Detection of Biomedically Relevant Stilbenes from Wines by Mass Spectrometry

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Veronica Andrei, Dana Copolovici, Florentina-Daniela Munteanu, Armand G. Ngounou Wetie, Iuliana Mihai, Costel C. Darie, and Alina Vasilescu

Abstract

Stilbenes represent a class of compounds with a common 1,2-diphenylethylene backbone that have shown extraordinary potential in the biomedical field. As the most well-known example, resveratrol proved to have anti-aging effects and significant potential in the fight against cardiovascular diseases and some types of cancer. Mass spectrometry is an analytical method of critical importance in all studies related to stilbenes that are important in the biomedical field. From the discovery of new natural compounds and mapping the grape metabolome up to advanced investigations of stilbenes' potential for the protection of human health in clinical studies, mass spectrometry has provided critical analytical information. In this review we focus on various approaches related to mass spectrometry for the detection of stilbenes—such as coupling with chromatographic separation methods and direct infusion—with presentation of some illustrative applications. Clearly, the potential of mass spectrometry for assisting in the discovery of new stilbenes of biomedical importance, elucidating their mechanisms of action and quantifying minute quantities in complex matrices is far from being exhausted.

Keywords

Stilbenes · Antioxidant · Wine · Mass spectrometry

V. Andrei · I. Mihai · A. Vasilescu (✉)
International Centre of Biodynamics, Bucharest, Romania
e-mail: avasilescu@biodyn.ro

D. Copolovici · F.-D. Munteanu
Faculty of Food Engineering, Tourism and Environmental Protection, "Aurel Vlaicu" University of Arad, Arad, Romania

A. G. Ngounou Wetie · C. C. Darie
Biochemistry & Proteomics Group, Department of Chemistry & Biomolecular Science, Clarkson University, Potsdam, NY, USA

40.1 Introduction

Consumption of wines in moderation can have a positive influence on the human body due to their high content in antioxidants. Antioxidants have the ability to scavenge free radicals species which are formed during metabolic processes in human cells which may inhibit normal functioning of the cells [1]. Ever since the French Paradox relating wine consumption to the low prevalence of cardiovascular diseases in spite of the unhealthy diet in France [2], wine antioxidants have been the focus of intensive research, with one compound, resveratrol, becoming particularly famous.

Resveratrol (3,5,4-trihydroxystilbene) is a stilbene, part of the phytoalexins group, an important class of *de novo* synthetized substances as response to pathogenic infections in plants [3, 4]. Resveratrol is found as a mixture of *cis* and *trans* isomers, synthesized in plants by stilbene-synthase [5]. The oxidative dimerization of resveratrol leads to oligomers called viniferins [6] (Fig. 40.1). Stilbenes (from stilbos—"shining" in Greek) are a class of substances with a common 1,2-diphenylethylene backbone. The *cis*- and *trans*- isomers of stilbenes have different pharmacological activities, the trans-isomer of resveratrol, for example, performs better in antioxidant and anticancer assays. Hydroxylated derivatives of stilbenes are called stilbenoids.

Resveratrol and resveratrol derivatives have been shown to be powerful antioxidants [3]. As many diseases develop due to the production of radical oxygen species in the human body, in the quest for new strategies against diseases, resveratrol and related stilbenes have been used in many *in vivo* and *in vitro* studies investigating their potential beneficial effect on human health [7–11]. A range of protective and preventive effects are currently widely attributed to resveratrol and its derivatives, including anti-aging [8], antioxidant [3], as an enhancer of NO production in endothelial cells [12], cardio protection [13], as a reducer of the invasion of breast cancer cells [14] etc.

