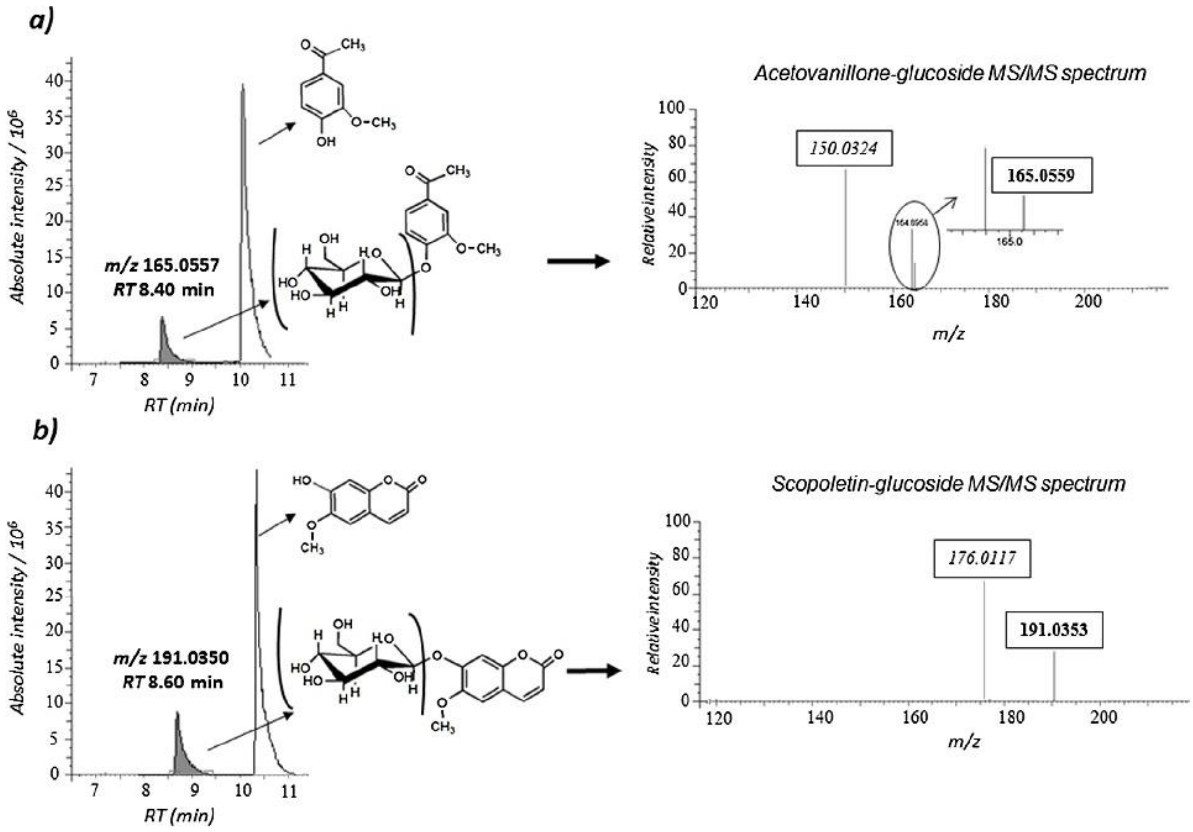

Fig. 1. Comparison of retention times for free and glucosidically bound forms. (*a*) The black peak corresponds to acetovanillone-glucoside after sugar loss in HESI, the hollow one to free acetovanillone. (*b*) The black peak corresponds to scopoletin-glucoside after HESI sugar loss, the hollow one to free scopoletin. On the right, the corresponding MS/MS spectrum of the glucosidically bound compound (in bold, the ion used for quantification; in italics, the ion used for identification).

their origin. Moisture ranged from 4.8% to 5.6%, while the elemental content of carbon and nitrogen was on average 60% (± 3%) and 2.5% (± 0.2%) respectively of dried sample weight. No significant differences (HSD Tukey's test, $p < 0.05$) were found between the origin macro-areas for these basic parameters, confirming the compositional homogeneity of the bean samples. Only CA and SA appeared to be significantly different in terms of nitrogen content.

3.2. Optimisation and validation of the analytical method

Considering the high selectivity of high-resolution mass detection (experimental Δ *m/z* values were below 3 ppm) and the chromatographic separation obtained, almost all the targeted compounds were individually quantified. The isomer pairs of aceto-/isoacetovanillone and *m*- and *p*-cresol were the only ones to be determined as an isomeric sum.

Negative ion mode ionisation allowed the greatest sensitivity. Normalised collision energy (NCE, Table 1) was tuned for each targeted compound in dd-MS/MS experiments.

As regards quantification, the linearity range varied from 2 to 6 orders of magnitude (2 orders of magnitude for 7% of compounds, 3 for 20%, 4 for 17%, 5 for 37%, and 6 for 17%). R^2 values were at least 0.99, with the exception of tyrosol (0.984), vanillic acid (0.977), acetovanillone-glucoside and scopoletin-glucoside (0.982). Linearity range, R^2 and LOQs are summarised in Table 1.

Acceptable recovery, ranging from 80% to 120%, was obtained for over 83% of compounds. The remaining recovery ranged from 122% to 134%, with one exception of 75% (see Table 1). Method precision (R.S.D.%) was always lower than 12% for all phenols with concentrations over the LOQs.

3.3. Method application

The analytical method described allowed the identification and quantification of 62 simple phenols, four of which were glucosidic precursors. Table 2 summarises the mean levels of targeted compounds in cocoa beans of different geographical origin and the standard deviations.

It also made it possible to tentatively identify 46 glycosilated simple phenols, both as mono- and disaccharidic derivatives, never previously described in this matrix as far as we know. Table 3 shows the retention time, accurate mass [M-H] – and fragment ions of the tentatively identified phenolic precursors.

3.3.1. Free simple phenols

Of the free targeted simple phenols, the most abundant were epicatechin (533–4787 mg kg^{-1}, Table 2; [32]), catechin (40.1–302 mg kg^{-1} [32]), hydroxybenzoic acids and their derivatives (gentisic acid, 18.6-59.4 mg kg^{-1}; protocatechuic acid, 2.20-22.2 mg kg^{-1}; vanillic acid, 4.57-20.5 mg kg^{-1}; salicylic acid, 2.37-21.1 mg kg^{-1}; protocatechuic aldehyde 9.27-32.9 mg kg^{-1} [32]), 4-vinylguaiacol (not detected-72.3 mg kg^{-1}) and coniferyl alcohol (not detected–29.2 mg kg^{-1}) and 4-ethylguaiacol (not detected-20.1 mg kg^{-1}). Pyrocatecol was found in 2 AS samples, aceto-/isoacetovanillone in 1 WAF and in 2 AS samples, and 4-ethylcatecol in 2 AS samples, while they were never detected in samples of other geographical origin. Homovanillic acid, 4-

Table 2
Phenolic compound content (min, median, max; mg kg^{-1}) in 60 cocoa beans (**SA** = South America, N = 14; **CA** = Central America, N = 9; **WAF** = West Africa, N = 21; **EAF** = East Africa, N = 8; **AS** = Asia, N = 8).

Compounds	SA			CA			WAF			EAF			AS		
	min	median	max	min	median	max	min	median	max	min	median	max	min	median	max
gallic acid	<0.039	<0.039	1.60	<0.039	<0.039	0.170	<0.039	<0.039	1.08	<0.039	<0.039	0.500	<0.039	<0.039	0.528
pyrocatecol	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	<0.199	0.780
p-carboxyphenol	0.558	1.44	2.37	2.24	2.52	3.01	0.684	2.53	3.66	0.635	1.25	1.72	0.885	2.55	4.39
gentisic acid	27.1	30.6	39.1	28.9	33.5	59.4	21.9	32.3	45.0	21.2	24.8	29.5	18.6	33.2	53.4
hydroxytyrosol	<0.206	<0.206	0.450	<0.206	<0.206	0.274	<0.206	<0.206	0.419	<0.206	<0.206	0.372	<0.206	<0.206	<0.206
vanillic acid	4.57	7.81	10.1	8.54	9.81	14.5	6.30	9.64	20.5	6.43	8.32	9.11	6.87	9.40	12.4
syringic acid	1.63	2.30	4.39	2.14	3.67	5.60	1.40	2.54	6.46	1.25	2.10	3.09	1.72	3.26	5.11
caffeic acid	<0.043	0.573	1.57	0.657	1.12	2.40	<0.043	0.419	1.27	<0.043	0.516	1.18	0.232	0.826	1.26
homovanillic acid	<0.396	<0.396	2.14	<0.396	<0.396	0.611	<0.396	<0.396	3.65	<0.396	<0.396	<0.396	<0.396	<0.396	1.46
tyrosol	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042
protocatechuic acid	2.78	9.29	16.8	5.19	7.99	14.7	4.75	10.2	22.2	2.20	7.82	21.6	4.50	7.35	13.8
protocatechualdehyde	14.3	16.3	21.2	15.4	17.9	32.9	11.2	17.2	24.6	10.9	12.9	15.7	9.27	17.7	29.4
p-coumaric acid	0.533	0.948	1.75	0.902	1.30	1.68	0.418	0.859	1.34	0.333	0.555	1.54	0.651	1.23	1.57
salicylic acid	2.97	8.48	12.6	2.37	9.41	21.1	3.13	8.81	19.7	5.48	5.82	11.4	3.93	7.35	11.4
phenol	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0	<42.0
catechin	87.2	170	245,0	102,0	146	302,0	46.1	95.6	197,0	40.1	115	134,0	84.7	146	193,0
ferulic acid	<0.050	0.075	0.168	0.065	0.096	0.160	<0.050	0.083	0.181	<0.050	0.051	0.137	<0.050	0.062	0.134
aesculetin	0.413	1.75	3.06	0.198	0.606	1.18	0.393	0.718	1.70	0.688	1.46	1.68	0.216	0.785	3.21
sinapic acid	<0.200	0.375	0.411	<0.200	0.374	0.394	<0.200	0.375	0.406	<0.200	0.386	0.396	<0.200	0.388	0.407
homovanillyl alcohol	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04	<2.04
epicatechin	1004	2700	4177	1206	1641	2110	1050	1524	2974	533	1601	2557	634	2222	4787
vanillin	<0.043	<0.043	0.806	<0.043	0.206	0.659	<0.043	0.159	0.631	<0.043	<0.043	0.145	<0.043	0.339	0.599
coniferyl alcohol	<4.28	10.4	22.1	<4.28	<4.28	23.4	<4.28	7.40	29.2	<4.28	8.71	13.8	<4.28	<4.28	9.77
4-methylcatechol	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208	<0.208
syringaldehyde	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300	<0.300
isopropiovanillone	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16	<2.16
scopoletin	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405	<0.405
aceto-/isoacetovanillone	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	<0.042	0.110	<0.042	<0.042	<0.042	<0.042	<0.042	0.184
isopropiosyringone	<0.439	<0.439	0.917	<0.439	0.578	0.861	<0.439	0.562	1.01	<0.439	0.478	0.889	<0.439	0.498	0.900
acetosyringone	<0.041	0.065	0.246	<0.041	<0.041	0.071	<0.041	0.059	0.240	<0.041	<0.041	0.103	<0.041	0.193	0.417
isoacetosyringone	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432	<0.432
syringol	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17	<5.17
coniferaldehyde	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	<0.040	0.086
ethylvanillin	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00
sinapinaldehyde	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403	<0.403
tryptophol	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1	<44.1
o-vanillin	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398	<0.398
methyl vanillate	<0.206	0.563	2.89	0.777	1.79	2.61	<0.206	0.466	4.14	<0.206	0.384	1.11	<0.206	0.625	2.13
(*m* + *p*)-cresol	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4	<40.4
4-ethylcatechol	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	<0.206	0.357
o-cresol	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8	<46.8
vanillyl ethyl ether	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407	<0.407
guaiacol	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39	<4.39
4-methylsyringol	<4.05	<4.05	<4.05	<4.05	<4.05	<4.05	<4.05	&lt4.05	<4.05	<4.05	<4.05	<4.05	<4.05	<4.05	<4.05
4-vinylphenol	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48	<4.48
ethyl vanillate	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220	<0.220
3.4-xylenol	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98	<3.98
4-vinylguaiacol	<2.18	<2.18	72.3	<2.18	<2.18	45.2	<2.18	<2.18	18.9	<2.18	<2.18	<2.18	<2.18	<2.18	25.1
4-ethylphenol	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9	<40.9
4-methylguaiacol	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22	<4.22
4-ethylguaiacol	<0.343	<0.343	19.5	<0.343	<0.343	19.4	<0.343	<0.343	20.1	<0.343	<0.343	<0.343	<0.343	<0.343	19.3
4-allylsyringol	<8.10	<8.10	<8.11	<8.10	<8.10	<8.10	<8.10	<8.10	<8.11	<8.10	<8.10	<8.10	<8.10	<8.10	<8.10
eugenol	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48	<3.48
isoeugenol	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06	<4.06
vanillic acid-glucoside	<4.20	53.7	104	<4.20	83.6	94.3	<4.20	76.2	136	<4.20	<4.20	87.5	<4.20	44.7	84.7
aesculetin-glucoside	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20	<2.20
acetovanillone-glucoside	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60	<5.60
scopoletin-glucoside	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60	<3.60

Note: min = minimum; max = maximun.

Table 3
UHPLC–MS parameters of glycosidically bound simple phenols tentatively identified in 60 samples of *Forastero* cocoa beans.

	Compounds	RT (min)	Exact mass $[M-H]^-$	MS/MS fragments	Δ *m/z*		Compounds	RT (min)	Exact mass $[M-H]^-$	MS/MS fragments	Δ *m/z*
	hexose derivatives					23–25	p-carboxyphenol-pent/protocatechuic aldeihyde-pent/salicylic acid-pent	5.57	269.0666	137.0244	0.9
1–3	p-carboxyphenol-hex/protocatechuic aldeihyde-hex/salicylic acid-glu	5.33	299.0772	137.0244	0.7	26	syringaldehyde-pent	5.57	313.0929	181.0506	0.8
4	gallic acid-hex	5.47	331.0670	169.0142	0.5	27	gallic acid-pent	5.67	301.0565	169.0142	0.6
5–6	gentisic acid-hex/protocatechuic acid-hex	5.51	315.0721	153.0193	0.6	28	ferulic acid-pent	5.77	325.0928	193.0506	0.7
7	syringic acid-hex	5.53	359.0983	197.0455	0.9	29	vanillin-pent	6.01	283.0823	151.0401	1.0
8	p-coumaric acid-hex	5.77	325.0928	163.0401	0.7	30–31	acetovanillone-pent/isoacetovanillone-pent	7.39	297.0979	165.0557	0.6
9	caffeic acid-hex	6.03	341.0878	179.0350	1.0	32	ethyl vanillate-pent	10.53	327.1085	195.0662	0.7
10	p-hydroxybenzaldehyde-all	6,10	283.0823	121.0295	0.8		*hexose-hexose derivatives*				
11	homovanillic acid-hex	6.26	343.1034	181.0506	0.8	33	4 methylcatechol-hex-hex	9.62	447.1508	285.0979, 123.0451	0.7
12	ethylvanillin-hex	6.36	327.1085	165.0557	0.7	34–36	p-carboxyphenol-hex-hex/protocatechuic aldeihyde-hex-hex/salicylic acid-hex-hex	5.55	461.1301	299.0772, 137.0244	0.7
13	catechin-hex	7.52	451.1245	289.0718	0.5	37	syringic acid-hex-hex	5.54	521.1512	359.0983, 197.0455	0.6
14	vanillin-hex	7.58	313.0928	151.0401	0.4	38	vanillic acid-hex-hex	5.51	491.1406	329.0878, 167.0350	0.8
15	epicatechin-hex	8.77	451.1245	289.0718	0.9		*hexose-pentose derivatives*				
16	4 methylsyringol-hex	10.53	329.1241	167.0713	0.8	39	caffeic acid-hex-pent	5.89	473.1301	341.0878, 179.0350	0.9
	pentose derivatives					40–41	gentisic acid-hex-pent/protocatechuic acid-hex-pent	5.53	447.1144	315.0721, 153.0193	0.5
17	vanillic acid-pent	5.33	299.0772	167.0350	1.1	42	vanillic acid-hex-pent	5.55	461.1301	299.0772, 167.0350	0.7
18	syringic acid-pent	5.47	329.0878	197.0455	0.5		*pentose-hexose derivatives*				
19–20	gentisic acid-pent/protocatechuic acid-pent	5.49	285.0615	153.0193	0.5	43	ethylvanillin-pent-hex	7.41	459.1508	297.0979, 165.0557	0.9
21	homovanillic acid-pent	5.57	313.0928	181.0506	0.7	44–45	gentisic acid-pent-hex/protocatechuic acid-pent-hex	5.53	447.1144	285.0615, 153.0193	0.5
22	methyl vanillate-pent	5.57	313.0928	181.0506	0.7	46	syringic acid-pent-hex	5.51	491.1406	329.0878, 197.0455	0.8

Note: hex = hexoside; glu = glucoside; all = allopyranoside; pent = pentoside; Δ *m/z* = difference between expected and experimental masses (ppm).

vinylguaiacol and 4-ethylguaiacol, never found in EAF samples, were detected only in a few samples (AS, N = 4, 2, 2; CA, N = 5, 2, 1; SA, N = 3, 5, 1; WAF, N = 9, 6, 1 respectively). Hydroxytyrosol, never found in AS samples, was detected only in a few samples (CA, N = 3; SA, 4; EAF, 1; WAF, 5).

According to the Kruskal-Wallis test ($p < 0.05$), SA samples were significantly different from WAF (for catechin, epicatechin and *p*-carboxyphenol), CA (for aesculetin and epicatechin) and EAF samples (for catechin). CA samples were significantly different from EAF (for gentisic, *p*-coumaric, protocatechuic and syringic acids) and WAF samples (for caffeic and *p*-coumaric acids). EAF samples were significantly different from WAF samples (for *p*-carboxyphenol).

Parametric statistical analysis (Honestly Significant Difference HSD Tukey's test, $p < 0.05$, and Forward Stepwise Discriminant Analysis) was performed on normally distributed compounds (caffeic acid, detected in 91.8% of samples; aesculetin, catechin, epicatechin, gentisic acid, *p*-carboxyphenol, *p*-coumaric acid, protocatechuic acid, protocatechuic aldehyde, salicylic acid, and syringic acid detected in 100% of samples) and on those normalised by applying Box-Cox transformation (ferulic acid, methyl vanillate and vanillic acid, detected in 70.5%, 73.8% and 100% of samples respectively; Kolmogorov-Smirnov test, $p < 0.05$).

According to HSD Tukey's test ($p < 0.05$), SA samples were significantly different from WAF (for aesculetin, catechin, epicatechin, *p*-carboxyphenol and vanillic acid), CA (for aesculetin, catechin, epicatechin and *p*-carboxyphenol), EAF (for catechin and epicatechin) and AS samples (for *p*-carboxyphenol). CA samples were significantly different from EAF (for *p*-carboxyphenol, caffeic and *p*-coumaric acids) and WAF samples (for caffeic and *p*-coumaric acids). EAF samples were significantly different from WAF and AS samples (for *p*-carboxyphenol).