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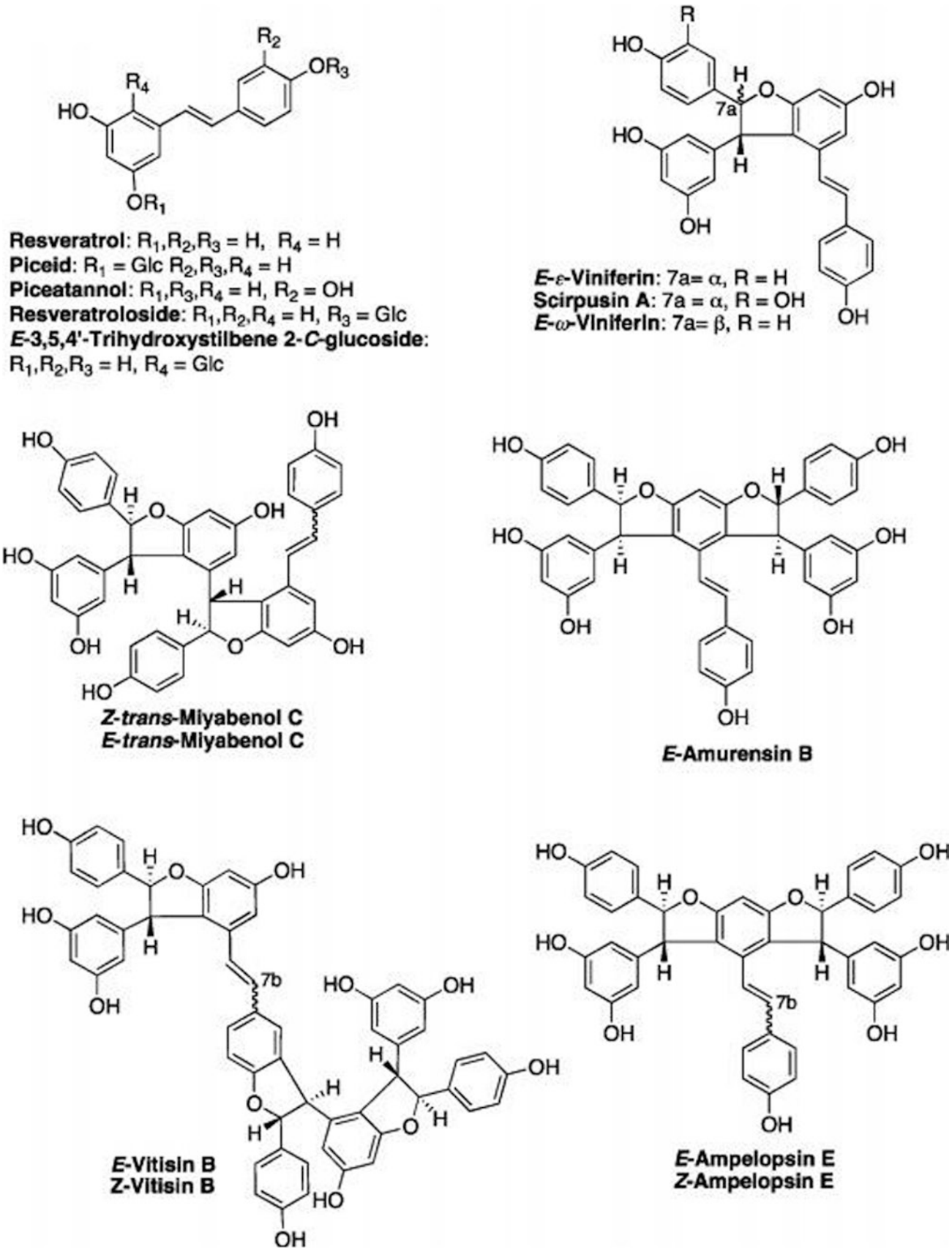

Fig. 40.1 Structures of compounds isolated from *V. amurensis* or *M. rotundifolia*. Reprinted with permission from [15]. Copyright (2013) American Chemical Society

This comprehensive area of well-known pharmacological properties of resveratrol exists due to a large research activity directed towards understanding the pharmacokinetics, the pharmacodynamics and the metabolism of this compound. Resveratrol was at the center of scientific world's attention in 2003 when a study published in the journal *Nature* showed that this molecule can increase the lifespan of *Saccharomyces cerevisiae* [16]. Later, in 2006, Professor Sinclair's group, found also that resveratrol can prolong the lifespan of obese mice, although it didn't have any effect on normal mice [17]. Clinical trials were launched, first based on resveratrol, than on some of its derivatives, examining the clinical efficiency of these compounds in managing diseases like cancer, diabetes, obesity, cardiovascular diseases and neurological affections such as Alzheimer's disease [18]. In 2013 it has been confirmed that resveratrol has anti-aging effects, being demonstrated that it activates the protein SIRT 1 through an allosteric mechanism [19]. Promising results were reported for the use of stilbenes in the prevention of cardiovascular disease, diabetes for delaying aging effects, moreover resveratrol appears to be well tolerated [18]. A review of clinical trials on resveratrol [18] summarizes the knowledge gathered so far: resveratrol is rapidly adsorbed in the human body after oral administration, with maximum levels in the human body being reached in approximately 30–60 min [20]. It is also metabolised very fast and the major issue in most clinical trials appears to be the low bioavailability. Besides resveratrol, pterostilbene was also studied with regards to its pharmacokinetics and safety. In May 2018, the initiation of a new clinical trial was announced, aiming to evaluate a novel pterostilbene formulation called "Nanostilbene", that relies on nanoparticles for an efficient drug delivery (www.therapeuticsolutionsint.com).

New stilbenoids have recently been identified [21, 22], hence the rising interest in developing new methods and strategies for their identification and quantification. Nowadays, there are more than 300 resveratrol oligomers identified and characterized in natural sources, mainly due to the advance of chromatographic methods coupled with mass spectrometry and NMR spectroscopy, respectively [23]. In addition, the challenges related to the analysis of very small volumes of samples led to different analytical approaches increasingly complex techniques and instrumentation.

Grapes and wines are a natural source of nutraceutical compounds, including stilbenes. The content of stilbenes in these natural matrices is influenced by a set of factors such as the wine making process, variety, climate etc. Different strategies are currently taken into consideration in the winemaking process for increasing not only the content of stilbenes in grapes but also the efficiency of their transfer to the obtained wines. For this purpose, a rapid screening of complex samples for the desired stilbenes is necessary and the analytical information has to be available in a timely manner. In this work we report on the application of mass spectrometry (MS), one of the most used analytical methods for the detection of biomedically-important stilbenes from wines.

40.2 Grape and Wine Stilbenes

Increased efforts have been directed in the biomedical field towards developing new strategies to prevent, treat or identify the causes of major diseases. The detection and quantification of biochemical compounds found in plants, that may act against various types of diseases is therefore very important. A tremendous amount of work has been done over the years to evaluate the chemical composition of grapes and wines. According to the literature, more than one thousand components have been identified in wine [24].

The antioxidant properties of grapes and wine are mainly due to the fact that they contain a large amount of polyphenols with the ability to scavenge reactive oxygen species. Resveratrol and its derivatives can be found in different parts of the grapevine plant such as grape canes [25, 26] or grape skin [27] and are also retrieved in wine [5, 15]. The evolution and synthesis of stilbenes in plant organisms depends on the occurrence of pathogenic infection [28] or exposure to various stimuli such as UV-C light [29]. One of the most common fungal infections which may affect the grapes and all the plant organs during growth is *Botrytis cinerea*. The pathways of stilbene formation in plants as response to fungal infections are not completely understood [4]. To gain more knowledge, *Vitis vinifera* cells were inoculated with methyl-β-cyclodextrin and methyl jasmonate, which lead to an increase in the concentration of resveratrol [30]. Both isomers of resveratrol, *cis*- and *trans*-, were found inside and outside of the cells, in contrast to another stilbene, piceid, which was found only inside the cells. Using the same elicitor (methyl jasmonate) in grape culture cells lead to the formation of *trans*-ε-viniferin, *trans*-δ-viniferin, *trans*-3-methylviniferin and *trans*-piceatannol [31].

The amount of stilbenes formed as a response to fungal infections depends on the grape variety [32]. Post-harvest irradiation with UV light can also stimulate the synthesis of stilbenoids in grapes [22, 33, 34]. Up to 15.25 mg/kg of total stilbenes (from which 10.89 mg/kg resveratrol) were found in grapes subjected to UV irradiation, compared to control where only 0.61 mg/kg have been found. In addition to resveratrol, p-viniferin (1.06 mg/kg), α-viniferin (0.34 mg/kg) and piceatannol (2.95 mg/kg) have been quantified in the UV-irradiated grapes samples. Similar results have been observed with white grapes, although the amounts of stilbenes were lower [35]. Besides increasing the concentration of stilbenes in wines, postharvest exposure of grapes to ultraviolet C was also found to result in the formation of new compounds (e.g. isorhapontigenin [22]).