Finally, the possibility of characterising SA, WAF, EAF and CA samples well in terms of composition, on the basis of their free simple phenol content, emerged from Forward Stepwise Discriminant Analysis (Rad. 1 explained 45.86% and Rad. 2 34.75% of total variability). As shown by the reclassification model, it was possible to reclassify over 70% of samples to the corresponding macro-areas of origin, with the highest rate for Central America (78%) and the lowest for West Africa (64%).

3.3.2. Glycosidically bound phenols

As regards targeted analysis of the 4 bound phenols, vanillic acid-glucoside was found in all cocoa bean samples at concentrations that ranged from not detected to 136 mg kg^{-1}, while aesculetin-glucoside, acetovanillone-glucoside and scopoletin-glucoside were never detected (Table 2).

As far as we know, this is the first time it has been possible to tentatively identify 32 new monoglycosilated simple phenol precursors (hexoside derivatives, N = 16; pentoside derivatives, 16) and 14 diglycosilated simple phenol precursors (hexoside–hexoside derivatives, N = 6; hexoside-pentoside derivatives, 4; pentoside-hexoside derivatives, 4) in cocoa beans using an untargeted approach. Compounds with no isobaric correspondence (2, 4, 7, 9, 11, 16, 17, 26, 27, 29, 33, 37, 39, 43; Table 3) were unequivocally identified. Compounds that have in common the precursor ion (8–28, 12–32, 14–21, vanillic acid-glucoside-18, 36–42, 38–46; Table 3) could be distinguished on the basis of the accurate mass of the corresponding aglycon, obtained after the loss of one or more sugar units in HESI. In the case of the 12–32 and 14–21 pairs, different peaks were detected for the same *m/z* value, and attribution was based on a similar shift in the retention time of the corresponding aglycons. In other cases the detected compounds were coeluted.

Finally, isobaric compounds (1–2-3, 5–6, 19–20, 21–22, 23–24-25, 34–35-36, 40–41, 44–45) having the precursor ion and aglycon in common could not be distinguished. The only exception were 13 and 15, which could be distinguished by the different retention times, tracing the elution order of the corresponding free forms. Furthermore, 40–41 and 44–45 could be distinguished on the basis of their fragmentation profile, due to the different *m/z* value of the ion corresponding to the loss of one sugar unit (315.0721 and 285.0615 *m/z* respectively; Table 3).

As regards untargeted profiling validation, the use of the synthesized standards made it possible to confirm the correct identification of salicylic acid-glucoside and *p*-hydroxybenzaldehyde-allopyranoside through the matching of accurate mass (Δ *m/z* = 0.53 and 0.51 ppm respectively), retention time (Δ = 0.02 and 0.05 min respectively), isotope pattern and characteristic fragmentation profile. As expected, the two characteristic ions [M-H] − and [M-H-$C_6H_{10}O_5$] −, *m/z* 299.0772 and *m/z* 137.0244 for salicylic acid-glucoside and *m/z* 283.0823 and *m/z* 121.0295 for *p*-hydroxybenzaldehyde-allopyranoside, were indeed detected in the mass spectrum of the extracted chromatogram peaks. As regards orcinol-glucoside, the precursor ion [M-H] − was not isolated, probably due to sugar loss in HESI, and the aglyconic form [M-H-$C_6H_{10}O_5$] − at *m/z* 121.0295 was used for identification, taking advantage of different RTs in the free and bound forms (8.67 and 6.10 min respectively). In conclusion, as it was impossible to detect the two characteristic ions [M-H] − and [M-H-$C_6H_{10}O_5$] −, it was confirmed that unfortunately any glycosidic precursors characterised by this mass behaviour would not be detected using this untargeted approach.

Quantification of these compounds using the untargeted approach was not possible. However, considering that all the samples were randomly processed in a single analytical batch, with one quality control sample for every 10 cocoa samples confirming the narrow repeatability of the analyte area (R.S.D. always <12%), peak areas were used to carry out statistical processing, as adopted in a similar metabolome approach [33].

According to the Kruskal-Wallis test ($p < 0.05$), AS samples were significantly different from EAF (for compounds 7, 12, 18, 43, 46 and vanillic acid-glucoside), CA (for compounds 12, 38 and 46), SA (for compounds 4, 5, 7, 9 and 40) and WAF samples (for compounds 42 and 46). CA samples were significantly different from EAF (for compounds 28 and 30), SA (for compounds 3, 5, 30, 42 and vanillic acid-glu) and WAF samples (for compounds 11 and 16). SA samples were significantly different from WAF (for compounds 4, 5, 9, 13, 18, 34, 36 and vanillic acid-glu) and EAF samples (for compounds 5, 18, 36, 43 and vanillic acid-glucoside).

As regards parametric statistical analysis (Honestly Significant Difference HSD Tukey's test, $p < 0.05$, and Forward Stepwise Discriminant Analysis), it was performed on the normally distributed compounds (32 detected in 55% of samples; 39 in 60%; 40, 41, 44, 45 in 68.3%; 33 in 78.3%; 43 in 90%; 23–25, 27, 34–36 in 93.3%; 38 in 91.7%; 42 in 95%; 14 in 96.7%; 4, 19–22, 26, 30, 31 and 46 in 98.3%; and 1–3, 7–8, 13, 15, 17–18, 28 and vanillic acid-glucoside detected in 100% of samples) and on those normalised by applying Box-Cox transformation (5/6, 9, 11, 12 and 16, detected in 100% of samples; Kolmogorov-Smirnov test, $p < 0.05$).

According to HSD Tukey's test ($p < 0.05$), AS samples were significantly different from EAF (for compounds 7, 12, 30, 42, 43, 46 and vanillic acid-glucoside), CA (for compounds 4, 15 and 46), SA (for compounds 4, 5, 7, 9, 30, 40, 43 and 44) and WAF samples (for compounds 7, 12, 46 and vanillic acid-glucoside). CA samples were significantly different from EAF (for compounds 12, 28 and 30), SA (for compounds 5, 30, 40, 44 and 46) and WAF samples (for compounds 4 and 11). SA samples were significantly different from WAF samples (for compounds 4, 5, 40, 44, 46 and vanillic acid-glucoside). SA samples were significantly different from EAF samples (for compounds 5, 40, 44 and vanillic acid-glucoside). EAF samples were significantly different from WAF samples (for compound 7). Finally, the possibility of characterising SA, CA, WAF, EAF and AS cocoa beans well in terms of composition, on the basis of the glycosylated simple phenol ionisation profile, emerged from Forward Stepwise Discriminant Analysis (Rad. 1 explained 45.15% and Rad. 2 30.14% of total variability). As shown by the reclassification model, it was possible to reclassify over 93% of samples to the corresponding macro-areas of origin, with the highest rate for Asia, South America and West Africa (100%) and the lowest for Central America (67%).

Surprisingly, the Neural Networks approach (training performance = 100; test performance = 89; validation performance = 100), was able to correctly reattribute 98.3% of the samples to the declared geographical area of production, although without providing indications of the most predictive compounds.

4. Conclusions

As a result of tandem high-resolution mass spectrometry detection and the efficiency of SPE pretreatment in reducing matrix interference, it was possible to extensively characterise the phenolic profile of a wide selection of cocoa beans produced in 5 international macro-areas. Using accurate mass, isotope pattern matching and the presence of specific fragmentation profiles, for the first time it was also possible to tentatively identify 46 new precursors of simple phenols as hexoside, pentoside, hexoside-hexoside, hexoside-pentoside and pentoside-hexoside derivatives. The free phenol content made it possible to provide individual descriptions of cocoa beans produced in SA, WAF, EAF and CA. However, the ionisation profile, obtained using an untargeted approach, was able to more specifically characterise cocoa beans produced in all the 5 geographical areas.

In conclusion, the proposed targeted and untargeted high-resolution mass approach represents a promising tool for detailed description of phenolic profiles and characterisation of the origin of cocoa products.

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Conclusion

In this work, the availability of a comprehensive method based on both a targeted and suspect screening approach allowed broad characterization of a wide selection of *Forastero* cocoa beans produced in 5 international macro-areas. In particular, it was possible to define their low-molecular-weight phenol content, together with their glycosylated phenolic profiles.

As regards sample preparation, cocoa beans underwent solvent extraction and were then directly analyzed, since use of an online SPE procedure ensured the elimination of matrix interference. This clean-up approach again proved to be an effective and well-performing tool for general application of the proposed analytical method, without any limitation in terms of analyzable matrices.

Finally, while the free phenolic profile allowed characterization of 4 out of 5 cocoa bean production macro-areas, the glycosylated profile distinguished all 5 macro-areas. Consequently, in the event of availability of analytical standards for low-molecular-weight phenolic glycosides, the proposed method could be used for routine investigation of cocoa bean and sub-product origin.

SECTION 2.2.8.

Polyphenolic profile, palynological analysis, mineral content and antioxidant properties of bee honeys from Uruguayan native plants

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Aim of work

Another attempt to extend the focus of my work to other non-oenological food matrices consisted in investigating the floral origin and the phenolic profile of several honeys typical of Uruguayan native plants. Honey composition is affected by the soil type and the climate condition of the area of production and its purity (mono-floral honey) is associated with major quality and highest commercial prices (Li *et al.*, 2017).

Honey is essentially a mixture of glucose, fructose, organic acids, amino acids, proteins, polyphenols, minerals and other less abundant compounds (Kečkeš *et al.*, 2013). However, its bioactivity is principally related to the presence of polyphenolic compounds that are commonly appreciated for their health-promoting effects (Bravo, 1998).

The aim of this work was to define the floral origin of selected Uruguayan honeys and to investigate the profile of free and glycosylated low-molecular weight phenolic compounds, by combining the Neutral Loss experiment with the suspect screening analysis.

Conclusion

In this work it was possible to define the floral origin of selected Uruguayan honeys through the pollen analysis, and to describe their free and glycosylated low-molecular weight phenolic profile through the combination of the Neutral Loss experiment and the suspect screening analysis.
The limit of this study was the impossibility to detect low abundant phenolic compounds or those with low ionization intensity, not for an intrinsic limit of the MS approach but for a problem caused by samples themselves. The composition of honeys and in particular the abundance of sugars caused a polarization of S-lens in the Q-Exactive instrument and a consequent reduction of its sensitivity (only the intervention of Service instrument solved the problem). Ionization in the negative polarity in fact is more instable than that in the positive polarity and is more affected by instrument cleaning status. For this reason, a general lowering of the ionization signal was observed in all samples, limiting the tentative identification of phenolic compounds only to those more abundant or with a higher ionization intensity. For the same reason, quantification of free phenolic compounds was prevented because the complexity of honey matrices caused suppression. The only way to overcome the problem was to normalize the ionization intensity of each signal to the ionization intensity of the total chromatogram. In this way in fact, the suppression effect of matrices was eliminated as well as the suppression caused by S-lens polarization. Consequently, this work could be considered a preliminary qualitative study about the free and glycosylated phenolic profile of selected Uruguayan honey, and so a starting point for a more promising study aiming at the quantification of markers for honey traceability in case of availability of standards and of more appropriate analytical condition

SECTION 2.2.9.

Targeted and untargeted profiling of alkaloids in herbal extracts using online solid-phase extraction and high-resolution mass spectrometry (Q-Orbitrap)

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Aim of work

In all previously reported works, tentative identification performed with the suspect screening approach was based on matching the experimental and theoretically supposed fragmentation and isotope pattern, together with detection of accurate mass with an error lower than 5 ppm. The aim of this work was to investigate an alternative suspect screening approach in which the tentative identification of compounds of interest was based on their detection in matrices known to be particularly rich in them in the literature. In particular, alkaloids in alpine herbal extracts were studied, since they are a group of nitrogenous basic compounds with hepatotoxic, mutagenic, and cancerogenic effects (Yanga et al., 2009; Schulz et al., 2015).

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Targeted and untargeted profiling of alkaloids in herbal extracts using online solid-phase extraction and high-resolution mass spectrometry (Q-Orbitrap)

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The biological activity of alkaloids (ALKs) and the different content of these natural products in herbs and plants have made them an attractive field for chemical studies.

A screening method automatically combining online solid-phase purification and concentration of samples with analysis using ultra-high performance liquid chromatography coupled with a hybrid quadrupole orbitrap mass spectrometer was developed and is reported in this paper. The proposed quantification method was validated for 35 ALKs with reference to pure analytical standards. A further 48 ALKs were identified on the basis of their accurate mass and characterised for chromatographic retention time and fragmentation profile, following their confirmation in extracts of herbs already well documented in the literature. More than 250 other untargeted ALKs were also tentatively identified using literature information, such as exact mass and isotopic pattern. The mass spectrometer operated in positive ion mode and mass spectra were acquired, with full MS-data-dependent MS/MS analysis (full MS–dd MS/MS) at a resolution of 140 000.

The method was linear up to an ALK concentration of 1000/3000 $\mu g\,l^{-1}$, with R^2 always >0.99 and limits of detection ranging between 0.04 and 10 $\mu g\,l^{-1}$. Accuracy, expressed as the recovery relative error, had a median value of 7.4%, and precision (relative standard deviation %) was generally lower than 10% throughout the quantitation range. The proposed method was then used to investigate the targeted and untargeted ALK profile of a selection of 18 alpine herbal plants, establishing that pyrrolizidine, pyrrolidine and piperidine ALKs were the most well represented.

Additional supporting information may be found in the online version of this article at the publisher's web site.

Keywords: alkaloids; herbal extracts; liquid chromatography; orbitrap; online solid-phase extraction

Introduction

In the last few decades, over 10 000 alkaloids [alkali-like; activity of alkaloids (ALKs)], an extremely varied group of natural, nitrogen-containing, basic organic compounds, have been isolated from natural sources, mainly in angiosperms (*Angiospermae* or *Magnoliophyta*).[1]

Activity of alkaloids have been classified into three principal classes depending on precursors and final molecular structures: atypical, typical and pseudo-ALKs. Typical and atypical ALKs derive from amino acids such as ornithine, arginine, lysine, histidine, phenylalanine and tyrosine.[2] Atypical ALKs are non-heterocyclic compounds, sometimes called 'proto-ALKs' or biological amines, while typical ALKs are heterocyclic compounds that can themselves be classified into the following main groups: pyrrole, pyrrolidine, tropane, pyrrolizidine, piperidine, quinoline, isoquinoline, aporphine, quinolizidine, indole, indolizidine, pyridine, imidazole and purine compounds, according to their ring structure.[3] The third class of molecules, pseudo-ALKs, are basic compounds not deriving from amino acids, to which diterpene and steroid groups belong.[4]

Although ALK functions in plants are not yet fully understood, and even if it has been suggested that they could simply be the waste products of plant metabolic processes,[5] their very differentiated chemical nature suggests that they fulfil various specific biological functions. In some plants, the concentration of ALKs increases just prior to seed formation and drops off later when the seed is ripe, suggesting that ALKs may play a triggering role in this process. Some evidence shows that ALKs actively protect plants against pathogen and herbivore attack[6,7] and that they can act as scavengers of reactive oxygen radicals, such as the singlet oxygen $^1O_2$, able to induce very damaging photodegradation processes in plant tissues.[8]

Moreover, in addition to fulfilling these specific functions in plants, ALKs often manifest a marked physiological action on humans and animals, acting very quickly on specific areas of their nervous system. Some of them are regarded as responsible for the beneficial effects of traditional medicines,[9–12] but some may instead have the harmful effects of poisons.[13,14] In particular,

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pyrrolizidine ALKs have hepatotoxic, mutagenic and carcinogenic effects, and in accordance with the German Federal Institute for Risk Assessment (BfR), a daily intake limit of 0.007 μg kg^{-1} body weight (0.42 μg for a 60-kg adult) was established for 1,2-unsaturated pyrrolizidine ALKs.[15]

Several determination methodologies have been developed to detect and quantify ALKs in different commodities. Meaningful examples include high-pressure liquid chromatography coupled with a diode-array detector[16] or a fluorimetric detector,[17] capillary electrophoresis[18] and also gas chromatography coupled with a mass spectrometer.[19,20] Methods using high-pressure liquid chromatography–diode-array detector/fluorimetric detector are not generally sensitive and selective enough to analyse ALKs in traces, while the main limitation of gas chromatography coupled with a mass spectrometer approaches is that ALKs cannot be directly analysed but require time-consuming preventive steps for derivatisation.

Last but not least, the complex matrix of plant or herbal extracts can definitely influence determination of ALKs, with suppression of the signal or false positive results. To overcome these problems, most analytical methods pretreat these samples using manual solid-phase extraction (SPE), although this purification step is time-consuming and cost-intensive.[21,22]

This work aimed to develop a method that would make it possible to investigate the broad profile of ALKs potentially present in herbal plants with a targeted and untargeted approach, by combining automatic online SPE clean up to reduce matrix interference and the rapid and selective detection ability of hybrid quadrupole orbitrap mass spectrometry.

Materials and methods

Reagents and solutions

LC-MS grade acetonitrile (ACN), LC-MS grade methanol (MeOH), MS grade formic acid (FA, 98%) and LC-MS grade ammonium acetate were purchased from Fluka (St. Louis, MO, USA), and ammonia solution 25% was purchased from Merk Millipore (Darmstadt, Germany). For mass calibration, a standard mix of n-butylamine, caffeine, MRFA and ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) were used. Deionised water was produced with an Arium Pro Lab Water System (Sartorius AG, Goettingen, Germany).

Table 1S shows the technical characteristics of commercial ALKs used to implement the target method. Individual stock solutions of each ALK were prepared by dissolving the standard in a 50% aqueous methanol solution to reach a final concentration of about 100 mg l^{-1}. An aliquot of 2 ml of the mix solution produced from the single stock solutions, with a final concentration of 3 mg l^{-1} of each single ALK, was transferred into an analytical vial and used for calibration in the range 0.02–3000 μg l^{-1}. The mix solution was prepared freshly before each analysis, while stock solutions were stored at −4 °C.

Plant sampling and sample extract preparation

Eighteen herbal plants of typical Italian alpine flora were collected directly from mountain pastures in northern Italy. Eight of them were selected on the basis of well-documented ALK composition in the literature. The whole plant was sampled and kept frozen (−10 °C) until required for analytical preparation. Table 1 summarises the botanical characteristics of the plant samples.

Table 1. Botanical characteristics of herbal samples

Species	Common name	Family
Cyclamen libanoticum	Cyclamen	Primulaceae
Convallaria majalis	Lily of the valley	Asparagaceae
Dryopteris filix-mas	Male fern	Dryopteridaceae
Phy0tolacca decandra	Pokeweed	Phytolaccaceae
Gelsemium sempervirens	Yellow jessamine	Gelsemiaceae
Hyoscyamus niger	Henbane	Solanaceae
Lactuca virosa	Wild lettuce	Asteraceae
Lobelia inflata	Indian tobacco	Campanulaceae
Solanum nigrum	Black nightshade	Solanaceae
Scrophularia nodosa	Figwort	Scrophulariaceae
Senecio vulgaris	Common groundsel	Senecionaeae
Datura stramonium	Jimson weed	Solanaceae
Arnica montana	Wolf's bane	Asteraceae
Trollius europaeus	Globeflower	Ranunculaceae
Ranunculus montanus	Mountain buttercup	Ranunculaceae
Rhododendron ferrugineum	Rhododendron	Ericaceae
Gentiana lutea	Great yellow gentian	Getianaceae
Hypericum perforatum	Perforate St John's-wort	Hypericaceae

Before ALK analysis, each solid plant sample was subjected to extraction using polyethylene 50 ml falcon tubes (Sartorius AG, Goettingen, Germany). A homogeneous aliquot of 2.5 g herb was added to 20 ml of extraction solution (H_2O/MeOH/FA; 44.5:44.5:1 v/v/v), sonicated for 10 min (LBS1 6Lt, FALC Instruments, Treviglio BG, Italy) and subjected to vertical shaking for 12 h at 20 rpm (Rotoshake 24/16, Gerhardt GmbH & Co. KG, Königswinter, Germany). The mixtures were once again sonicated for 10 min, and the methanolic extract was separated after centrifugation (10 min at 4100 rpm; IEC CL31 Multispeed, Thermo Scientific, Sunnyvale, CA, USA). Finally, the extract was filtered with a 0.45 μm cellulose filter cartridge (Sartorius AG, Goettingen, Germany) and diluted two times with an ammonia solution (pH = 10) before analysis.

Method development

SPE and chromatographic separation

Chromatographic separation was obtained using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA). A Rheodyne 6-port diverter valve allowed control of two independent fluid systems. The first system was dedicated to online SPE sample processing, while the second controlled chromatographic separation on the analytical column. Chromeleon 7.2 Chromatography Data System software (Thermo Scientific™ Dionex™) automatically piloted the switching valve and the chromatographic separation gradient. The autosampler was set at a temperature of 5 °C and the column at 35 °C.

In order to remove matrix interference, according to Barnaba *et al.*,[23] different SPE cartridges were tested: HyperSep Retain PEP, 3.0 mm × 10 mm, 40–60 μm; HyperSep Retain CX, 3.0 mm × 10 mm, 40–60 μm; HyperSep Hypercarb, 3.0 mm × 10 mm, 40–60 μm (Thermo Scientific, Sunnyvale, CA, USA) and SolEx HRP, 2.1 mm × 20 mm, 12–14 μm, hydrophilic divinylbenzene (ThermoFisher, Sunnyvale, CA, USA).

In order to improve the chromatographic separation of many isomeric ALK compounds, four columns were tested: Raptor Biphenyl, 3 mm × 150 mm, 2.7 μm particle size (Restek, Bellefonte, PA, USA),

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Kinetex PFP, 3 mm × 150 mm, 2.6 μm particle size; Synergi Fusion-RP, 2 mm × 100 mm, 2.5 μm particle size (Phenomenex, Torrance, CA, USA) and Acclaim Trinity P1, 2.1 mm × 100 mm, 3 μm particle size (Thermo Scientific, Sunnyvale, CA, USA).

Initially, the online SPE UHPLC system operated an injection of sample (1 μl) into the sample loop, being the Rheodyne 6-port diverter valve in position 1–6, while pump 1 flushed the SPE online cartridge with 100% eluent A (4% MeOH with ammonia to pH = 9) at 1 ml min^{-1} in order to promote the retaining of ALKs and discharge as much as possible of the interfering matrix. After 1 min, pump 1 switched to 100% eluent B (0.1% FA) and flushed the cartridge at 1 ml min^{-1} for another minute to complete matrix interference removal. In the meantime, pump 2 conditioned the analytical column at 0.7 ml min^{-1} with 70% of eluent C (0.1% FA with 5 mM ammonium acetate) and 30% of eluent D (MeOH/ACN, 95:5 v/v, with 0.1% FA and 5 mM ammonium acetate). Subsequently, the diverter valve was switched to position 1–2, and pump 2 eluted the retained analytes from the SPE cartridge to the analytical column in reverse-flow. Chromatographic separation was achieved with eluent D set at 30% from 2 to 4 min, then it was linearly increased to 80% from 4 to 25 min, and to 100% from 25 to 26 min. After 3 min at 100%, eluent D was linearly reduced to 30% in 0.5 min. Before each injection, the analytical column was equilibrated for 2.5 min at 30% eluent D with the initial conditions, meanwhile pump 1 flow was set to 0.1 ml min^{-1} and connected to the waste port. During the column rinse step, at 27 min, the valve was switched again to the initial 1–6 position, and pump 1 flushed the SPE cartridge with 100% eluent E (MeOH with 1% FA) at 1 ml min^{-1} in order to wash it, and then with 100% eluent A to re-equilibrate it before the next analysis.

Mass spectrometry

A Q-Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionisation (HESI-II) interface was used for ALK analysis. In the HESI-II source, nitrogen was used as the drying and collision gas in positive ion mode.

Heated electrospray ionisation tune parameters were set according to the literature, aiming to find an acceptable compromise for optimisation of all ALK molecules.[24] The heated capillary temperature was set at 330 °C, while the sheath gas flow rate was set at 30 arbitrary units, auxiliary gas flow rate at 10 arbitrary units, spray voltage at 3.5 kV and auxiliary gas heater temperature at 300 °C.

Mass spectra were acquired in profile mode through full MS data-dependent MS/MS analysis (full MS–dd MS/MS). Full mass spectra were recorded at a resolution of 140 000 full width at half-maximum (calculated for *m/z* 200, 1.5 Hz). The automatic gain control target was set at $5 \cdot 10^6$ ions, the maximum inject time at 100 ms, while data-dependent mass spectra were recorded at a resolution of 17 500 full width at half-maximum (defined for *m/z* 200, 12 Hz; automatic gain control target of $2 \cdot 10^5$ ions, inject time of 50 ms). In order to obtain high-quality data-dependent spectrograms, which could be used to compare the fragments generated with the reference ALK standards and in-house database confirmation fragments, an exclusion duration of 5 s was set in the dd-MS/MS experiment. This was the best compromise in order to avoid any loss of ionic fragment detection, the medium chromatographic peak width being generally 15–20 s. In order to obtain the most informative MS/MS spectra, containing both precursor ion and fragments, normalised collision energy for higher-energy collisional dissociation was optimised by direct infusion of each target compound. Accurate mass calibration was performed with the calibration solution, consisting of n-butylamine (*m/z* 74.09643), caffeine (*m/z* 195.08765), MRFA peptide (*m/z* 524.26496) and ultramark 1621 (characteristic masses: *m/z* 922.01035, 1022.00397, 1121.99758, 1221.99119, 1269.97235, 1321.98481, 1421.97842, 1521.97203, 1621.96564, 1721.95926, 1821.95287, 1921.94648, 2021.94013). Chromeleon 7.2 Chromatography Data System software was used for acquisition control and target ALK data processing. In addition, Thermo Fisher Scientific TraceFinder software (Thermo Scientific, San Jose, CA, USA) was used for untargeted ALK data processing.

Activity of alkaloids molecules were identified as having a mass accuracy of below 5 ppm. Variability in isotopic pattern recognition was required not to exceed 20%, with at least one of the expected ion fragments being present.

Target method validation

The characteristics of the target ALK method were studied using 35 pure standards. The linearity range was evaluated considering the linear regression between the signal response (peak area) and the nominal concentration of 11 increasing levels from 0.02 to 3000 μg l^{-1}, each replicated with seven different injections, for each ALK. The linearity range was defined as the maximum concentration allowing a correlation coefficient (R^2) higher than 0.99, starting from 0.02 μg l^{-1}. The limit of detection (LOD) was estimated as three standard deviations of ten replicated blank samples according to EURACHEM,[25] and similarly, the limit of quantification (LOQ) was estimated as ten standard deviations of the same replicates.

Precision was estimated as the relative standard deviation (RSD %) of seven analytical replicates of a blank sample spiked at three increasing concentration levels covering the quantitation range of each ALK: 1×LOQ (Low), 2×LOQ (medium) and 20×LOQ (high concentration) for protoveratrine A; (1×LOQ), (10×LOQ), (100×LOQ) for aconitine, α solamargine, α solanine, harmaline, senkirkin, sipeimine, solasodine, striknine and tomatidine/tomatine; (5×LOQ), (100×LOQ), (1000×LOQ) for coniine, echimidine, erucifoline, erucifoline-N-oxide, jacobine, jacobine-N-oxide, jervine, lycopsamine, retrorsine N-oxide and veratramine; (10×LOQ), (200×LOQ), (2000×LOQ) for α solasonine, heliotrine, hyoscyamine/atropine, lasiocarpine, monocrotaline, retrorsine, scopolamine, senecionine N-oxide, senecionine/seneciverine, senecionine, senecionine/seneciphylline and veratridine; (20×LOQ), (500×LOQ), (5000×LOQ) for gramine.

Method accuracy, expressed as relative error %, was estimated as the percentage difference between the expected and the returned mean concentration of the same blank sample, spiked at low, medium and high concentration levels, each one analytically replicated seven times.

Untargeted study

In order to develop an untargeted method useful for ALK profiling of commercial herbal products, initial putative confirmation of the retention time and fragmentation of 48 non-commercially available ALKs was performed, using eight plant samples with a well-documented ALK composition. Moreover, from systematic survey of the literature, it was possible to implement the untargeted method with detailed mass information, producing a final database of 305 ALKs.[24,26–37] ALK name, molecular

formula, parent *m/z* mass and, if present, *m/z* fragments are detailed in Tables 2S and 3S.

Results and discussion

SPE cartridge and analytical column optimisation

Different SPE columns and loading/cleaning phases were tested. As regards ALK loading on SPE, a methanolic aqueous phase adjusted with ammonia to pH 9.0 was used.[21] HyperSep Retain CX, HyperSep Retain PEP and HyperSep Hypercarb did not adequately retain ALKs with MeOH concentrations greater or equal to 4%, while SolEx HRP stopped all ALKs at 4%, although it did not retain coniine, monocrotaline and lasyocarpine at concentrations greater or equal to 5%. Moreover, the elution of ALKs from HyperSep Retain CX, HyperSep Retain PEP and HyperSep Hypercarb led to chromatographic separation with asymmetric and broad peaks, SolEx HRP being on the contrary characterised by the best chromatographic performance. As regards column cleaning and conditioning for preventing possible carryover between samples, effective SPE cartridge washing was obtained by fluxing for 2 min with a methanolic solution at 1% FA after ALK elution.

As regards chromatographic separation, Synergi Fusion-RP did not adequately retain ALKs, while with Acclaim Trinity P1, many chromatographic peaks were broad and irregular. Raptor Biphenyl and Kinetex PFP showed relatively similar performance and adequate chromatographic separation, but the former was more efficient and allowed better separation of isomeric compounds, although not all the target compounds could be individually isolated. The two structural isomers senecionine and senecivernine both eluted at 12.63 min, the two enantiomers atropine and hyoscyamine both eluted at 12.65 min, and tomatidine and its glycosylated precursor tomatine both eluted at 24.55 min. In particular, the parent ion of tomatine was not detected, probably because of sugar loss in HESI or because of a hydrolysis reaction already occurring in the vial, forcing its identification as its aglyconic form.

Targeted method

The method allowed the quantification of 35 ALKs, using linear calibration curves that always had correlation coefficients (R^2) higher than 0.99. The range of quantitation went from the quantification limits to 500 $\mu g\,l^{-1}$ for echimidine and α-solanine; to 1000 $\mu g\,l^{-1}$ for monocrotaline, lycopsamine, coniine, erucifoline, senecionine N-oxide, erucifoline-N-oxide, heliotrine, senkirkin, sipeimine, veratramine, α-solasonine and solasodine; to 1500 $\mu g\,l^{-1}$ for gramine, jacobine-N-oxide, retrorsine, retrorsine N-oxide, senecionine/senecivernine, jacobine α-solamargine, protoveratrine A, veratridine, aconitine, lasiocarpine and striknine; to 2000 $\mu g\,l^{-1}$ for scopolamine, seneciphylline, hyoscyamine/atropine, jervine, harmaline and tomatidine/tomatine.

Detectability was strongly dependant on the specific ionisation efficiency of each compound and the LOD ranged from the lowest values for heliotrine (0.04 $\mu g\,l^{-1}$), monocrotaline, senecionine N-oxide and gramine (0.05 $\mu g\,l^{-1}$), to the highest for harmaline, tomatidine/tomatine and protoveratrine A (3.49, 5.99 and 10.71 $\mu g\,l^{-1}$, respectively). The method characteristics, namely, linearity, LOD and LOQ determined for each target ALK compound, are shown in Table 2.

Within-run precision (RSD %) was investigated for each ALK at low, medium and high concentration levels (low, medium and high), covering the entire quantitation range (Table 2). Considering the overall group of ALKs, the median precision values were 2.99, 1.60 and 1.02% at the corresponding low, medium and high concentration, respectively, always being lower than 10%, with the exception of protoveratrine A and α-solamargine (15.4 and 13.3%, respectively, at the lowest concentrations).

Accuracy, evaluated in terms of relative errors for all ALKs, had median values of 17, 3.3 and 7.5, respectively, at the three increasing concentration levels (low, medium and high), with an overall figure of 7.4% over the entire range of quantitation (Table 2).

Untargeted ALK confirmation

To confirm the correct identification of tropane ALKs, analysis of *Datura stramonium* and *Hyoscyamus niger* was performed. *D. stramonium* is a very toxic plant in the Nightshade family (Solanaceae). The literature documents the presence of tropane ALKs: 3.6-diacetyltropine, 3-acetyltropine, apohyoscyamine, hyoscyamine and Tropinone.[34] In our experiments, hyoscyamine represented the highest signal and the retention time of 12.65 min, and fragmentation ions (*m/z* 124.1122 and *m/z* 93.0702) found in the untargeted approach were confirmed by standard analysis. The other molecules mentioned earlier, with the exclusion of apohyoscamine, were also detectable and chromatographically well separated, allowing the quadrupole to selectively isolate the masses of interest and ascertain the correct fragmentation pattern, while it was only possible to verify the retention time of 17.88 min for apohyoscyamine. The retention times confirmed for 3-acetyltropine, 3.6-diacetyltropine and tropinone were 3.79, 3.86 and 14.08 min, respectively.

H. niger, commonly known as henbane, black henbane or stinking nightshade, is another poisonous plant of the Solanaceae family, widely cultivated in Europe and Asia.[38] *H. niger* is also a known source of tropane ALKs, such as atropine, anisodamine and scopolamine.[37] We were able to confirm the presence of atropine and scopolamine using the analytical standards (RT = 12.65 min and 10.25, respectively), while for anisodamine, the retention time (10.36 min) and fragmentation profile were defined.

Steroidal and glycosteroidal ALKs were investigated in extracts of *Solanum nigrum* (Morella), common name Nightshade. *S. nigrum*, a weed native to Eurasia, belongs to the Solanum genus, the largest and most important in the Solanaceae family,[39] and was traditionally used for many disorders,[33] although recent studies have highlighted the acute toxicity of solanine, a neurotoxic glyco-ALK.[40] The ALKs β-solamargine, α-solamargine, α-solasonine and α-solanine are generally present in this plant at a level of a few mg g^{-1}.[33] Our plant extract analysis, also in comparison with the standard compounds, confirmed the presence of α-solamargine (20.00 min), α-Solanine (21.77 min) and α-solasonine (RT = 19.83 min). The retention time of β-solamargine was identified as 21.75 min, although the low intensity of the signal did not make it possible to confirm the fragmentation.

For piperidine-type ALKs, we analysed an extract of *Lobelia inflata*, a plant belonging to the Campanulaceae family. The most important ALK of this plant is lobeline, but it also contains 8,10-diethyllobelidiol, 8-ethyl-10-phenylnorlobelidione, 8-ethylnorlobelol, 8-methyl-10-phenyllobelidiol, allosedamine, isolobinanidine, isolobinine, lelobanidines I and II, lobelanine, lobelanidine, lobinaline, lobinanidine, lobinine, norallosedamine, norlelobanidine, norlobelanidine and norlobelanine.[29] However, in our study on *L. inflata* extracts, we could not detect 8-ethyl-10-henylnorlobelidione, allosedamine and norlobelanidine. The extracted ion chrogmatogram (EIC) referred to the two isomers,

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Table 2. Performance characteristics of the targeted alkaloids method

Compound	Linearity range (μg L^{-1})	R^2	LOD[a] (μg l^{-1})	LOQ[b] (μg l^{-1})	Precision (RSD%[c])			Accuracy (RE%[d])		
					Low conc.	Medium conc.	High conc.	Low conc.	Medium conc.	High conc.
Monocrotaline	0.17–1000	0.998	0.05	0.17	3.0	0.6	0.7	14.2	2.7	5.7
Lycopsamine	0.40–1000	0.998	0.12	0.4	1.0	0.6	1.0	15.8	3.4	5.1
Coniine	0.71–1000	0.998	0.21	0.71	3.2	1.4	1.2	27.7	3.3	5.3
Erucifoline	0.25–1000	0.998	0.07	0.25	2.0	0.8	0.6	7.4	2.1	6.8
Senecionine N-oxide	0.17–1000	0.997	0.05	0.17	2.3	1.9	1.2	11.9	5.0	8.2
Gramine	0.16–1500	0.998	0.05	0.16	3.0	1.8	1.3	21.4	1.6	2.0
Scopolamine	0.65–2000	0.996	0.20	0.65	1.6	1.0	1.1	16.4	3.3	9.1
Jacobine-N-oxide	0.94–1500	0.996	0.28	0.94	1.9	0.9	0.8	11.8	1.1	9.5
Erucifoline-N-oxide	1.18–1000	0.996	0.35	1.18	2.5	0.5	0.7	7.2	0.6	7.6
Heliotrine	0.14–1000	0.998	0.04	0.14	2.6	1.0	0.6	16.0	3.4	7.4
Retrorsine	0.83–1500	0.996	0.25	0.83	2.4	0.8	0.8	21.1	6.9	18.2
Seneciphylline	0.58–2000	0.996	0.14	0.56	1.7	1.1	1.0	10.4	3.1	7.8
Retrorsine N-oxide	1.43–1500	0.996	0.43	1.43	3.1	3.1	0.8	25.3	7.1	5.3
Senecionine/Senecivernine	0.46–1500	0.993	0.14	0.46	1.5	0.7	0.8	23.1	4.0	13.1
Hyoscyamine/Atropine	0.51–2000	0.994	0.15	0.51	1.4	1.1	0.6	20.6	1.9	8.6
Echimidine	0.25–500	0.999	0.08	0.25	4.9	1.3	0.7	18.0	1.7	5.8
Senkirkin	2.22–1000	0.998	0.66	2.22	1.5	0.9	0.8	13.6	1.8	6.5
Jacobine	0.95–1500	0.997	0.28	0.95	2.5	0.9	0.6	12.6	1.2	8.2
Lasiocarpine	0.41–1500	0.998	0.12	0.41	2.4	0.9	1.1	18.3	2.7	6.3
Striknine	1.86–1500	0.993	0.59	1.86	6.3	1.9	1.3	39.2	6.9	13.2
Harmaline	11.63–3000	0.996	3.49	11.6	8.3	6.0	2.0	34.7	5.9	8.7
Sipeimine	3.92–1000	0.998	1.18	3.92	4.2	2.9	0.8	9.9	4.7	8.1
Veratramine	0.59–1000	0.995	0.18	0.59	6.7	6.3	2.3	17.9	1.9	10.4
α-Solasonine	0.25–1000	1000	0.1	0.35	3.0	1.8	0.9	17.6	3.1	2.3
Jervine	0.86–2000	0.995	0.26	0.86	4.5	2.4	1.3	32.4	4.7	14.2
α-Solamargine	0.11–1500	0.999	2.73	9.1	13.3	2.9	1.1	28.6	2.4	1.0
Protoveratrine A	35.71–1500	0.996	10.7	35.7	15.4	6.4	3.7	45.0	13.7	2.9
Veratridine	0.45–1500	0.998	0.14	0.45	9.9	6.3	1.9	7.7	5.2	4.4
α-Solanine	4.26–500	0.998	1.28	4.26	5.4	2.5	4.1	53.0	8.6	7.2
Solasodine	2.16–1000	0.993	0.65	2.16	6.0	8.6	3.2	14.6	4.4	10.4
Aconitine	4.12–1500	0.999	1.24	4.12	2.3	1.8	0.7	14.6	1.6	3.3
Tomatidine/Tomatine	19.97–3000	0.991	5.99	20.0	5.0	7.9	2.5	2.9	5.2	12.5

[a]LOD, limit of detection.
[b]LOQ, limit of quantitation.
[c]RSD, relative standard deviation.
[d]RE%, relative error.