A 2018 report shows moreover that spraying grape berries with chitosan leads to an increased concentration of stilbenes in the skin of the grapes [36], with 1.71- to 3.84-fold concentration changes for resveratrol, ε-viniferin, δ-viniferin, and piceid, values determined by using the LC/MS-MS.

In a recent work of Pugajeva et al. [37], it was demonstrated by means of HPLC/QqQ-MS that the highest amount of stilbenes is found in the stems and skins of the grapes, while the grape juice proved to contain a high amount of *cis*- and *trans*-piceids.

The concentration of resveratrol in wines is influenced not only by grape variety [26], geographical region, climate [25], the chemical treatments applied to the vine to prevent pathogenic infections, the occurrence of fungal infections in grapes, postharvest treatments etc. but also by the winemaking procedures [38, 39]. Evolution and stability of resveratrol and its derivatives during wine making and maturation is of great interest [39, 40]. It has been observed that, during wine maturation, the amount of both isomers of resveratrol decrease, from 0.37 mg/L of resveratrol found in the grape juice, to less than the detection limit in the final product, the same trend being also observed for piceid. The decrease of stilbenoids in wines exposed to UV irradiation [33] was proportionally much lower, from 6.52 mg/L resveratrol and 1.90 mg/L picetannol initially to 4.12 mg/L and 1.05 mg/L respectively in bottled wines.

Wines from all over the world have been shown to contain stilbenes. Red grapes have a higher content in resveratrol than white and rose grapes [35, 41]. A review published in 2007 [42] has shown that Canada produces wines with some of the highest resveratrol contents (3.2 ± 1.5 mg/L), calculated as mean between lowest and highest concentration in cited articles. In the same study the highest level of resveratrol, 3.6 ± 2.9 mg/L was found in Pinot Noir wines from France, while wines of Agiorgitiko variety grown in Greece displayed the lowest contents (0.6 ± 0.2 mg/L). In Italy, a total of 1.31 mg/L of trans-resveratrol have been found in Nero d'Avola, the main red wine variety from grapes grown in Sicily [43]. North African red wines also contain stilbenoids. A quantity of 1.20 mg/L of trans-viniferin has been found in Merlot wine, 0.69 mg/L in Cabernet Sauvignon from Algeria [44]. In Brazil, 2.27 mg/L of *cis*-resveratrol were determined in Cabernet Sauvignon, however, the highest level was observed in a local variety, Tannat, which contained 5.49 mg/L. Macedonian wines analyzed by HPLC-DAD–ESI-MS and MS/MS were found to contain higher amounts of stilbenes, compared to the Brazilian wines, namely *cis*- and *trans*-resveratrol, and *cis*- and *trans*-piceid [45]. In this study, the total stilbene amount for the wines produced in 2008 was found to be 43.9 μmol/L for Vranec, 14.0 μmol/L for Cabernet Sauvignon, and 19, 2 μmol/L for Merlot. A content of 7.81 mg/L trans-resveratrol was found in an Australian Pinot Noir [46]. Stilbene levels in wines from the Idaho Valley region in the US, expressed as trans-resveratrol ranged from 0.97 mg/L (Riesling) to 12.88 mg/L (Cabernet Sauvignon) [47].

40.3 Mass Spectrometry Analysis of Grapes and Wines Samples

Identification and detection of stilbenes are typically performed directly by electrochemistry [48], nuclear magnetic resonance (NMR), mass-spectrometry (MS, [49]) and capillary electrophoresis [50] or, most of the time, by coupling chromatographic separation with sensitive detection procedures. High performance liquid chromatography (HPLC) was coupled with DAD, fluorescence, electrochemical or mass spectrometry detectors [51–53]. The coupling of HPLC or GC with MS allows assessing in detail complex matrices with high sensitivity, being successfully used for both analytical applications and basic research [54]. The mass fragmentation patterns acquired by mass spectrometry can help to identify a wide range of compounds, either by comparison with an external standard solution or based on mass spectral data available in MS libraries.