8-methyl-10-phenyllobelidiol and norlelobanidine in the literature, corresponded to three peaks, suggesting the existence of a possible unidentified isomer. In consideration of the strong similarity of the molecular ion fragmentation spectra with the most abundant fragment accurate mass *m/z* 156.1378, the three peaks were tentatively labelled as 8-methyl-10-phenyllobelidiol/norlelobanidine (RT = 15.53, 16.08 and 16.6 min). Similarly, retention times were putatively assigned to the two pairs of isomers isolobinine/lobinine and lelobanidine I/lelobanidine II (16.84 and 19.99; 16.91 and 17.41 min). The EIC of the two isomers isolobinanidine and lobinanidine corresponded to three peaks having similar data-dependent spectra and retention times – 16.23, 17.40 and 17.64 min, respectively – but the existence of a third isomer, β-lobinanidine, has also been confirmed by the literature.[41] The EICs of lobelanidine, lobelanine and lobeline showed two peaks for each of these ALKs (RT = 20.17, 20.86 min; 20.66, 21.15 and 20.23, 20.54, respectively). The presence of two *cis/trans* forms for lobelanidine is reported by Felpin and Lebreton,[29] making it reasonable to also surmise the existence of similar *cis/trans* isomers for lobelanine and lobeline. The EIC of 8,10-diethyllobelidiol showed two peaks at 12.05 and 13.07 min, suggesting the existence of other possible isomers, while the EICs of 8-ethylnorlobelol, lobinaline, norallosedamine and norlobelanine had only one peak at 5.60, 28.49, 19.76 and 20.89 min, respectively.

To confirm the correct identification of pyrrolizidine and pyrrolidine ALKs, *Senecio vulgaris* and *Arnica montana extracts* were analysed. *S. vulgaris*, a tenacious annual herb present in worldwide habitats, belongs to the Asteraceae family. It contains toxic pyrrolizidine ALKs such as integerrimine, integerrimine-N-oxide, retrorsine, riddelline, riddelline-N-oxide, senecionine, seneciphylline, seneciphylline N-oxide, spartioidine, spartioidine-N-oxide, usamarine and usamarine-N-oxide.[21,42] Retrorsine (RT = 10.73 min), seneciphylline (11.40 min) and senecionine (12.63 min) were confirmed in comparison with the analytical standards, while

riddelline-N-oxide and usamarine-N-oxide were not found. Because of the presence of several isobaric compounds, assignment of the correct retention times to other ALKs was sometimes very complicated. The EIC corresponding to senecionine, integerrimine and senecivernine showed only one chromatographic peak at 12.63 min in this extract, possibly of three coeluted ALKs. The EIC of the six isomers senecionine N-oxide (RT = 8.97 min), retrorsine (10.73 min), jacobine (14.05 min), integerrimine-N-oxide, senecivernine-N-oxide, and usamarine showed four peaks, the one at 11.22 min being different to those of the target compounds. Riddelline, seneciphylline N-oxide and spartioidine-N-oxide, with the same mass as erucifoline (RT = 8.44 min), showed a single chromatographic peak with a retention time of 9.52 min. Spartioidine showed one peak at a retention time of 11.39 min.

The medicinal herb *A. montana* belongs to the Asteraceae family and is endemic in Europe.

A study of the flower heads of different Arnica species showed the presence of two pyrrolidines, 2-pyrrolidineacetic acid and 2-pyrrolidineacetic methyl ester, and of eight pyrrolizidines, tussilagine, isotussilagine, 1-epimers of tussilagine and 1-epimers of isotussilagine, tussilaginic acid, isotussilaginic acid, 1-epimers of tussilaginic acid and 1epimers of isotussilaginic acid,[35] but unfortunately, only 2-pyrrolidineacetic acid was detected in our sample of *A. montana* at 3.51 min.

In order to confirm the correct identification of indole ALKs, we investigated extract of *Gelsemium sempervirens*, a climbing plant indigenous to the southern United States belonging to the Loganiaceae family. It contains extremely toxic ALKs, with gelsemine being the most abundant, but Gelsemicine the most toxic.[3] Other ALKs are 14,15-dihydroxygelsenicine, 16-epi-voacarpine, 19Z-16-epi-voacarpine, gelsemoxonine, gelsempervine-A, gelsempervine-B, gelsempervine-C, gelsempervine-D, gempervilam and sempervirine.[30,31] Our extract analysis did not find sempervilam and sempervirine. The EICs of the pair of isomers gelsempervine-A and gelsempervine-C, gelsempervine-B and gelsempervine-D, 19Z-16-epi-voacarpine and 16-epi-voacarpine each showed two peaks (RT = 15.32 and 15.62 min; 18.54 and 18.91; 16.30 and 17.40, respectively), whilst 14,15-dihydroxygelsenicine and gelsemoxonine showed a single peak at 28.37 min. Unfortunately, because of insufficient quadrupole selectivity, the fragmentation spectra of the latter two ALKs could not be defined. The EICs of gelsemine and gelsemicine showed a single peak at 11.85 and 19.59 min, respectively.

Finally, to confirm quinoline-type ALKs, we studied an extract of *Ranunculus montanus*, a plant in the Ranunculaceae family common in alpine meadows at high altitude. Magnoflorine, a positively charged molecule, previously quantified at 24 $\mu g\,g^{-1}$ in dry rhizomes of *Ranuncolus*,[36] was also confirmed to be present in our experiment, with a single peak at 15.04 min in the selected EIC. Fig. 1(a) and (b) shows the MS fragmentation profiles of ALKs that were not previously documented in literature.[43–47]

Table 3 shows accurate masses of the selected precursor ion and fragments, and the normalised collision energy of the detected targeted ($N = 35$) and untargeted (49) ALKs.

ALK characterisation of a selection of 18 alpine herbs

Targeted profile

Table 4 shows the content of the 35 ALKs quantified in the herbal extracts. The concentration of ALKs is relatively diversified in the plant species. Pyrrolizidines were the most commonly present ALKs (44% of samples), with concentrations generally ranging from 0.01 to 0.5 mg kg^{-1}, with the exception of retrorsine, senecionine/senecivernine and seneciphylline, which showed higher content in *S. vulgaris* (87, 179 and 246 mg kg^{-1}, respectively), confirming the documented presence of pyrrolizidine ALKs in many *Senecio* spp..[21,42,48] The most well represented was echimidine (present in 39% of samples) with an average concentration of 0.02 mg kg^{-1}, whereas erucifoline, erucifoline-N-oxide, jacobine-N-oxide, lasiocarpine, monocrotaline, seneciphylline and senkirkin were never present.

Important concentrations of tropane ALKs were detected in Solanaceae family plants, in particular, hyoscyamine/atropine and scopolamine (204 and 136 mg kg^{-1}, respectively) in *H. niger* and hyoscyamine/atropine (34 mg kg^{-1}) in *D. stramonium*.[34,37,49]

Glycosteroidal and steroidal ALKs were detected in 30% of samples, with concentrations ranging from 0.1 to 1.3 mg kg^{-1}, while jervine, tomatine/tomadine, sipeimine, veratrine, veratramine and protoveratrine A were never detected. The highest concentrations were found for solasodine (24 mg kg^{-1}) in *S. nigrum*.[50]

For indole ALKs, gramine was found in quantifiable amounts only in *H. niger* (0.05 mg kg^{-1}), whilst strychnine and harmaline were never present.

As regards diterpene ALKs, Aconitine was quantified only in *Phytolacca decandra*, while the piperidine ALK coniine was never present in our samples.

Untargeted profiling

In order to carry out characterisation of the herbal samples, tentative identification of other ALKs was performed through comparison with the previously mentioned database. The study of untargeted ALKs was limited to the compounds providing a sufficient detectable response (area > 100000 area units). For compounds whose recognition was based only on parent ion accurate mass and isotopic pattern, many chromatographic peaks at different retention times were sometimes found.

As many as 101 different ALKs were detected in untargeted analysis of the 18 herbs based on our 305-ALK database. The results are shown in Table 5, summarising the ALKs, sorted by chemical/botanical group. ALKs (at least one) belonging to the pyrrolizidine, pyrrolidine and piperidine groups were present in all the samples. Pyridines, quinolines, tropanes, protoALKs, indoles and quinazolines were widely present (from 83% to 50% of analysed samples), whilst isoquinoline, steroidal, pyrrole, imidazoline, azepine and aconitine-related ALKs were detected in less than 22% of plant samples.

In Solanaceae family plants (*D. stramonium*, *H. niger* and *S. nigrum*), 39 different ALKs were detected. Of these, piperidines ($N = 10$ ALKs), pyrrolizidines (7) and tropanes (4) were the most well-represented groups. For the single ALK, 2-pyrrolidineacetic acid, 3-acetyltropine, lobinaline, one isomer of lobinanidine/isolobinanidine/β-lobinanidine, magnoflorine, pycnarrhine and tropinone were always present in the three plants.

Lobelia inflata (Campanulaceae family) was the richest of these nitrogen active compounds, having as many as 37 ALKs. The most well-represented groups were piperidine ($N = 22$ ALKs; 8-ethylnorlobelol, 3 isomers of 8-methyl-10-phenyllobelidiol/norlelobanidine, two isomers of 8,10-diethyllobelidiol, two isomers of lelobanidine I/lelobanidine II, three isomers of lobinanidine/isolobinanidine/β-lobinanidine, two isomers of *cis*-lobelanidine/*trans*-lobelanidine, two isomers of

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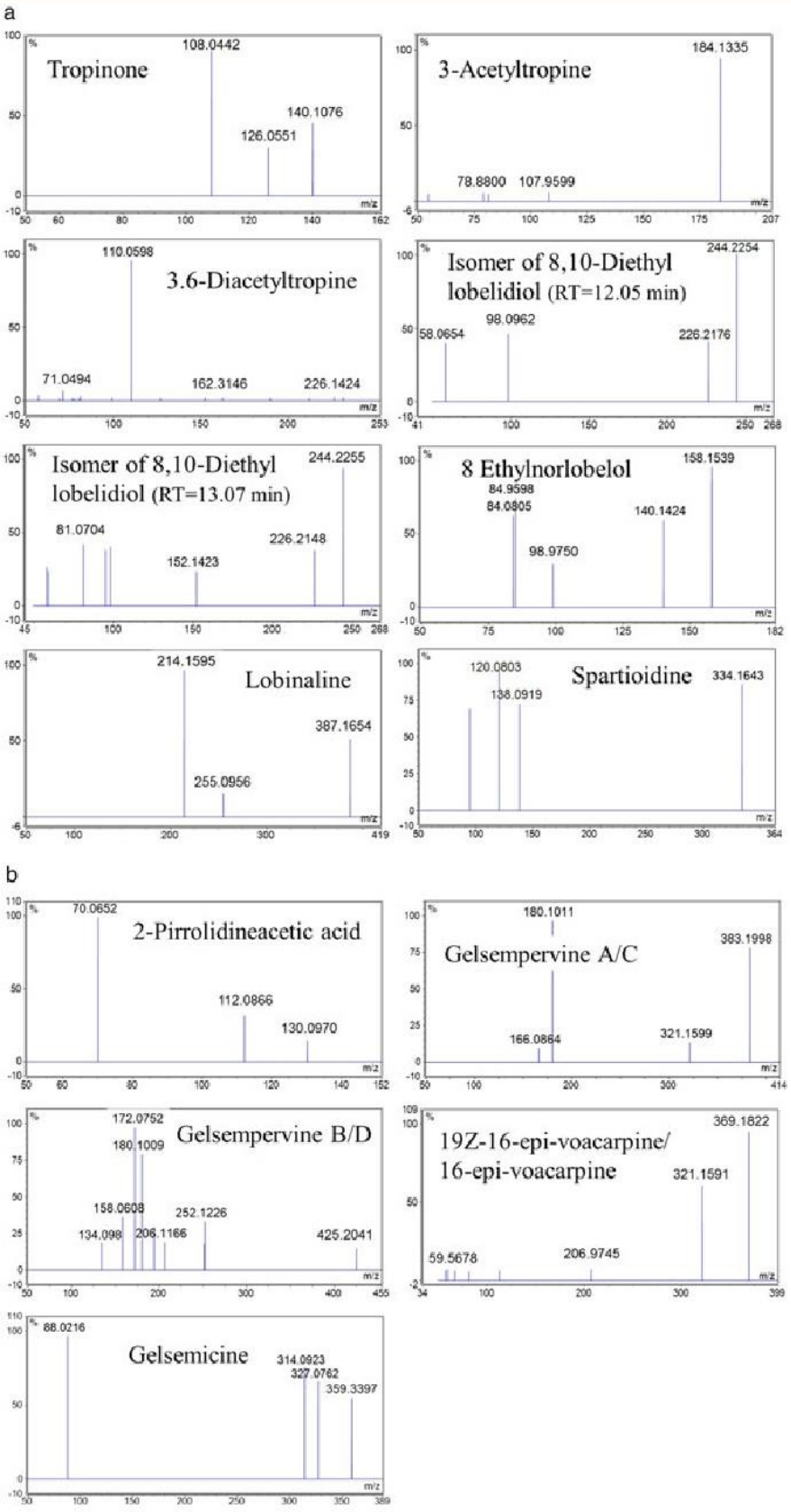

Figure 1. (a) MS fragmentation profiles of activity of alkaloids detected in eight alpine herb extracts. (b) MS fragmentation profiles of activity of alkaloids detected in eight alpine herb extracts.

Table 3. Retention time, precursor ion and fragments accurate masses of targeted and untargeted ALKs

Compound name	RT[a] (min)	$[M+H]^+$ (*m/z*)	Δ *m/z*[b] (ppm)	NCE[c]	MS/MS fragments (*m/z*)
Target compounds					
Monocrotaline	5.25	326.1594	1.23	60	120.0801; 121.0889; 94.0653
Lycopsamine	6.96	300.1796	3.00	30	138.0903; 94.0652
Coniine	7.59	128.1433	0.78	45	69.0700
Erucifoline	8.44	350.1587	3.06	60	20.0802; 94.0652; 67.0549
Senecionine N-oxide	8.97	352.1750	1.42	50	120.0802; 155.1054; 122.0960
Gramine	9.28	175.1228	1.14	30	130.0655; 113.0590
Scopolamine	10.25	304.1537	1.97	30	138.0903; 156.1018; 121.065
Jacobine-N-oxide	10.52	368.1694	2.72	50	120.0804; 296.1477; 119.0732
Erucifoline-N-oxide	10.66	366.1540	1.91	60	94.0652; 118.0654; 119.0730
Heliotrine	10.71	314.1956	1.91	30	138.091; 156.1017
Retrorsine	10.73	352.1750	1.42	50	120.0803; 94.0652; 165.0136
Seneciphylline	11.40	334.1641	2.39	50	120.0804; 94.0653; 306.1704
Retrorsine N-oxide	11.89	368.1694	2.72	60	94.0652; 120.0803
Senecionine/Senecivernine	12.63	336.1795	3.24	50	120.0803;138.0902; 94.0652
Hyoscyamine/Atropine	12.65	290.1746	1.72	45	124.1122; 93.0702
Echimidine	13.64	398.2169	1.00	30	120.0803; 84.0491; 220.1317
Senkirkin	13.94	366.1906	1.37	30	168.1023; 122.0595; 150.0937
Jacobine	14.05	352.1749	1.70	50	118.0652; 120.0803; 136.0759
Lasiocarpine	15.80	412.2325	1.21	30	120.0803; 220.1314; 336.1808
Striknine	16.33	335.1746	2.39	60	184.0703; 222.0962
Harmaline	16.42	215.1175	1.86	50	200.0931; 174.0902
Sipeimine	17.55	430.3309	1.63	50	138.1274; 214.1384
Veratramine	19.68	410.3050	0.97	30	295.2022; 84.0824
α-Solasonine	19.83	884.4976	2.94	50	85.0288; 71.0498; 157.1012
Jervine	19.85	426.3000	0.70	30	126.1372; 313.2073
α-Solamargine	20.00	868.5041	1.38	45	85.0288; 71.0498
Protoveratrine A	20.62	740.4309	1.51	50	658.3609; 436.6457
Veratridine	21.65	674.3515	2.97	60	456.2718; 165.0534; 438.2591
α-Solanine	21.77	868.5041	1.38	50	98.0966; 398.3385
Solasodine	23.60	414.3355	2.90	60	157.1017; 70.0658; 159.1158
Aconitine	23.68	646.3195	4.02	50	105.0330; 368.1839
Tomatidine/Tomatine	24.54	416.3520[d]	1.44	60	161.1319; 70.0658; 147.1161
Untargeted compounds					
2-Pyrrolidineacetic acid	3.51	130.0861	1.54	30	70.0652; 112.0866
3-Acetyltropine	3.79	184.1329	1.63	30	107.9599; 78.8800
3,6-Diacetyltropine	3.86	226.1433	2.21	30	110.0598; 71.0494
8-Ethylnorlobelol	5.60	158.1536	1.90	30	84.9598; 140.1424; 98.9750
Usamarine/Integerrimine-N-oxide/Senecivernine-N-oxide/Senecionine N-oxide	8.97	352.1748	1.99	50	120.0805; 324.1392; 138.0918
Riddelline/Seneciphylline N-oxide/Spartioidine-N-oxide	9.52	350.1591	2.00	30	120.0809; 138.0901; 322.1627
Anisodamine	10.36	306.1690	3.27	50	140.1060; 122.0965
Usamarine/Integerrimine-N-oxide/Senecivernine-N-oxide/Retrorsine	10.73	352.1747	2.27	50	94.0660; 138.0918; 120.0805
Usaramine/Integerrimine-N-oxide/Senecivernine-N-oxide	11.22	352.1747	2.27	50	120.0815; 352.1751; 138.0918
Spartioidine	11.39	334.1642	2.09	30	120.0803; 138.0919
Gelsemine	11.85	323.1743	3.47	30	70.0654; 236.1065
8,10-Diethyllobelidiol	12.05	244.2262	3.69	60	98.0962; 226.2176; 58.0654
Integerrimine/Senecionine/Senecivernine	12.63	336.1798	2.38	50	120.0815; 138.0919; 94.0659
8,10-Diethyllobelidiol	13.07	244.2264	2.87	60	81.0704; 226.2148; 152.1423
Usamarine/Integerrimine-N-oxide/Senecivernine-N-oxide/Jacobine	14.05	352.1750	1.42	50	94.0652; 120.0805; 138.0919
Tropinone	14.08	140.1067	2.14	30	108.0442; 126.0551
Magnoflorine	15.04	342.1692[e]	2.34	30	297.1140; 265.0847; 58.0657
Gelsempervine-A/Gelsempervine-C	15.32	383.1958	1.83	30	180.1011; 321.1599; 166.0864
8-Methyl-10-phenyllobelidiol/Norlelobanidine	15.53	278.2108	2.52	30	156.1376; 138.1270

(Continues)

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Table 3. *(Continued)*

Compound name	RT[a] (min)	$[M+H]^+$ (*m/z*)	Δ *m/z*[b] (ppm)	NCE[c]	MS/MS fragments (*m/z*)
Gelsempervine-A/Gelsempervine-C	15.62	383.1958	1.83	30	180.1015; 321.1600; 166.0864
8-Methyl-10-phenyllobelidiol/Norlelobanidine	16.08	278.2110	1.80	30	156.1378; 171.1374; 202.1578
Lobinanidine, Isolobinanidine, β-Lobinanidine	16.23	290.2108	2.41	30	168.1375; 96.0807; 272.1996
19Z-16-epi-Voacarpine/16-epi-Voacarpine	16.30	369.1802	1.90	30	321.1591; 206.9745; 112.7368
8-Methyl-10-phenyllobelidiol/Norlelobanidine	16.60	278.2109	2.16	30	156.1378; 171.1370; 138.1269
Lobinine/Isolobinine	16.84	288.1952	2.08	30	162.0893; 111.0802; 99.0440
Lelobanidine I/Lelobanidine II	16.91	292.2263	2.40	40	170.1540; 202.1580; 98.0961
Lobinanidine, Isolobinanidine, β-Lobinanidine	17.40	290.2109	2.07	30	50.0655; 164.1063; 200.1426
19Z-16-epi-Voacarpine/16-epi-Voacarpine	17.40	272.1638	1.35	30	321.1591; 206.9745; 112.7370
Lelobanidine I/Lelobanidine II	17.41	425.2063	2.74	40	170.1538; 202.1580; 98.0959
Lobinanidine, Isolobinanidine, β-Lobinanidine	17.64	425.2064	2.07	30	50.0655; 168.1375; 200.1425
Apohyoscyamine	17.88	359.1957	2.57	50	—
Gelsempervine-B/Gelsempervine-D	18.54	206.1536	1.88	40	172.0752; 180.1009; 158.0608
Gelsempervine-B/Gelsempervine-D	18.91	288.1952	1.65	40	172.0752; 180.1009; 158.0608
Gelsemicine	19.59	340.2264	2.23	30	88.0216; 314.0923
Norallosedamine	19.76	338.2107	1.46	30	84.0384; 122.0964; 105.0699
Lobinine/Isolobinine	19.99	338.2107	2.08	30	162.0893; 111.0802; 99.0440
cis-Lobelanidine/*trans*-Lobelanidine	20.17	336.1952	2.06	30	202.1584; 218.15441; 98.0962
cis-Lobeline/*trans*-Lobeline	20.23	340.2263	2.37	30	216.1361; 96.0808
cis-Lobeline/*trans*-Lobeline	20.54	322.1794	2.37	30	216.1511; 216.1386; 96.0808
cis-Lobelanine/*trans*-Lobelanine	20.66	336.1956	1.78	30	96.0814; 216.1378; 290.1744
cis-Lobelanidine/*trans*-Lobelanidine	20.86	868.5055	2.35	30	218.1544; 202.1583; 98.0961
Norlobelanine	20.89	359.1612	2.48	30	202.1223; 82.0657; 171.1392
cis-Lobelanine/*trans*-Lobelanine	21.15	387.2787	0.59	30	96.0813; 216.1380
β-Solamargine	21.75	292.2263	0.23	45	—
Gelsemoxonine/14,15-Dihydroxygelsenicine	28.37	290.2109	2.78	30	—
Lobinaline	28.49	272.1638	2.07	30	214.1595; 255.0956

[a]RT, retention time.
[b]Δ *m/z* (ppm), error of accurate mass respect to exact mass.
[c]NCE, normalised collision energy.
[d]Tomatine, $[M+H\text{-}C_{23}H_{38}O_{19}]^+$.
[e]$[M]^+$.

cis-lobelanine/*trans*-lobelanine, two isomers of *cis*-lobeline/*trans*-lobeline, lobinaline, two isomers of lobinine/isolobinine, norallosedamine and norlobelanine), as already reported in the literature[29] and tropane ALKs (6; 3,6-diacetyltropine, 3-acetyltropine, anisodamine, apohyoscyamine, bellendin and tropinone).

In *S. vulgaris* (Senecionaeae family), 36 ALKs were found. According to the literature,[21,42,48] the most well-represented groups were pyrrolizidine (*N* = 16 ALKs; acetylerucifoline-N-oxide, dehydrojaconine, jacoline, jacoline-N-oxide, jacozine-N-oxide, junceine, monocrotaline N-oxide, riddelline-N-oxide, spartioidine, trichodesmine, one isomer of riddelline/seneciphylline N-oxide/spartioidine-N-oxide, four isomers of usamarine/integerrimine-N-oxide/jacobine/retrorsine/senecionine/senecivernine-N-oxide and one isomer of integerrimine/senecionine/senecivernine) and tropane (8; 3,6-diacetyltropine, 3-acetyltropine, arecoline, ecgonine, ferruginine, scopine, tropinone and valerine).

Twenty-nine ALKs were detected in *Convallaria majalis* (Asparagaceae family), these being pyperidine (*N* = 5 ALKs; one isomer of lelobanidine I/lelobanidine II, one isomer of lobinaline, one isomer of lobinanidine/isolobinanidine/β-lobinanidine, one isomer of lobinine/isolobinine and one isomer of lobinine/isolobinine), tropane ALKs (*N* = 4; 3-acetyltropine, anisodamine, ferruginine and tropinone) and pyrrolizidine (*N* = 3; jacoline-N-oxide, monocrotaline-N-oxide and spartioidine) being the most well represented.

Ranunculaceae (*R. montanus* and *Trollius europaeus*) and Asteraceae (*A. montana* and *Lactuca virosa*) had the same number of ALKs (*N* = 27), albeit with different profiles. The most well-represented groups in Ranunculaceae herbs were piperidine (*N* = 5 ALKs), quinoline (5), and pyrrolizidine (4) compounds, reticuline, 2-pyrrolidineacetic acid, chavicine, indigotin, indirubin, one isomer of integerrimine/senecionine/senecivernine, lobinaline, nicotine, piperine, pycnarrhine and spartioidine being present in both plants, whilst in Asteraceae herbs, the most well represented were pyrrolizidine (9) and tropane (4) groups, 2-pyrrolidineacetic acid and lobinaline being common to the two plants.

Twenty-two ALKs were detected in *G. sempervirens* (Gelsemiaceae family), indole ALKs (*N* = 6 ALKs; gelsemicine, gelsemine, one isomer of gelsempervine-A/gelsempervine-C, one isomer of gelsempervine-B/gelsempervine, one isomer of 14,15-dihydroxygelsenicine/gelsemoxonine and one isomer of 19Z-16-epi-voacarpine/16-epi-voacarpine), as previously reported in the literature,[30,31] and piperidine ALKs (5; 8-ethylnorlobelol, lobinaline, one isomer of lelobanidine I/lelobanidine II and two isomers of lobinanidine/isolobinanidine/β-Lobinanidine) being the most well represented.

Table 4. Activity of alkaloids contents (mg kg^{-1}) of 18 alpine plant extracts

Herb sample	Acontitine	α-Solanine	α-Solasonine	α-Solamargine	Echimidine	Erucifoline	Gramine	Heliotrine	Hyoscyamine/Atropine	Jacobine	Lycopsamine	Retrorsine	Retrorsine N-oxide	Scopolamine	Senecionine N-oxide	Senecionine/Sefffnecivernine	Solasodine
Cyclamen libanoticum	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Convallaria majalis	<0.07	0.64	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	0.528	<0.013	0.024	<0.01	<0.003	<0.007	<0.04
Dryopteris filixn.d.mas	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Phytolacca decandra	0.09	<0.07	<0.006	<0.15	0.016	<0.004	<0.003	<0.002	0.103	<0.015	<0.006	<0.013	<0.023	<0.01	0.006	<0.007	<0.04
Gelsemium sempervirens	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Hyoscyamus niger	<0.07	<0.07	0.221	0.37	0.017	<0.004	0.049	0.088	204	<0.015	<0.006	<0.013	0.024	136	<0.003	<0.007	<0.04
Lactuca virosa	<0.07	<0.07	<0.006	<0.15	0.017	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Lobelia inflata	<0.07	<0.07	<0.006	<0.15	0.019	<0.004	<0.003	<0.002	1.31	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Solanum nigrum	<0.07	<0.07	0.103	<0.15	0.017	<0.004	<0.003	0.021	<0.008	<0.015	0.252	<0.013	<0.023	<0.01	<0.003	<0.007	24.0
Scrophularia nodosa	<0.07	0.13	<0.006	<0.15	0.017	<0.004	<0.003	0.019	1.50	<0.015	<0.006	<0.013	0.027	0.18	<0.003	0.012	0.13
Senecio vulgaris	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	0.116	<0.006	87.3	0.064	<0.01	<0.003	179	<0.04
Datura stramonium	<0.07	<0.07	0.376	1.31	0.029	<0.004	<0.003	<0.002	34.2	<0.015	<0.006	<0.013	<0.023	2.21	<0.003	<0.007	<0.04
Arnica montana	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Trollius europaeus	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Ranunculus montanus	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	0.018	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Rhododendron ferrugineum	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Gentiana lutea	<0.07	<0.07	<0.006	<0.15	<0.004	<0.004	<0.003	<0.002	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04
Hypericum perforatum	<0.07	<0.07	<0.006	<0.15	<0.004	0.009	<0.003	0.011	<0.008	<0.015	<0.006	<0.013	<0.023	<0.01	<0.003	<0.007	<0.04

Table 5. Summary of the untargeted ALK profile of 18 alpine plant extracts, sorted by chemical/botanical groups

Herb sample	Family	Aconitine related	Azepine	Imidazoline	Indole	Isoquinoline	Piperidine	Protoalkaloid	Pyridine	Pyrrole	Pyrrolidine	Pyrrolizidine	Quinazoline	Quinoline	Steroidal	Tropane
Convallaria majalis	Asparagaceae	✖	✖	✖	✓	✖	✓	✓	✓	✖	✓	✓	✓	✓	✓	✓
Arnica montana	Asteraceae	✖	✖	✖	✖	✖	✓	✖	✓	✖	✓	✓	✖	✖	✖	✖
Lactuca virosa	Asteraceae	✖	✖	✖	✓	✖	✓	✓	✖	✖	✓	✓	✓	✓	✖	✓
Lobelia inflata	Campanulaceae	✖	✖	✓	✓	✖	✓	✖	✓	✖	✓	✓	✓	✓	✖	✓
Dryopteris filix-mas	Dryopteridaceae	✖	✖	✖	✓	✖	✓	✓	✓	✖	✓	✓	✓	✖	✖	✓
Rhododendron ferrugineum	Ericaceae	✖	✖	✖	✖	✖	✓	✓	✓	✖	✓	✓	✖	✓	✖	✓
Gelsemium sempervirens	Gelsemiaceae	✖	✖	✖	✓	✓	✓	✓	✓	✖	✓	✓	✖	✓	✖	✓
Gentiana lutea	Gentianaceae	✖	✖	✖	✖	✖	✓	✓	✓	✖	✓	✓	✓	✖	✖	✓
Hypericum perforatum	Hypericaceae	✖	✖	✖	✖	✖	✓	✖	✖	✖	✓	✓	✖	✓	✖	✓
Phytolacca decandra	Phytolaccaceae	✖	✖	✖	✖	✓	✓	✓	✓	✖	✓	✓	✓	✓	✖	✓
Cyclamen libanoticum	Primulaceae	✖	✖	✖	✓	✖	✓	✖	✓	✖	✓	✓	✖	✓	✖	✖
Ranunculus montanus	Ranunculaceae	✖	✖	✖	✓	✓	✓	✓	✓	✖	✓	✓	✖	✓	✖	✓
Trollius europaeus	Ranunculaceae	✖	✓	✖	✓	✓	✓	✖	✓	✖	✓	✓	✖	✓	✖	✖
Scrophularia nodosa	Scrophulariaceae	✖	✖	✖	✓	✖	✓	✖	✓	✖	✓	✓	✓	✓	✓	✓
Senecio vulgaris	Senecionaeae	✖	✖	✖	✖	✖	✓	✓	✓	✖	✓	✓	✓	✓	✖	✓
Datura stramonium	Solanaceae	✓	✖	✖	✖	✖	✓	✓	✖	✖	✓	✓	✓	✓	✖	✓
Hyoscyamus niger	Solanaceae	✖	✖	✖	✓	✖	✓	✓	✓	✓	✓	✓	✖	✓	✓	✓
Solanum nigrum	Solanaceae	✖	✖	✖	✓	✖	✓	✓	✓	✖	✓	✓	✖	✓	✖	✓

✓, detected.
✖, not detected.

Twenty-one ALKs were identified in *P. decandra* (Phytolaccaceae), nine of them belonging to the piperidine group (8-ethylnorlobelol, lobinaline, two isomers of 8,10-diethyllobelidiol, one isomer of *cis*-lobelanidine/*trans*-lobelanidine, one isomer of lelobanidine I/lelobanidine II, two isomers of lobinanidine/isolobinanidine/*β*-Lobinanidine and one isomer of 8-methyl-10-phenyllobelidiol/norlelobanidine).

Scrophularia nodosa (Scrophulariaceae) and *Dryopteris filix-mas* (Dryopteridaceae) had 18 different ALKs. The most well represented in the Scrophulariaceae herb belonged to pyrrolizidine ALKs ($N = 3$ ALKs; monocrotaline N-oxide, one isomer of integerrimine/senecionine/senecivernine and one isomer of usamarine /integerrimine-N-oxide/senecivernine-N-oxide/jacobine), indole ALKs (3; gelsemicine, gelsemine and yohimbine) and tropane ALKs (3; 3-acetyltropine, anisodamine and tropinone), whilst in the Dryopteridaceae herb, the most well represented was the indole group (5; corynoxin B, gelsemicine, gelsemine, isorhynchophyllin and rhynchophylline).

Rhododendron ferrugineum (Ericaceae) and *Hypericum perforatum* (Hypericaceae) had 13 different ALKs. The most well represented in the Ericaceae herb were piperidine ALKs ($N = 3$ ALKs; lobinaline, tuberostemonine and one isomer of 8-methyl-10-phenyllobelidiol/norlelobanidine) and quinoline ALKs (3; magnoflorine, quinidine and quinine), whilst in the Hypericaceae herb, the most well represented was the pyrrolizidine group (4; europine N-oxide, florosenine, neo-senkirkine and usaramine-N-oxide).

The herbs showing the lowest number of ALKs turned out to be *Gentiana lutea* (Gentianaceae) and *Cyclamen libanoticum* (Primulaceae), with seven ALKs (2-pyrrolidineacetic acid, 5-methoxyvascicinol, lobinaline, jacozine, mefenamic acid, scrophularianine C and tropinone) and six ALKs (2-pyrrolidineacetic acid, gelsemine, isomer of lelobanidine I/lelobanidine II, lobinaline, magnoflorin and nicotine), respectively.

Conclusions

The proposed method, using liquid chromatographic separation coupled with a high-resolution mass with targeted and untargeted approaches, made it possible to define the alkaloid profile in more detail. The quantification of 35 alkaloids and untargeted screening of a further 305 alkaloids in herbal extracts was possible with reduced analysis times and automation, through SPE online pretreatment of herbal extracts in order to minimise the matrix effects on instrumental response.

This broad and rapid ALK characterisation can represent a useful tool for assessing the healthiness of human.

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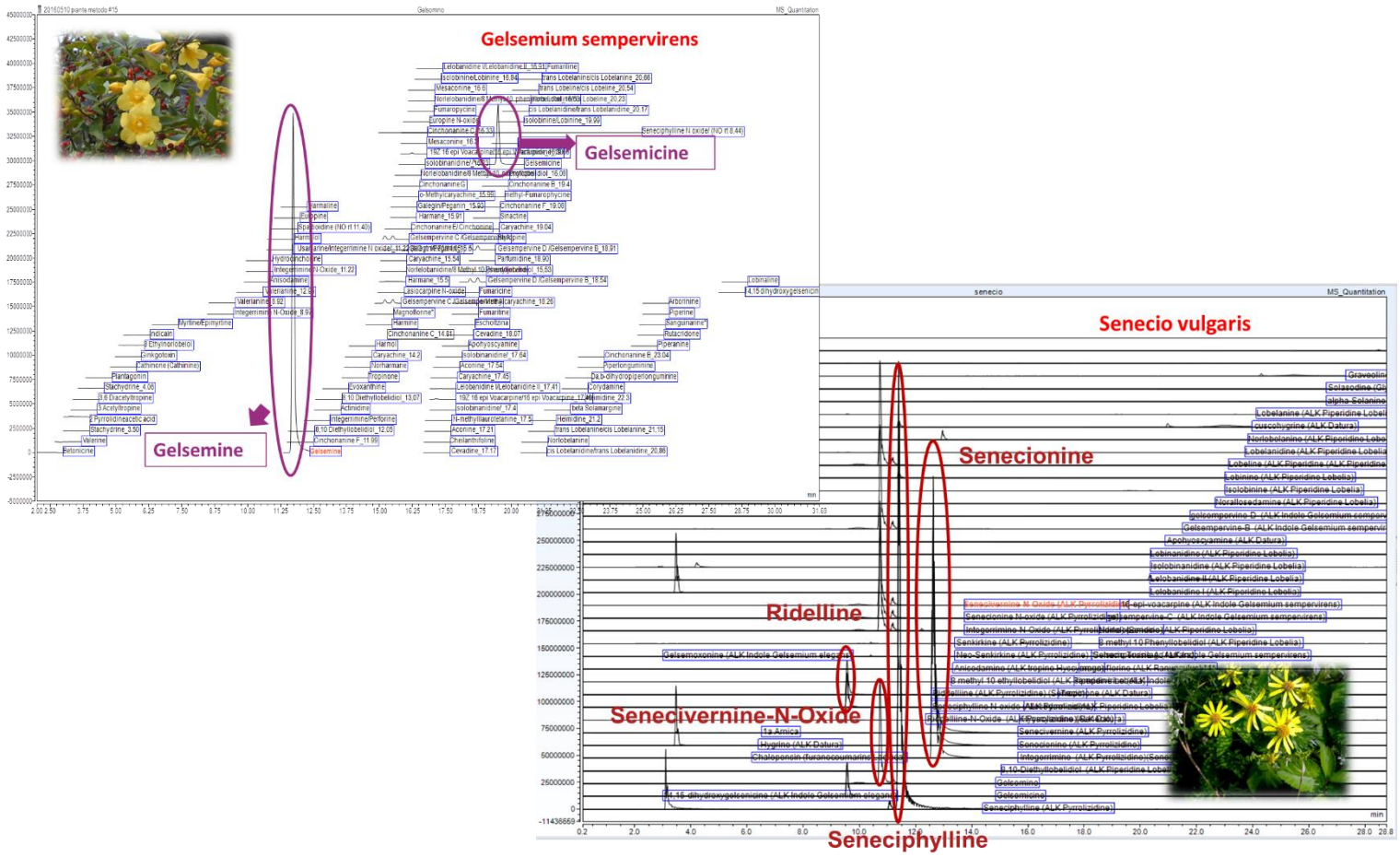
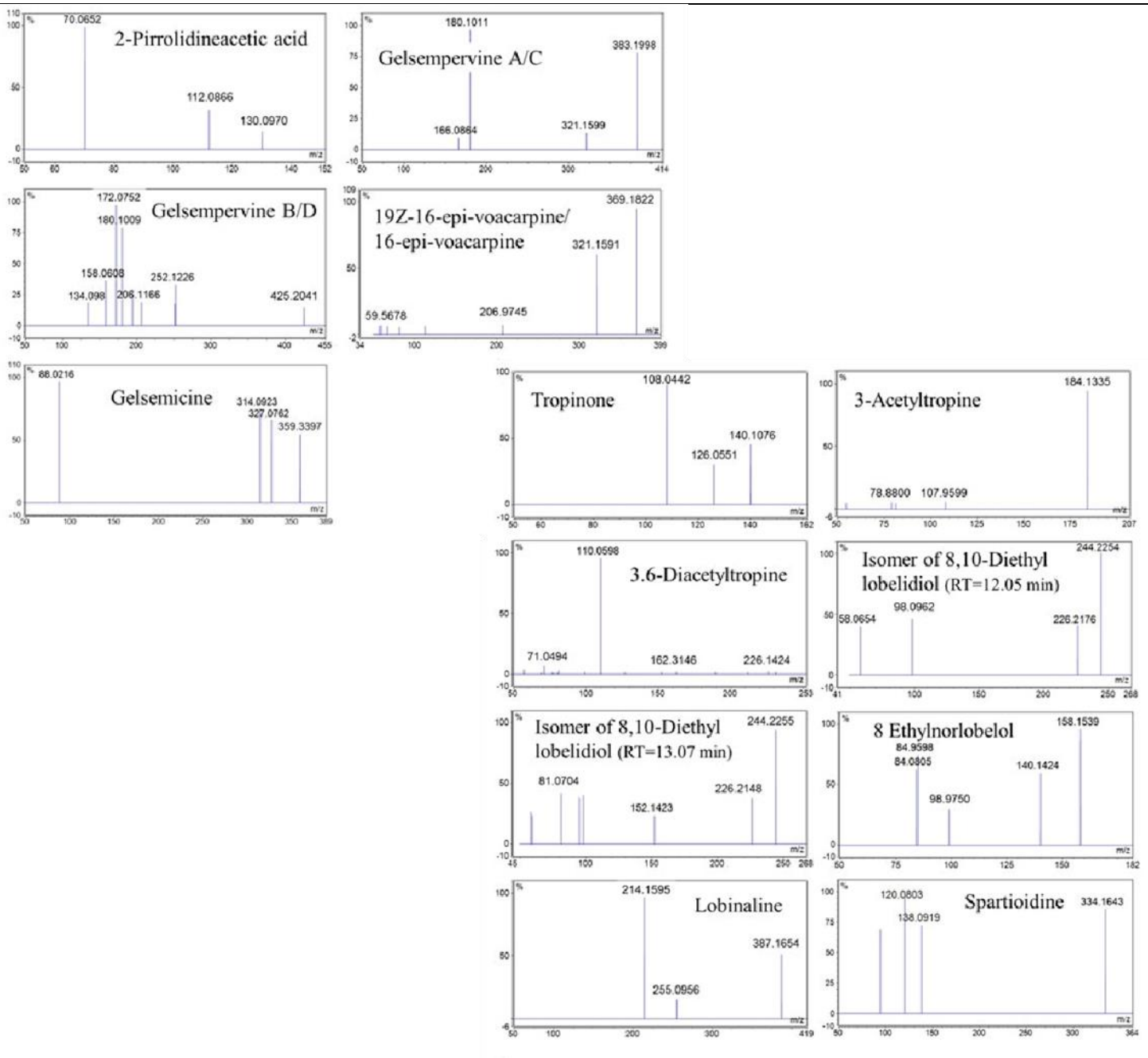
Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.