The mass spectrometer is more than just another analytical instrument. Currently, it is a very important analytical tool in many fields such as chemistry, biochemistry and medicine and the complexity of this instrument has increased tremendously. A small glimpse into the power of mass spectrometry is offered by the 2015 report on the analysis of a 170 years old champagne bottle from a shipwreck in the Baltic sea [55], where the analytical techniques used included inductively coupled plasma sector field mass spectrometry (ICP-sf-MS), Fourier transform ion cyclotron resonance/mass spectrometry (FTICR/MS), ultrahigh-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (UPLC-Q-ToF-MS) and stir bar sorptive extraction-liquid desorption-gas chromatography-mass spectrometry (SBSE-LD-GC-MS).

Besides identification and quantitative detection of various classes of compounds, mass spectrometry is also very powerful for imaging, especially when correlated with other methods. For example, mass spectroscopy imaging (MSI) performed in parallel with fluorescence imaging enabled the in situ analysis of stilbenes on grapevine leaves for mapping their spatial distribution and monitoring their evolution in time as the leaves were subjected to either infection by *Plasmopara viticola* or to UV-C light [29] (Fig. 40.2).

The range of applications based on mass spectrometry with various types of mass analyzers and ionization methods continues to widen [56–58] due to the interest in discovering new compounds in the field of biomedicine with improved bioactivity and bioavailability that can be used to fight major diseases.