Supplematary Figure 1: Example of typical chromatogram in case of analysis of herbal extract, known to be particularly rich in several ALKs.



Supplematary Figure 2: Typical fragmentation spectra of ALKs never previously documented in literature (obtained by the analysis of 8 known herbal extracts).

Conclusion

In this work, both the targeted and suspect screening approaches were employed to characterize the alkaloidic profile of alpine herbal extracts. In particular, tentative identification with the suspect screening approach was shown to be achievable both through matching of experimental and theoretically supposed fragmentation and isotope patterns, and through comparison of retention times with those of certainly-identified peaks in matrices particularly rich in the compounds studied. Obviously, in both cases detection of accurate masses is always required.

Furthermore, this work showed that the HRMS screening approaches can also be well employed in positive polarity and allow the detection of chemically very different compounds.

SECTION 2.2.10.

Cissampelos pareira extract effects in envenomation induced by Bothropsdiporus snake venom

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Aim of work

In this work it was intended to give chemical answers to questions related to the use of a reputed plant capable to neutralize the effects of snake venom. In Corrientes Province (Argentina) local knowledge justify the use of *Cissampelos pareira* extracts on envenomation induced by *Bothrops diporus* snake venom. But, even traditional healers continue to play an important role in primary snake venom treatment in the rural areas, there was not previous biological nor chemical evidence about. Our research group, multidisciplinary in nature, investigated if *C. pareira* extracts possesses inhibitory effects in both *in vitro* and *in vivo* models against the venom of *B. diporus*. Moreover, the UHPLC-MS data allowed examination that certain flavonoids may mitigate some venom-induced local tissue damage.

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The effects of *Cissampelos pareira* extract on envenomation induced by *Bothropsdiporus* snake venom☆

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ABSTRACT

Ethnopharmacological relevance: Ophidian accidents are a serious public health problem in Argentina; the *Bothrops* species is responsible for 97% of these accidents, and in particular, *B. diporus* is responsible for 80% of them. In the northeast of the country (Corrientes Provinces), *Cissampelos pareira* L. (Menispermaceae) is commonly used against the venom of *B. diporus;* its use is described in almost all ethnobotanical literature from countries where the plant grows.

Aim of the study: In this study, the *in vitro* and *in vivo* antivenom activities of *C. pareira* extracts were evaluated against *B. diporus* venom, with a particular focus on the local effects associated with envenoming. The seasonal influence on the chemical composition of the active extracts was also studied, in order determine the associated range of variability and its influence on the antivenom activity.

Materials and methods: This research was conducted using aerial parts (leaves, flowers, tender stems) and roots of *Cissampelos pareira* collected from two different phytogeographic regions of Corrientes (Argentina); Paso de la Patria and Lomas de Vallejos. In addition, to perform a seasonal analysis and to evaluate the metabolic stability, material was collected at three different growth stages. *In vivo* and *in vitro* anti-snake venom activities were tested, and a bio-guided chromatographic separation was performed in order to determine the active chemicals involved. The fractions obtained were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the chemical profile of the most active constituent was analyzed by ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap). (UHPLC-MS).

Results: The alcoholic extract was found to be the most active The bio-guided fractionation allowed selection one fraction to be analyzed by UHPLC-MS in order to identify the components responsible for the activities found; this identified five possible flavonoids.

Conclusions: Our studies of the activity of *C. pareira* against the venom of *B. diporus* have confirmed that this species possesses inhibitory effects in both *in vitro* and *in vivo* models. Moreover, the present data demonstrate that certain flavonoids may mitigate some of the venom-induced local tissue damage.

1. Introduction

Cissampelos pareira L. (Menispermaceae) is commonly known as *ka'apeva, ka'á-peva, ysypó-morotí, caápebá, zarza, pareirabrava,* or *mil hombres*. Its popular uses are mentioned in almost all ethnobotanical literature from countries where the plant grows, including South America, Asia, and Africa (Semwal et al., 2014; Singh et al., 2010). As per traditional knowledge, it is used as a carminative, a febrifuge, for the alleviation of liver disorders, and for constipation, menstrual pain, colic, and rheumatism (Arora et al., 2012; Semwal et al., 2014).

Ethnopharmacological surveys have established that *Cissampelos pareira* decoctions containing leaves and roots, as well as aqueous or

☆ Contributions to the study: phytochemistry and chromatography (B. Ricciardi, G. Ricciardi, E. Dellacassa), *in vitro* experiments (A.M. Torres), *in vivo* experiments (P. Teibler, S. Maruñak), MS experiments (C. Barnaba, E. Dellacassa), spectra interpretation (R. Larcher, G. Nicolini).

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alcoholic infusions, are traditionally used for ophidian antivenom in Paraguay (González Torres, 2005; Jolis, 1789; Manfred, 2014; Montenegro, 2007), India and Pakistan (Chakraborty and Bhattacharjee, 2006; Dey and De, 2013; Jabeen et al., 2009; Kadel and Jain, 2008; Katewa and Galav, 2006; Sankaranarayanan et al., 2010), and Mexico (Ramos Hernandez et al., 2007). Its use also extends to the Amazon (Ecuador and Peru) and Central America (Barranco Pérez et al., 2010; Giovannini and Howes, 2017).

Some of its pharmacological properties have been scientifically investigated; specifically, anti-inflammatory, antispasmodic, and muscle and uterine relaxant properties in rats and rabbits (Feng et al., 1962), and diuretic and curare mimetic effects (Basu, 1970). Further, the activities of some of its isolated components have been investigated, including tetrandrine (analgesic, antipyretic, anti-inflammatory, cardioactive, and hypotensive effects), pareirubines A and B (antileukemic), alkaloids (febrifuges and curarizers), berberine (hypotensive, antimicrobial, and antifungal effects) (Sanchez-Medina et al., 2001), and cissampelin (muscle relaxant) (Semwal et al., 2014). In particular, in Costa Rica it has been shown that a 10% aqueous infusion of the whole plant has anti-hemorrhagic and anti-proteolytic activity against *B. asper* venom (Badilla et al., 2008). Snakebites are seriously under-reported worldwide. This is especially true in countries where agricultural activities are predominant, since this is one of the occupations most often affected by snakebites. Moreover, studies show not only a higher incidence in men but also a reasonably high incidence in children. This may be related to the fact that in rural areas of many under developed countries, where snakebites represent a major health issue, children take part in agricultural activities or are attacked due to their innate curiosity (Chippaux, 1998).

Generally, local effects of a snakebite occur in the first 10–30 min; there may be numbness around the bite with bleeding, or a purpuric rash, and/or necrosis or gangrene (Cavazos et al., 2012). These local reactions are not effectively neutralized by conventional antivenom serum therapy, as revealed by animal models and clinical studies (Avila-Aguero et al., 2001; Lomonte et al., 1994). In severe cases, local effects of envenoming may lead to permanent tissue loss, disability, or amputation (Gutierrez, 2002).

In Argentina, ophidian accidents are a serious public health problem. *Bothrops* is responsible for 97% of accidents and, in particular, *B. diporus* accounts for 80% of these (Boletín Epidemiológico Periódico, 2009). As a consequence of this situation, and given the context of the use of *Cissampelos* sp. for the treatment of snakebites in traditional medicine, in this study, we investigated the antivenom activity of *C. pareira* against the *B. diporus* species from northeast Argentina.

In order to understand the limits of variability associated with a bioactive natural product, such as the plant extracts prepared in this work, we studied the chemical fingerprint of these plant extracts, and its association with the factors influencing such variability.

2. Material and methods

2.1. Venom

Bothrops diporus venom was obtained by personnel of Corrientes Serpentario, Argentina. Captured specimens were milked, resulting in a representative pool of snakes; the venom was then dried under vacuum. All experimental procedures were performed in accordance with the guidelines of the institutional animal care and use committee of the Universidad Nacional del Nordeste (UNNE), in accordance with the legislation on animal care. *In vivo* studies were developed following protocol 056, and were approved by the Ethics and Biosafety Committee of the Faculty of Veterinary Sciences of UNNE.

2.2. Plant material

Leaves, flowers, tender stems (A), and roots (B)of *Cissampelos pareira* were collected from two different phytogeographic regions of Corrientes (Argentina); Paso de la Patria (PP, San Cosme Department, 27°22'58.6'S, 58°34'56.4'W, 65 masl) and Lomas de Vallejos (LV, General Paz Department, 27°45'37.8'S, 57°55'56.6"W, 59masl). In addition, to perform a seasonal analysis and to evaluate the metabolic stability, material was collected at three different growth stages: autumn (I, May 2013), spring (II, November 2013), and summer (III, February 2014). The species was identified by Prof. Tressens (Instituto de Botánica del Nordeste (IBONE/UNNE)), and specimens were deposited in the IBONE herbarium (CTES 17 Torres, CTES 19 AM and B. Ricciardi).

2.3. Extract preparation

The plant material, divided into aerial parts (leaves, flowers, and tender stems) and roots, was dried by aeration at approximately 66 °C and humidity within 10–15%; the material was turned over during the drying process. Three extracts were prepared: (1) aqueous (maceration in distilled water, 24 h), (2) ethanolic (48 h), and (3) hexane (48 h). Next, all extracts were filtered and evaporated under reduced pressure. Extracts were kept in a refrigerator in closed containers until use.

2.4. Screening of antivenom activity

2.4.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

The protein composition of snake venom was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-Protean IV Electrophoresis Cell equipment. SDS-PAGE was performed on a slab according to the method of Laemmli (1970), with 4% (w/v) stacking gel (pH 6.8; Tris 6%, SDS 0.4%) and 12% (w/v) buffer gel (pH 8.8; Tris 18.2%, SDS 0.4%). The solutions for resolving gels and stacking gels for Tris-Glycine-SDS-Polyacrylamide Gel Electrophoresis were prepared as previously reported (Pardo and Natalucci, 2002; Pilosof and Bartholomai, 2000). Gels were stained for 3–4 h at room temperature with 0.25% (w/v) Coomasie brilliant blue R in 9.2% (v/v) acetic acid and 55.4% (v/v) methanol, and were then destained for 24 h with several changes of 7% acetic acid and 30% (v/v) methanol (Camargo et al., 2011).

Modification of the pattern of bands obtained from the standard venom compared to the extracts is considered an indicator of antivenom activity.

2.5. In vitro activities

2.5.1. Inhibition of proteolytic activity

The neutralization of the proteolytic activity of *B. diporus* venom was performed following an adaptation of the SDS-PAGE technique, as previously reported by Torres et al. (2014).

Venom + casein solution (1 g/100 mL in 100 mM Tris-HCl buffer; pH 8) was used as the complete hydrolysis standard. Solutions of venom + casein + plant extract were prepared in order to observe the inhibition of proteolysis by the plant extracts. The venom solution was pre-incubated for 60 min at 37 °C (0.25 mg/mL in Tris-HCl buffer; pH 8) with the extract solution (venom:extract ratio of 1:30). They were then incubated with casein (10 mg/mL) for 60 min at 37 °C. Solutions of plant extracts were incubated with casein to discard the presence of plant proteases. Urea(4 M) was added to the sample buffer solution to improve resolution.

2.5.2. Inhibition of indirect hemolytic activity

The ability of plant extracts to neutralize the enzymes of *B. diporus* venom was evaluated by an indirect hemolysis assay on blood phosphatidylcholine agar plates (Gutiérrez et al., 1988; Otero et al., 1995) using a ratio venom:extract of 1:20.

Plant extracts (1500 g in 0.2 mL solvent: water, alcohol, or hexane) were incubated with 1 mL of venom solution (50 g/mL, minimum

indirect hemolytic dose (MIHD) of venom produces a 10 mm halo diameter after 20 h of incubation). The ratio of venom:extract was 1:20. Next, 10 µL of these solutions were incorporated into each wells of agar. The plates were incubated for 20 h at 37 °C, and the hemolysis halo was measured and compared with the MIHD. Reductions in the diameter indicated inhibition of phospholipase A_2 and its *in vitro* activity.

2.5.3. Inhibition of coagulant activity

A minimum coagulant dose (MCD) was defined as the amount of *Bothrops* venom which clots 0.2 mL of plasma in 60 s. The method used was that described by Iovine and Selva (1985) with a slight modification, by which 0.2 mL of plasma and 0.2 mL of 0.025 M $CaCl_2$ were added to 10 µL of saline solution, venom solution, or supernatant from the incubation of the venom + extract for 30 min at 37 °C. Inhibition of coagulant activity was expressed by the normal coagulation time restitution percentage after addition of extract incubated with venom.

2.6. Bio-guided fractionation

2.6.1. Fractionation

According to the results obtained in Section 2.5, the ethanolic extract of the aerial parts collected in summer from Paso de la Patria (A_2 III PP) resulted in the most active components, and was fractionated by introducing 500 mg on a chromatographic column (24 × 400 mm, silica gel flash 60 0.04–0.063 mm, MN) connected to a CX-1000 air pump. A solvent eluotropic series of increasing polarity (toluene 100; toluene:ethyl acetate 50:50; ethyl acetate 100; ethyl acetate:methanol 60:40; ethyl acetate:methanol 40:60; methanol 100) was used. The fractions were collected in the test tubes, grouped by their composition profile on thin layer chromatography (toluene:ethyl acetate 9:1, visualization at UV 254/365 nm and by spraying with anisaldehyde in sulfuric acid), and evaporated under reduced pressure. Then, the grouped fractions were analyzed by SDS-PAGE to evaluate their alexiteric activity.

2.6.2. In vitro study of fractions

The fractions obtained were analyzed by SDS-PAGE under the same conditions as described in Section 2.4. Next, *in vitro* tests of the inhibitory capacities on venom activities were carried out, as described in Sections 2.5.1, 2.5.2, and 2.5.3.

2.7. Ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap) (UHPLC-MS)

The active fraction (fraction F_6) was analyzed by UHPLC-MS. Chromatographic separation was carried out using a Thermo Ultimate R3000 UHPLC (Thermo Scientific, Sunnyvale, CA, USA), equipped with a Rheodyne 6-port automated switching valve used for on-line clean-up, adopting the method recently proposed by Barnaba et al. (2015). Identification of phenolic compounds in the active fraction was performed using a Q-Exactive TM hybrid quadrupole-orbitrap mass spectrometer (HQ-OMS, Thermo Scientific, Bremen, Germany) equipped with heated electrospray ionization (HESI-II). Mass spectra were acquired in negative ion mode through full MS-data dependent MS/MS analysis (full MS-dd MS/MS), recording full mass spectra at a mass resolving power of 140,000 full width at half-maximum (FWHM), and data-dependent mass spectra at 17,500 FWHM. The mass spectrometer operated as reported by Barnaba et al. (2016).

2.8. In vivo studies

2.8.1. Inhibition of lethal activity

The inhibition of lethal activity was performed according to the Spearman-Karber method (World Health Organization, 1981) using groups of four CF1 mice (18–20 g) injected intraperitoneally, and recording the results after 48 h. Literature values of LD_{50} for *Bothrops diporus* were used; 38.18 µg per mouse (Maruñak et al., 2010). The reagents were sterilized with filters (0.2 µm) and the solutions were prepared under laminar flow hood. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as described by the European Community guidelines (EEC Directive of 1986; 86/609/EEC) and the US guidelines (NIH publication #85-23, revised in 1985).

Working groups were as follows: Group 1: challenge dose $4LD_{50}$ of venom in 0.12 M NaCl, 0.04 M phosphate buffer, pH 7.2 (PBS); Group 2: 5 mg A_2 III PP in PEG 400:ethanol:PBS (10:10:80) with 153 µg of venom incubated for 30 min at 37 °C; Group 3: extract control group, 3 mg and 5 mg A_2 III PP in PEG 400:ethanol:PBS (10:10:80) and 3 mg F_6 in PEG 400:ethanol:PBS (10:10:80); Group 4: 5 mg F_6 in PEG 400:ethanol:PBS (10:10:80) with 153 µg of venom incubated for 30 min at 37 °C; Group 5: 3 mg F_6 in PEG 400:ethanol:PBS (10:10:80) with 153 µg of venom incubated for 30 min at 37 °C. Injection volume, 0.5 mL.