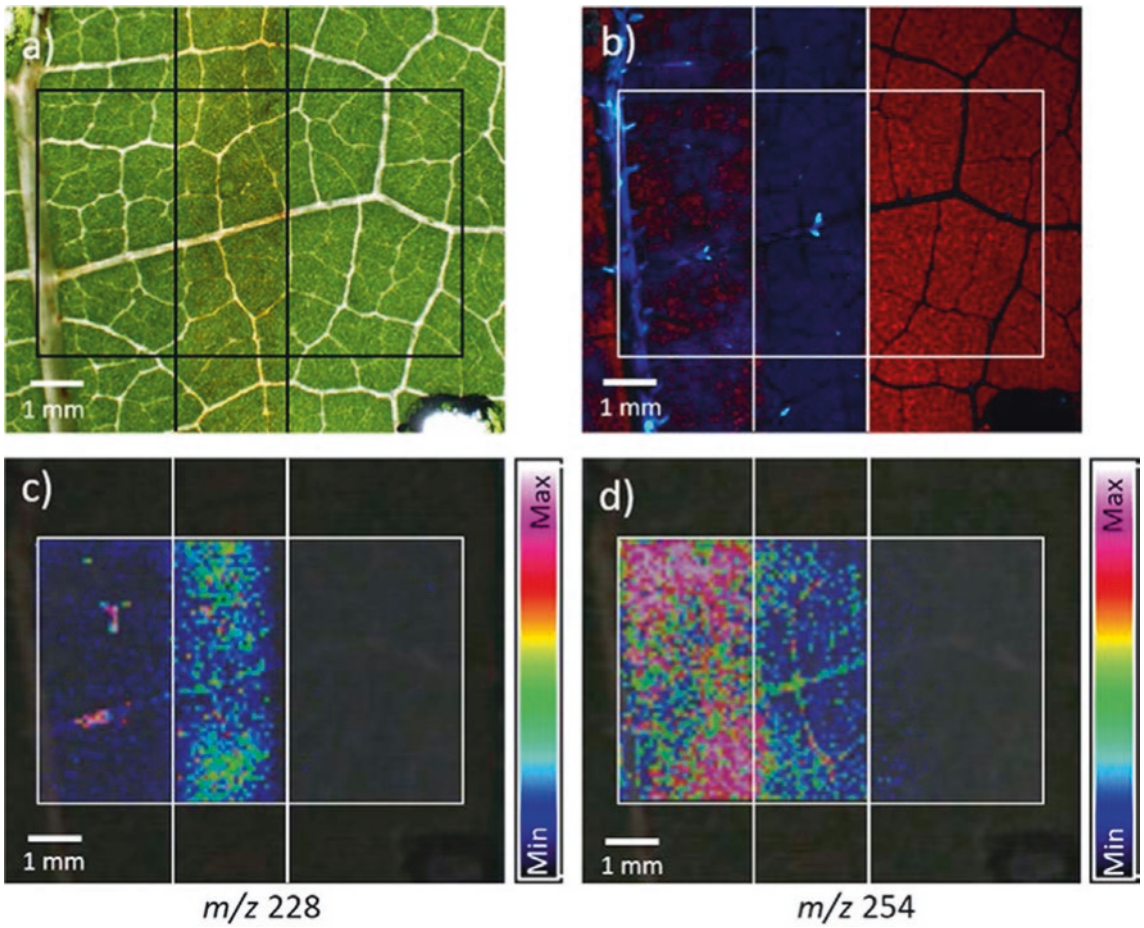

Fig. 40.2 Analysis of grapevine leaf: non irradiated (the area in the right) or submitted to the UV-C radiation for either 180 s (area in the middle) or 45 s (area on the left), by: (**a**) transmission microscopy. (**b**) fluorescence microscopy; (**c**, **d**) mass spectrometry imaging: molecular maps for resveratrol and piceids (**d**, m/z = 228)_and pterostilbene (**d**, m/z = 254), respectively. The color scales in (**c**, **d**) represent the relative intensity. Reproduced with permission from [29]. Copyright (2017), the American Chemical Society

Mass spectrometry has been shown to be invaluable in the analysis of stilbenes from grapes, grape cell cultures and wines, as well as for food supplements. For example, although more than 400 stilbenes from various plants are known in the present [59] other 23 new stilbenes were only discovered in 2013 with the help of MS [21]. Some cosmetics and numerous dietary supplements that contain stilbenoids and especially resveratrol can be found today on the market under many presentations: cremes, capsules, beverages, chocolate bars etc. (e.g. Resveratrol WINETIME bar™) [8]. The metabolic pathways and the rate of adsorption of active stilbenes from such products are not well investigated. Pharmacokinetics of stilbenes and the effect of various compounds from this class on various cell lines are two important research domains where the use of MS has a critical importance [60].

A variety of approaches have been used with mass spectrometry for discovering and quantifying stilbenoids in different types of matrices including: HPLC-ESI-MS for the detection of two important stilbenes, trans-resveratrol and trans-piceid in chocolate [61], the identification of a new resveratrol hexoside by reversed phase-HPLC coupled with atmospheric-pressure chemical ionization (APCI) tandem mass spectrometry (MS/MS) in cocoa liquor [62], the use of high resolution electrospray ionization mass spectrometry (HR-ESI-MS) in defining the chemical structure of novel stilbenoids from *Polygonum cuspidatum* [63] or from *Rumex bucephalophorus* roots [64] (compounds with a very important biological activity against α-glucosidase, important in controlling hyperglycemia). Mass spectrometry was either used in conjunction with separation techniques—typically GC or HPLC—or was directly applied to the samples (direct infusion ESI-MS).

Both targeted and non-targeted metabolomics rely quite heavily on mass spectrometry. The "suspect screening analysis" described in 2013 by Flamini et al. [65] is one of the tools that allow stilbene profiling in grapes and wines, which targets compounds with known structural formula and isotopic pattern. By using UHPLC-MS-TOF [66, 67] the group of Flamini et al. has developed "GrapeMetabolomics"—a

database of grape and wine metabolites that contains more than 1100 compounds Stilbene derivatives were identified in diverse varieties of grapes, such as Primitivo, Raboso Piave, Aglianico, Barbera, Cannonau, Cesanese d'Affile, Corvina, Enantio, Grignolino, Lambrusco Grasparossa, Montepulciano, Nebbiolo, Negro amaro, Nero d'Avola, Refosco dal Peduncolo Rosso, Rossese, Sagrantino, Sangiovese, Terrano, and Uva di Troia [65, 66, 68], Corvina Veronese, Carvinone, Raboso Piave [69]. In follow-up studies that took advantage of the GrapeMetabolomics database, *suspect screening* metabolomics based on high-resolution mass spectrometry allowed to identify markers that may be applied to assess the identity of diverse varieties of grapes used to obtain high-quality wines [66, 70].