3. Results

The polyacrylamide gel electrophoresis for *C. pareira* extracts collected in summer in the area of Paso de la Patria is shown in Fig. 1; the bands corresponding to the molecular weight pattern proteins of the venom and studied extracts can been seen.

The variation in the protein profile of venom compared to the plant extract is chosen as an indication of alexiteric activity; as can be seen in row A_2, the venom bands were completely erased. SDS-PAGE (Table 1) results indicated that the ethanolic extract of the aerial parts, collected in summer in Paso de la Patria, had the most active constituents.

The alexiteric activity was enhanced in summer, particularly in the ethanolic extract; this is likely related to the presence of more polar

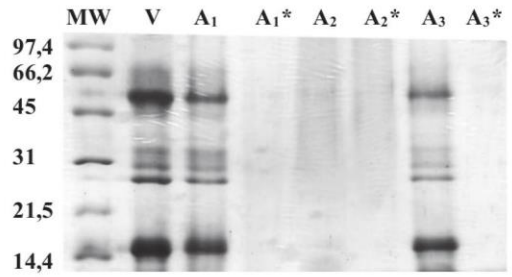

Fig. 1. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) for Paso de la Patria collected in summer (PP III) extracts. Venom:extract ratio 1:10.

Table 1
Screening of antisnake venom activity of *C. pareira* extracts by sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE.

	Autumn		Spring		Summer	
Paso de la Patria	**Aerial parts**	**Roots**	**Aerial parts**	**Roots**	**Aerial parts**	**Roots**
Aqueous extract	–	–	++	+	–	–
Ethanolic extract	++	+	+++	+	++++	++
Hexane extract	–	–	+	+	–	–
Lomas de Vallejos						
Aqueous extract	–	+++	–	–	–	++
Ethanolic extract	–	+++	+	+++	–	+++
Hexane extract	–	++	+	–	++	+++

Venom:extract ratio 1:10.
(-), No activity; (+), little activity; (++), moderately active; (+++), very active.

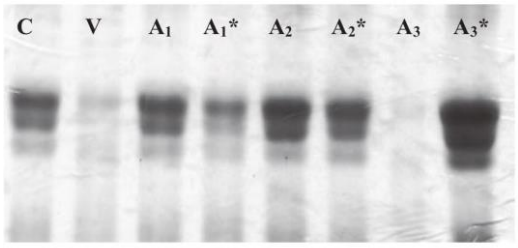

Fig. 2. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) with casein for Paso de la Patria collected in summer (PP III) extracts. Venom:extract ratio 1:30.

compounds, suggesting that the time of plant collection has important effects on the alexiteric activity of *C. pareira*.

When the results were evaluated from an edaphological point of view, the extracts from Paso de la Patria showed higher activity than those of Lomas de Vallejo. In addition, variation in activity was also found between the extracts from the different parts of the plants, that is, the leaves were more active in Paso de la Patria and the roots in Lomas de Vallejos were more active.

In order to investigate its proteolytic activity, an extract is considered active when it inhibits the proteolysis produced by venom, and the bands corresponding to casein remain intact. Fig. 2 shows that the ethanolic extract of leaves produced the most active inhibition of the proteolysis of the venom; this activity was verified for all the ethanolic extracts from all the regions and seasons studied (Table 2). The inhibition of indirect hemolytic activity of venom was found to be concentration dependent (Table 3), and higher activity was found in Paso de la Patria extracts collected in spring, while those from Lomas de Vallejo did not present any activity.

Regarding the inhibition of blood coagulant activity, neither the extracts nor the solvents modified the coagulation time (Torres, 2012). The results of coagulant activity of extracts when incubated with venom are shown in Table 2, where the main activity was found in spring and summer.

Ethanolic extract obtained from aerial parts collected in summer from the Paso de la Patria region (11% w/w) was responsible for all inhibition activities, and thus was fractionated by flash chromatography in order to identify the active fraction. Six fractions were obtained and analyzed by SDS-PAGE in order to monitor the antivenom activity. Fractions 4, 5, and 6 (25%, 38%, and 22% w/w, respectively) completely erased the protein profile of venom on SDS-PAGE, and were thus the most active (Fig. 3), whereas *in vitro* activities were 100% for all fractions (Table 4).

In order to evaluate the lethal activity and acute toxicity of the ethanolic extract and fraction F_6, five groups of four mice each were prepared: Group 1, venom control group (100% lethality); Group 2, 5 mg A_2 III PP + venom (0% lethality, 100% protection); Group 3, extract control group (0% lethality); Group 4, 5 mg F_6 + venom (0% lethality, 100% protection); and Group 5, 3 mg F_6 + venom (25% lethality, 75% protection).

At all concentrations tested, the plant extracts showed no acute toxicity and, in addition, we verified that the extract and the active fraction provided very good protection *in vivo*.

The structural elucidation of flavonoids was carried out by UPLC-MS. The following flavonoids were identified: quercetin 3-O-sophoroside [quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside] (1), naringenin 7-O-β-D-glucoside (2), eriodictyol-7-O-beta-D-glucoside (3), galangin-7-glucoside (4), and baicalein-7-O-glucoside (oroxin A) (5) (Fig. 4). Phenolic compounds, especially complex polyphenols such as some tannins, can bind to proteins acting directly upon venom constituents, thus avoiding the reaction on receptors, or provoking competitive blocking of receptors (Soares et al., 2005). However, only quercetin has been previously identified in *C. pareira* (Amresh et al., 2007a). These findings support the traditional use of the ethanolic extracts of this plant in the treatment of snake venom attacks at rural areas.

Table 2
Screening of hemolytic, coagulant, and proteolytic activities of the aqueous, ethanolic, and hexanic extracts.

Activity			Neutralization of hemolytic activity 1:30 (V:E) % Restitution		Neutralization of coagulant activity 1:15 (V:E) % Restitution		Neutralization of proteolytic activity 1:30 (V:E)	
Area			LV	PP	LV	PP	LV	PP
Autumn								
Extract	A	1	21	0	6	7	(-)	(-)
	R		8	0	8	0	(-)	(-)
	A	2	12	0	**41**	**43**	(+)	(+ + +)
	R		12	5	6	6	(+ + +)	(+ + +)
	A	3	17	20	7	**70**	(-)	(+ + +)
	R		8	0	5	**80**	(-)	(+ + +)
Spring								
Extract	A	1	4	0	**100**	13	(-)	(-)
	R		0	0	17	15	(-)	(-)
	A	2	19	**100**	**40**	**43**	(+)	(+ +)
	R		0	20	22	22	(+ + +)	(+ +)
	A	3	23	0	11	16	(+)	(+)
	R		8	0	11	29	(+)	(+)
Summer								
Extract	A	1	0	0	**49**	**40**	(-)	(+ +)
	R		10	0	**35**	**43**	(-)	(-)
	A	2	0	**35**	**66**	**94**	(+ + +)	(+ + +)
	R		0	0	22	**39**	(+ + +)	(+ + +)
	A	3	0	0	**43**	18	(-)	(-)
	R		0	0	27	28	(-)	(+)

V:E, venom:extract ratio; LV, Lomas de Vallejos; PP, Paso de la Patria; A, aerial parts; R, roots; 1, aqueous extracts; 2, ethanolic extracts; 3, hexanic extracts. (-), No activity; (+), little activity; (+ +), moderately active; (+ + +), very active.

4. Discussion

For most *Bothrops* species, bites lesions will vary from minor to life threatening. Whether life threatening or of no clinical significance, bites are invariably painful and are usually accompanied by local swelling and inflammation, bruising, blistering, necrosis, and abscess formation.

Medicinal plants and plant-based natural products have been reported to possess antivenom properties; laboratory assays correlate with ethnopharmacological studies (Soares et al., 2005). Natural inhibitors of snake venoms, particularly polyphenols, have been studied in the search for the most efficient wound treatments (Al Asmari et al., 2016; Caro et al., 2017; De Moura et al., 2015; Emmanuel et al., 2014; Gomes et al., 2016; Nirmal et al., 2008; Omale et al., 2012; Pithayanukul et al., 2004; Sánchez and Rodríguez-Acosta, 2008; Urs et al., 2014; Vale et al., 2011). Amresh et al. (2007a, 2007b, 2007c) suggest that internal use of *C. pareira* root preparations may counteract the inflammatory response following snakebites. The aerial parts from this species also show anti-inflammatory and analgesic effects in animal models of pain and inflammation, when administered orally; this may explain their use for treatment of some symptoms of snakebites. It is also interesting that a 70% ethanol extract of *C. pareira* leaves was found to be effective against anxiety-like behaviors (Thakur and Rana, 2013), explaining why the leaves are often used for snakebites, in order to calm the victims.

The present study found that the aqueous extracts of both root and aerial parts of *C. pareira* do not inhibit the proteolytic activity of *B. diporus* venom, as previously reported by Badilla et al. (2008) using

Table 3
Inhibition of indirect hemolytic activity at different ratios (VenomExtract) of the active alcoholic extracts of aerial parts (A_2) of *C. pareira*.

Paso de la Patria					Lomas de Vallejos				
V:E ratio	X_1	X_2	X_p	%Restitution	V:E ratio	X_1	X_2	X_p	%Restitution
Spring (A_2 II)									
1:5	3	3	3	(+) 74%	–	–	–	–	–
1:10	2	2	2	(+) 83%	–	–	–	–	–
1:20	0	0	0	(+) 100%	–	–	–	–	–
1:30	0	0	0	(+) 100%	1:30	10	11	10.5	(-)
1:40	0	0	0	(+) 100%	–	–	–	–	–
1:50	0	0	0	(+) 100%	1:50	11	12	11.5	(-)
V	11	12	11,5		V	11	12	11.5	
Summer (A_2 III)									
1:5	8	7	7.5	(-) 25%	1:5	10	10	10	(-)
1:10	7	6	6.5	(+) 35%	1:10	10	10	10	(-)
1:20	6	6	6	(+) 40%	1:20	10	10	10	(-)
1:30	6	7	6.5	(+) 35%	1:30	10	10	10	(-)
1:40	4	4	4	(+) 60%	1:40	10	10	10	(-)
1:50	4	4	4	(+) 60%	1:50	10	10	10	(-)
V	10	10	10		V	10	10	10	

(-), No inhibitory activity; %Restitution > 30% was considered (+); $\times_1$, $\times_2$: diameter in mm of hemolysis halo of ethanolic extract pre-incubated with venom or venom alone (V); X_p: average of $\times_1$ and $\times_2$; %Hemolysis = (X_p extracts × 100)/X_p V.%Restitution = 100 - %Hemolysis.

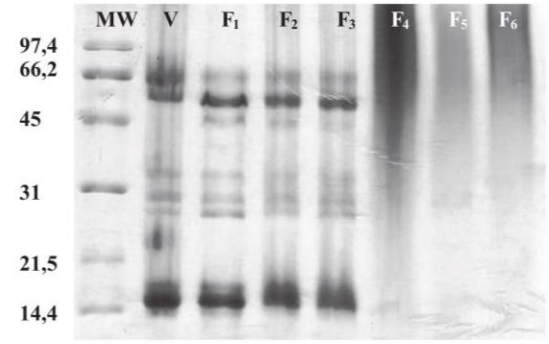

Fig. 3. Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) of fractions of the active extract; ethanolic extract from Paso de la Patria collected in summer (A_2 III PP).

Table 4
Inhibition of *in vitro* activities of venom by A_2 III PP fraction, as obtained by flash chromatography.

Inhibited activity	F_1	F_2	F_3	F_4	F_5	F_6
Proteolysis	(++)	(+++)	(++)	(++++)	(++++)	(++++)
Indirect hemolysis	(-)	(-)	(-)	**100%**	**100%**	**100%**
Coagulation	**34%**	(-)	(-)	**100%**	**100%**	**100%**

F_1, fraction 1; F_2, fraction 2; F_3, fraction 3; F_4, fraction 4; F_5, fraction 5; F_6, fraction 6. Fractions were obtained by column chromatography from the ethanolic extract of aerial parts collected in summer in the area of Paso de la Patria (A_2 III PP); (-), no inhibition activity; (++), moderate inhibition activity; (+++), strong inhibition activity; (++++), total inhibition activity.

aqueous root extracts on *Bothrops asper* venom. Similarly, and in agreement with the work of Saravia-Otten et al. (2015), we found no anti Phospholipases A2 (PLA_2) activity in the ethanolic extract of *C. pareira* roots. However, the ethanolic extract of *C. pareira* aerial parts, collected in spring and summer, was very active, suggesting the aerial parts should be used for plant based drugs, instead of the entire plant.

Ethanolic extract activity was observed for plants collected from Paso de la Patria in all seasons. This is in agreement with the results of Barranco Pérez et al. (2010) who investigated leaves and root liquor. Our study found that the activity of *C. pareira* varied according to the part of the plant used, the vegetative state, and the place of harvest; as such, the aerial parts collected in summer in Paso de la Patria are a source of active metabolites against *B diporus*.

Although many medicinal plants traditionally used against snakebites have been investigated pharmacologically, there is a large number yet to be evaluated. *C. pareira* has been reported as one of the anti-ophidian ethnomedicinal plants used in India (Dey and De, 2013) but, to our knowledge, ours is the first study where phytochemical constituents were identified as responsible for the antisnake venom activity.

We isolated and characterized a flavonoid enriched fraction responsible for the antivenom activity: quercetin 3-O-sophoroside [quercetin 3-O-β- D-glucosyl-(1,2)-β-D-glucoside], naringenin 7-O-β-D-glucoside, eriodictyol-7-Obeta-D-glucoside, galangin-7-glucoside, and baicalein-7-O-glucoside (oroxin A). Although the mechanisms of action of these active compounds have not been elucidated, their activity could be attributed to their ability to bind to biological polymers. Flavonoids can form hydrogen bonds with proteins due to the proximity and coplanarity between the carbon 5 phenolic hydroxyl and the pyrronic carbonyl (Grassmann et al., 1956; Jin et al., 1990). This explains the ability of flavonoids to act as inhibitors of inflammatory, hepatotoxic, hypertensive, and allergic processes and, more importantly, as enzymatic inhibitors. In addition, flavonoids have the ability to act as metal binders (chelating agents), particularly with zinc, which has the potential to affect the functionality of metalloproteinases present in snake venom (Badilla Baltodano et al., 2006; Houghton, 1993; Mors et al., 2000).

This study supports the preparation of a protocol for extraction and fractionation of the plant species. Once its pharmacological and/or toxic properties (toxicokinetic and toxicodynamic mechanisms) have been validated, this fraction, or the isolated active compounds, could be applied as an alternative to the conventional treatment of snakebite accidents by *B. diporus*; this would provide a possible solution to people living in rural areas, away from health care sites.

5. Conclusion

The current study scientifically explains the ethnobotanical use of *C. pareira* against venom of *B. diporus* in our region, by identifying the compounds responsible for the alexiteric activity.

The findings suggest that collection of this species should be performed in spring-summer. After an adequate investigation of the drug-toxicological characteristics, the current study suggests that an

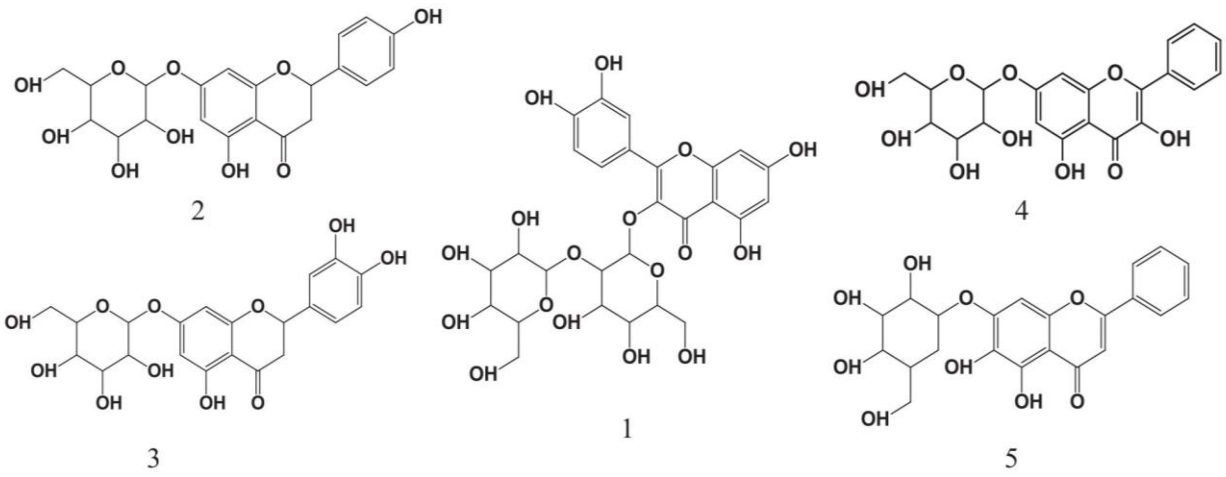

Fig. 4. Flavonoids identified by quadrupole/high-resolution mass spectrometry (UHPLC-MS/Q-Orbitrap).

ethanolic extract composed of aerial parts of the plant has potential to form a phytopharmacological product that could be used for first aid in cases of snakebite accidents.

Author contributions

All authors participated in the design (B. Ricciardi Verrastro, G. Ricciardi, C. Barnaba, P. Teibler, S. Maruñak,), interpretation of the studies (A.M. Torres, R. Larcher, G. Nicolini) analysis of the data (A.M. Torres, E. Dellacassa) and review of the manuscript (E. Dellacassa). All authors read and approved the final manuscript.

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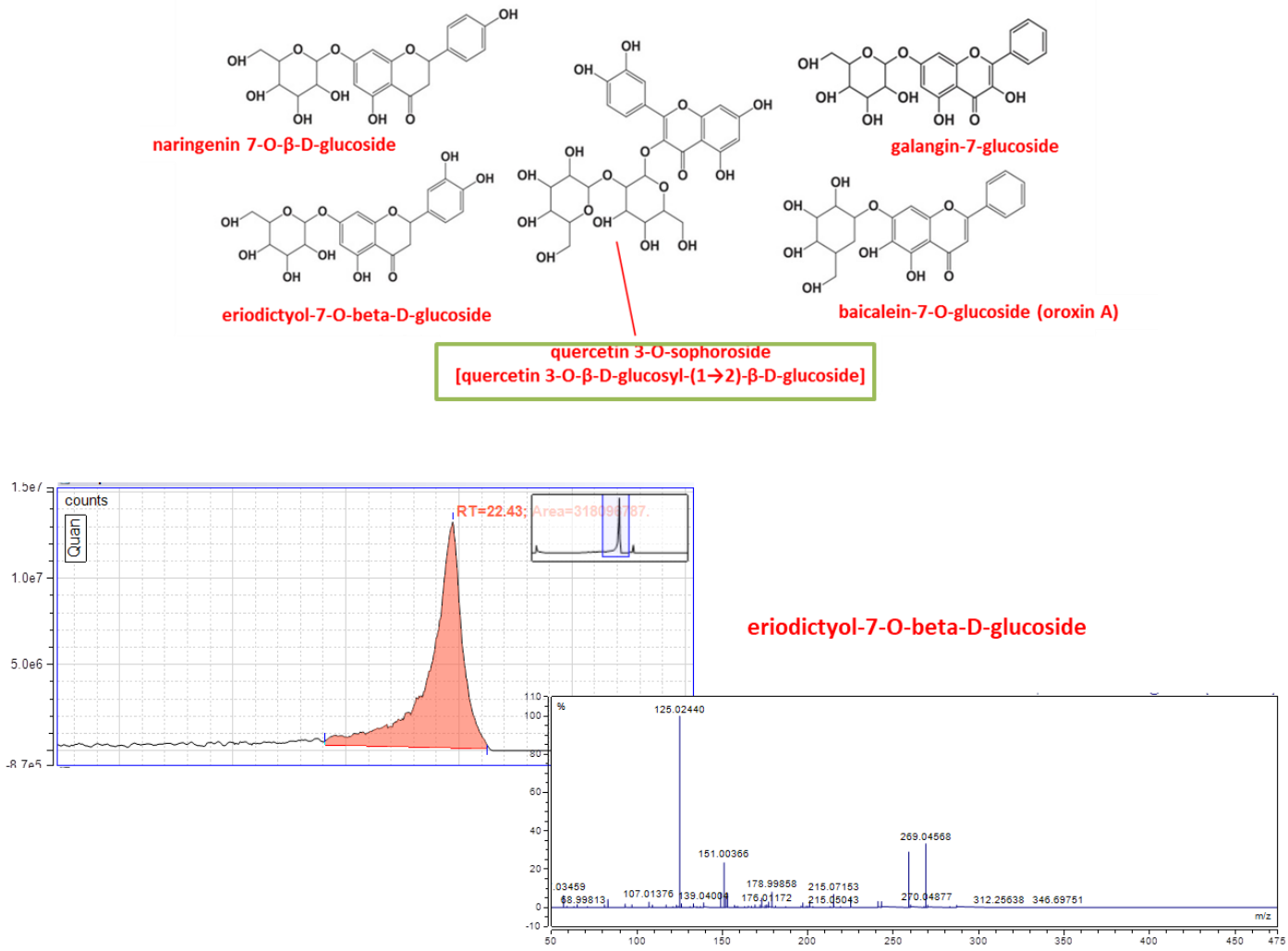
Glossary

(UHPLC-MS): Ultra high-performance liquid chromatography coupled to quadrupole/high-resolution mass spectrometry (Q-Orbitrap).
(SDS-PAGE): Sodium dodecyl sulfate polyacrylamide gel electrophoresis



Supplementary Fig. 1: Chemical structure of flavonoidic glycosides tentatively identified in sample and one specific chromatographic peak and MS/MS spectrum, as example.

Conclusion

The results found in this work showed how natural products (the so-called secondary metabolites) can be held responsible for the neutralizing effect of *C. pareira* against the action of snake venoms, in popular use. The evidence is relevant scientifically explaining the ethnobotanical use of C. *pareira* identifying the compounds responsible for the alexiteric activity.

Moreover, the correlation between the activity results and chemical profiles followed by the HRMS screening approaches suggests that collection of this species should be performed in spring-summer. The results found in this work showed how natural products (the so-called secondary metabolites) can be held responsible for the neutralizing effect of C. *pareira* against the action of snake venoms, as stated in popular use. The evidence is relevant scientifically in explaining the ethnobotanical use of C. *pareira* identifying the compounds responsible for the alexiteric activity.

Moreover, the correlation between the activity results and chemical profiles followed by the HRMS screening approaches suggests that collection of this species should be performed in spring-summer. Suggesting, in addition, that extract of aerial parts of the plant has potential to form a phytopharmacological product that could be used for first aid in snakebite accidents.

2.3. References

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3. Conclusions to the thesis

This work allowed the development of new analytical strategies, one for each HRMS approach, for the investigation and characterization of several of the most widespread food commodities globally, such as grape, wine, spirits, vinegar, cocoa and honey. It focused on this analytical technique in view of its increasing and noteworthy role in food safety and quality control, and led to three new analytical methods: The first was based on a non-targeted screening approach, while the others combined suspect and targeted screening analysis. However, in all cases the possibility of studying analytes characterized by considerable chemical and structural diversity was related to the high sensitivity and selectivity of HRMS detection. In particular:

- The Neutral Loss experiments proved to be a valid and well-performing analytical tool for non-targeted investigation of glycosidic compounds, despite the great variability of corresponding aglycones and sugar moieties. Only in-source non-ionizable or instable glycosides could not be detected with the current method, due to the lack of precursor/product ion association;
- Suspect screening analysis proved to be an efficient method for profiling compounds of interest in many different matrices, despite their chemical differences and regardless of ionization polarity. It allowed tentative identification both by matching accurate masses and experimental MS/MS fragmentation and isotope patterns with those reported in the literature or theoretically surmised, and by matching retention times, accurate masses and experimental MS/MS fragmentation with those detected in natural matrices known to be particularly rich in the analytes studied;
- The targeted approach allowed the development of comprehensive analytical methods for the quantification of low-molecular-weight phenolic compounds and naturally occurring alkaloids. It provided fast, efficient and sensitive routine procedures for quality control and safety assessment of a large selection of natural matrices;
- In all the analytical approaches developed, SPE sample pre-treatment proved to be an efficient clean-up procedure for removing matrix interference and isolating the compounds of interest. In the non-targeted approach it allowed isolation of the glycosidic fraction of each sample, contributing to reducing false positives. With the suspect and targeted screening approaches, the use of online SPE clean-up improved the selectivity and sensitivity of detection and reduced the risk of false negatives attributed to matrix suppression.

Organic analysis of a wide selection of oenological matrices, cocoa beans, honeys, alpine herbal extracts and Argentine flowering plant extracts led to broad characterization of content and a

detailed profile of the nature and occurrence of the compounds of interest in selected samples. In particular:

- The non-targeted approach allowed detailed description of glycosides naturally occurring in several international monovarietal wines and provided an innovative tool for their characterization and classification;
- Application of non-targeted and suspect screening approaches to the analysis of *Vitis vinifera* color-rich grapes potentially important for the Uruguayan viti-oenology allowed a first, as far as I know, description of their free and glycosylated low-molecular-weight phenolic profiles;
- The targeted approach furnished a comprehensive description of the low-molecular-weight phenolic content of several matrices ascribable to the oenological industry, such as wine, spirits and vinegar;
- Application of suspect and targeted screening approaches to the analysis of hybrid and *Vitis vinifera* grapes and wines produced from hybrid grapes provided in-depth characterization of their free and glycosylated low-molecular-weight phenolic content and profiles. In addition, it allowed comprehension of the impact of alcoholic fermentation on the occurrence and distribution of the compounds examined;
- Study of tannins of different botanical origin though the suspect screening approach furnished a detailed description of the nature and occurrence of low-molecular weight phenolic glycosides. Consequently, it supplied a new instrument for botanical classification of tannins and a useful tool for satisfying the industry's request to verify product labels. Furthermore, it constituted a suitable instrument for evaluating the possible impact of tannin on the phenolic profile of wine before its addition, and its contribution towards increasing the natural glycosylated stock of low-molecular-weight phenolic compounds;
- Targeted screening analysis performed on wines during 3-month ageing in barrels allowed the evolution of the phenolic profile of wines to be studied, both in terms of the kinetics of exchange between wine and wood, and the quantitative and qualitative composition of the finished wines. Furthermore, the results also provided confirmation that barrel ozone treatment, as well as being effective in eradicating spoilage microorganisms, did not affect the aroma profile of wines or alter their phenolic profile;
- Application of suspect and targeted screening approaches to the analysis of two principal varieties from southern Italy – Primitivo di Manduria and Negroamaro wines – made it possible to define the free and glycosylated low-molecular-weight phenolic profile of selected wines for

the first time and to describe the compounds that could be released after acidic hydrolysis during ageing;

- In the case of cocoa bean investigation, the use of both suspect and targeted screening methods provided a broad profile of free and glycosylated low-molecular-weight phenolic compounds for each international area of production. Consequently, this analytical approach could be an efficient and well-performing tool for cocoa bean classification and for checking their origin;
- Application of non-targeted and suspect screening approaches to the analysis of honeys of Uruguayan production allowed a deep characterization of free and glycosylated low-molecular-weight phenolic profile for each mono-floral variety, and furnished interesting tool for honey traceability;
- In the case of alpine herbal extract analysis, the application of suspect and targeted screening approaches furnished a detailed description of the nature and occurrence of different classes of alkaloids, providing a useful tool for plant food safety control;
- In the case of Argentine flowering plant exctract analysis, the use of the suspect screening approach allowed to define the flavonoidic profile of *C. pareira* extracts and demonstrating that certain flavonoids may mitigate some venom-induced local tissue damage.

The high selectivity of high resolution mass spectrometry in terms of compound identification and the efficiency and time-saving of online SPE clean-up in reducing matrix interference allowed definition of broad free and glycosylated phenolic profile for a wide selection of matrices and samples. Despite the changing chemical characteristics of the compounds under investigation, the approaches considered can be tuned and optimized in order to correctly identify, and when possible quantify new molecules, as in the case of alkaloids. Finally, by combining non-targeted, suspect and targeted approaches it was possible to obtain efficient research tools and a broad description of different oenological and food products.

4. Other scientific contributions

Scientific Papers

- Nardin T.; **Barnaba C.**; Abballe F.; Trenti G.; Malacarne M.; Larcher R. (2017). Fast analysis of quaternary ammonium pesticides in food and beverages using cation-exchange chromatography coupled with isotope-dilution high-resolution mass spectrometry. *Journal of Separation Science*, doi: 10.1002/jssc.201700579.
- Nardin T.; Piasentier E.; **Barnaba C.**; Larcher R. (2017). Alkaloid profiling of herbal drugs using high resolution mass spectrometry. *Drug Testing and Analysis*, doi: 10.1002/dta.2252.

Conference

Poster

- Arrieta-Garay Y., Boido E., Fariña L., Carrau F., **Barnaba C.**, Larcher R., Nicolini G., & Dellacassa E. Metabolismo volátil y polifenólicocomomarcadores de variedades de Vitisviníferas poco frecuentes, Quinto Encuentro National de Química, Montevideo, 18-20 Ottobre 2017.
- Godoy A., Fariña L., Bonini A., Danners G., **Barnaba C.**, & Dellacassa E. Uso de quimiomarcadores en mielesmonofloralesproducidas de la flora nativa del Uruguay, Quinto Encuentro National de Química, Montevideo, 18-20 Ottobre 2017.
- **Barnaba C.**, Larcher R., Nardin T., Serra M., Dellacassa E., & Nicolini G. Untargeted high-resolution tandem mass approach for a putative profile of glycosylated phenols in international wines. X In Vino Analytica Scientia Symposium (IVAS), Salamanca, 17-20 July 2017.
- **Barnaba C.**, Larcher R., Nardin T., Dellacassa E., & Nicolini G. Glycosylated simple phenolic profiling of oenological tannins by high-resolution tandem mass spectrometry (Q-Orbitrap). X In Vino Analytica Scientia Symposium (IVAS), Salamanca, 17-20 July 2017.
- Larcher R., **Barnaba C.**, Dellacassa E., Roman Villegas T., Nardin T., & Nicolini G. Targeted and untargeted-high resolution tandem mass approach for a glycosylated simple phenols putative profiling of hybrid grapes. X In Vino Analytica Scientia Symposium (IVAS), Salamanca, 17-20 July 2017.
- Nardin T., **Barnaba C.**, Nicolini G., Malacarne M., & Larcher R. Simultaneous determination of amino acids and biogenic amines after derivatization using hybrid quadrupole-orbitrap mass spectrometer. X In Vino Analytica Scientia Symposium (IVAS), Salamanca, 17-20 July 2017.

- **Barnaba C.**, Dellacassa E., Nardin T., Nicolini G., & Larcher R. Untargeted high-resolution mass approach (Q-Orbitrap) for glycosylated simple phenol profiling in food commodities. III Congreso Argentino de espectrometria de masas, Rosario, November 22-24 2016.
- **Barnaba C.**, Dellacassa E., Nardin T., Serra M., Nicolini G., & Larcher R. First description of sugar units of wine glycosylated simple phenols. The 1st Food Chemistry Conference, October 30 – November 01 2016.
- Larcher R., **Barnaba C.**, Nardin T., & Nicolini G. Untargeted tannins glycosylated simple phenol profile by high resolution mass (Q-Orbitrap). The 1st Food Chemistry Conference, October 30 – November 01 2016.
- Nardin T., **Barnaba C.**, Larcher R. Herbal infusion alkaloid profiles by high resolution mass spectrometry. The 1st Food Chemistry Conference, October 30 – November 01 2016.
- **Barnaba C.**, Nardin T., Giacomelli M., Nicolini G., & Larcher R. Characterization of free and glycosidically bound simple phenols in hybrid grape varieties using liquid chromatography coupled to high resolution mass (Q-Orbitrap), Macrowine, Changins, June 27-30 2016.
- Roman T.; **Barnaba C.**; Nicolini G.; Debiasi L.; Larcher R.; Nardin T. The commercial yeast strain as a significant source of variance for tyrosol and hydroxytyrosol in white wine, Macrowine, Changins, Giugno 27-30, 2016.
- Nardin T.; Piasentier E.; **Barnaba C.**; Romanzin A.; Larcher R. (2016). Targeted and untargeted alkaloid characterisation of pasture herbs and milk from eastern Italian Alps using high resolution mass spectrometry. In: Casasús, I.; Lombardi, G. (eds) Mountain pastures and livestock farming facing uncertainty: environmental, technical and socio-economic challenge: proceedings of the 19th Meeting of the sub-network on Mediterranean pastures of the FAO-CIHEAM International network for the research and development of pastures and fodder crops, Zaragoza, Giugno 14-16, 2016.
- **Barnaba C.**, Nardin T., Nicolini G., & Larcher R. Untargeted analytical method for glycosylated simple phenol profiling by on-line solid phase extraction and LC-high resolution mass spectrometry (Q-Orbitrap), 40th International Symposium on Capillary Chromatography and 13th GCxGC Symposium, Riva del Garda (TN), May 29 - June 03 2016.
- **Barnaba C.**, Nardin T., Nicolini G., & Larcher R. Untargeted high resolution mass analytical method (SPE-LC- Q-Orbitrap) for glycosylated simple phenol profiling in natural food matrices, Incontro di Spettroscopia Analitica, Matera, May 29–June 01 2016.

- **Barnaba C.**, Dellacassa E., Nicolini G., Nardin T., Malacarne M., & Larcher R. First identification of glycosylphenols in Apulian Italian wines, 34th Informal Meeting on Mass Spectrometry, Fiera di Primiero (TN), May 15-18 2016.
- **Barnaba C.**, Nardin T., Perini M., Nicolini G., & Larcher R. Untargeted glycosylated simple phenol profiling in oenological tannins by high resolution mass analytical method (SPE-LC-Q-Orbitrap), Food Integrity, Prague, April 6-7 2016.
- **Barnaba C.**, Dellacassa E., Nicolini G., Nardin T., Malacarne M., & Larcher R. A new comprehensive method for simple phenols in wine, vinegar and spirit using SPE/UHPLC/high resolution tandem mass spectrometry. XV Congresso Latino-Americano de Viticultura y Enologia, XIII Congresso Brasileno de Viticultura y Enologia, Bento Gonçalves, Brasile, 3-7 November 2015.
- Nardin T., Piasentier E., **Barnaba C.**, & Larcher R. Rapid target and untarget analytical method for alkaloids analysis in herbal extracts, 7th International Symposium on recent advances in food analysis, Prague, November 3-6 2015.
- **Barnaba C.**, Nardin T., Pierotti A., Camin F., Nicolini G., & Larcher R. Characterization of simple phenolic compounds in cocoa beans using high-performance liquid chromatography coupled to High Resolution Mass Spectrometry (Q-Orbitrap), 4th MS Food Day, Foggia, 7-9 Ottobre 2015.
- Nardin T., **Barnaba C.**, Nicolini G., Malacarne M., & Larcher R. A new comprehensive method for the characterisation of simple phenols in alcoholic beverages by high resolution mass spectrometry. IX In Vino Analytica Scientia Symposium (IVAS), Trento, 14-17 Luglio 2015.
- Román Villegas T., Nicolini G., Nardin T., **Barnaba C.**, & Larcher R. The yeast strain as a source of variance exploitable for healthiness. The case of hydrosoluble vitamins, tyrosol and hydroxytyrosol in white wines. 38th World Congress of Vine and Wine, Mainz, Germania, 5-10 Luglio 2015.
- Nicolini G., Román Villegas T., Larcher R., Ingrassia S., **Barnaba C.**, & Nardin T. Contenuto in vitamine idrosolubili dei vini bianchi in relazione al ceppo di lievito, Enoforum 2015: innovazione ed eccellenza, Vicenza, 5-7 Maggio 2015.

Oral presentation (Underlined the speaker)

- **Barnaba C.**, Nardin T., Larcher R. High resolution mass approaches for wine and oenological product analysis. 2nd MS-Wine Day, MS for grapes, wine, spirits. 9-10 May, 2017. (**Invited Talk**)

- Nardin T.; Barnaba C.; Larcher R. Food profiling: new horizons of high-resolution mass spectrometry application, International conference MASSA 2016, Rome, Septembre 6-8, 2016.
- Nardin T., Piasentier E., **Barnaba C.**, Romanzin A., Larcher R., Targeted and untargeted alkaloids characterization of pasture herbs in eastern Italian alps using high resolution mass spectrometry, 19th Meeting of the FAO-CIHEAM Mountain Pastures sub-network, Zaragoza, June 14-16, 2016.
- Nardin T., Piasentier E., **Barnaba C.**, Larcher R., Targeted and untargeted alkaloids profiling of alpine flora using high resolution mass spectrometry (Q-Orbitrap), Incontro di Spettroscopia Analitica, Matera, May 29 –June 01 2016.
- Nardin T., **Barnaba C.**, Larcher R., Target and untargeted analytical method for alkaloids analysis in herbal extracts by high resolution mass spectrometry, 4th MS Food Day, Foggia, October 7-9 2015.

Workshop

Oral presentation (Underlined the speaker)

- Nardin T., Larcher R., **Barnaba C.**, "Food profiling": nuovi orizzonti applicativi per la spettrometria di massa ad alta risoluzione, Sicurezza alimentare ed autenticità dei cibi, Bologna, 23 giugno 2016.
- **Barnaba C.**, Pierotti A., Nardin T., Larcher R., Characterization of free and glycosidically bound simple phenolic compounds in cocoa beans with UHPLC-HRMS (Q-Orbitrap), Seminario Cromatografia Liquida, Thermo Scientific, Pomezia, 9 dicembre 2015.
- Nardin T., **Barnaba C.**, Larcher R., Caratterizzazione degli alcaloidi da estratti vegetali tramite LC Orbitrap e gestione dati con software Chromeleon, Seminario Cromatografia Liquida, Thermo Scientific, Pomezia, 9 dicembre 2015.
- Nardin T., **Barnaba C.**, Larcher R., Malacarne M., SPE On-line-UHPLC: possibili applicazioni in enologia, Seminario Tecniche analitiche innovative nell'analisi del vino, Thermo Scientific, Asti, Italia, 26 Marzo 2015.