40.3.1 HPLC-MS for the Analysis of Stilbenes

HPLC is the most commonly used separation technique coupled with mass spectrometry for the quantification and detection of stilbenoids. This coupling combines the strength of chromatographic separation with the specificity and resolution achieved with mass spectrometry. Ions produced from the various sample components are identified and quantitated by different approaches. Various types of ionization methods (electrospray ionization ESI [71, 72]; electron ionization [73] atmospheric pressure chemical ionization APCI [74]; atmospheric pressure photoionization (APPI–MSn) [75] and mass analyzers (TOF [76], QTOF, triple quadrupole (QQQ) [77], ion trap [46]) were used for the analysis of stilbenes. The MS data was acquired either in the positive or negative mode and compared with authentic markers for identification. Some reports claimed that the negative ionization mode offered more information related to the chemical structure of the compounds that could be used for confirming peak identity, as compared to the positive ionization mode [46]. Most authors preferred selected ion monitoring (SIM) over multiple ions monitoring as MS analyzing mode, in order to reach the best sensitivity and reproducibility [78].

Several examples of the practical application of mass spectrometry are detailed further below, to underline the power of this technique, pertaining in particular to the identification of stilbenoids in complex matrices or to explain the pharmacokinetics of these compounds.

In vitro adsorption of resveratrol and its derivatives from the dietary source of roasted and boiled peanuts has been studied in details using HPLC-UV and HPLC coupled with a quadrupole MS [60]. The assay has been done on a human adenocarcinoma cell line (Caco-2). The separation of the compounds was made by reversed-phase LC on a classical C18 column using gradient elution and the spectra acquisition in the range of 200–600 nm. The LC eluent was transferred in the MS interface without stream splitting. This method uses an APCI source (positive ionization) and the ion abundance was acquired in the range of 50–600 amu. MS was used to distinguish the aglycone and glycosidic forms of resveratrol after gastro-intestinal digestion. The MS spectra analysis revealed indeed the presence of resveratrol diglycosides in peanuts. Further the transepithelial transport of resveratrol has been investigated and the results of this study have shown that the hydrolytic products of resveratrol glycosides are transported at a higher rate than the glycosidic forms. They can be found in a higher amount in roasted peanuts in comparison with the boiled sample. The same type of ionization source but operating in the negative mode has been successfully used also for stilbene detection in wines with great sensitivity [74].

A study aiming at the characterization of some newly discovered stilbenes from downy mildew-infected grapevine leaves found that APPI lead to cleaner MS spectra and allowed to determine resveratrol oligomers with higher sensitivity compared to ESI ionization. MS^n spectra obtained were used to propose chemical structures for the unknown stilbenes [75].

The relationship between stilbene composition of grape skins or stems and that of the corresponding wine has been studied by HPLC-ESI-MS [71]. The MS spectra were recorded in both positive and negative modes. The MS data allowed to conclude that although both grape stems and skin contain high amounts of stilbenes, their transfer rates to wine are very low: only 4% of resveratrol and 14% of piceid in stem-contact wine were contributed by stem tissue while the transfer rate for grape skin is lower that 11%. The HPLC-ESI-MS technique has been successfully used to determine compounds like trans-resveratrol and δ-viniferin [32], piceid metabolites in rats [79], isomers of resveratrol dimer and their analogues [80] or for the analysis of polyphenols (including resveratrol) in order to classify wines according to their geographic origin, grape variety and vintage [76].

Other examples included the identification of trans-stilbenes from different commercial grape juices (containing also other juice extracts, such as pomegranate, blackcurrent, strawberries) and grape pomace by HPLC-DAD-MS/MS [81, 82] and the analysis of stilbenes from different matrices by coupling UHPLC to triple-quadrupole mass spectrometer (UHPLC-QqQ) [83]. In the later work, trans-resveratrol, trans-piceid, trans-piceatannol, trans-pterostilbene, and trans-ε-viniferin were analysed in extracts from *Vitis vinifera* cells, bioconversion reaction media and red wine. First, the chromatographic conditions for separation and MRM conditions in positive mode were optimized in order to quantify the stilbenes. Thereafter, quantifier transition chromatograms were used for each compound to obtain the quantitative MRM (*m/z* 391.1 → 229.1 for piceid, *m/z* 245.08 → 135.1 for piceatannol, *m/z* 229.09 → 107.1 for resveratrol, *m/z* 455.15 → 107.1 for viniferin, and m/z 257.12 → 133.1 for pterostilbene) [83] (Fig. 40.3).

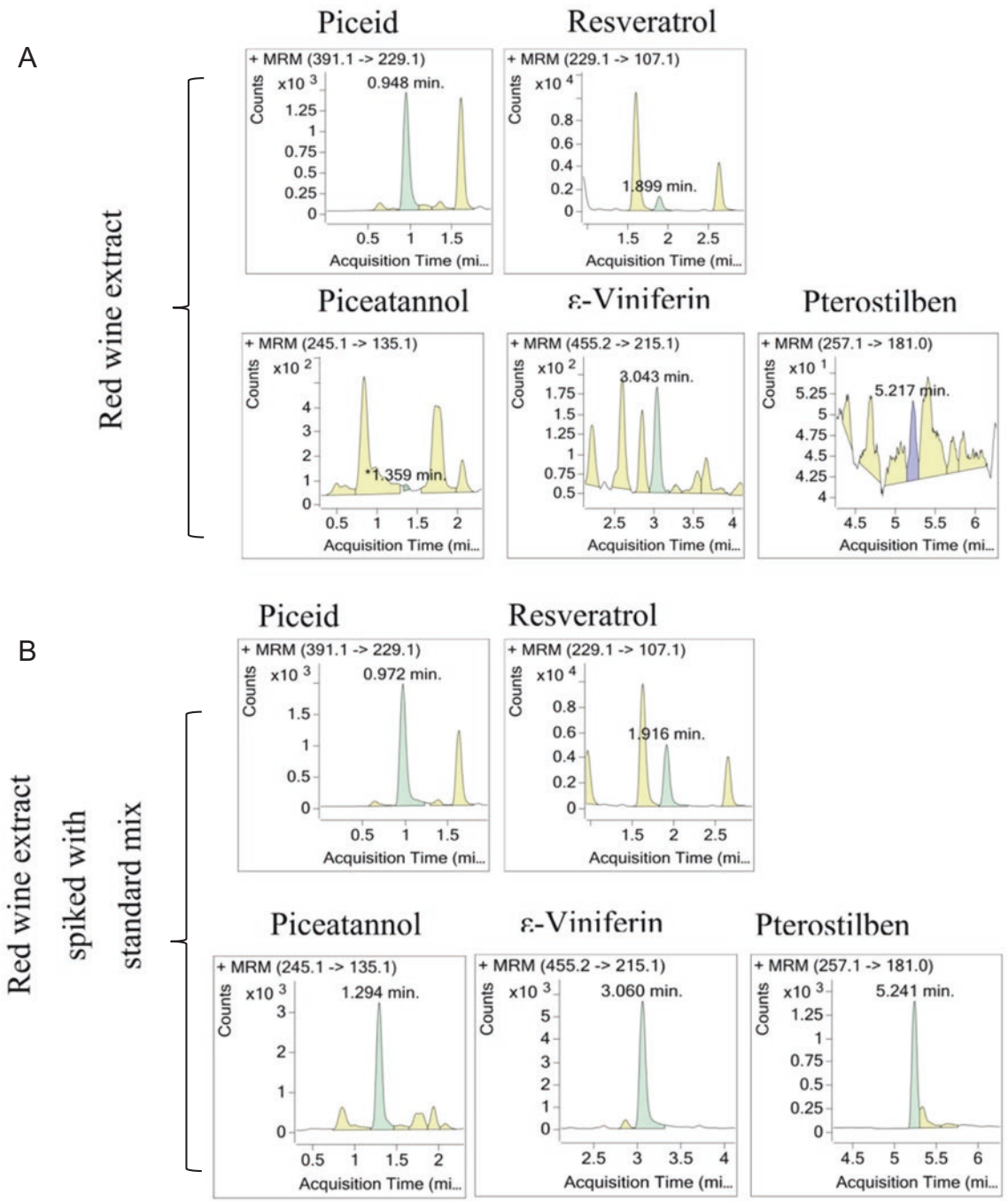

Fig. 40.3 Quantitative analysis of stilbenes by UHPLC-QqQ in a red wine extract before and after spiking with stilbene standards. Showing the MRM transition chromatograms for piceid, resvetratrol, piceatannol. Ɛ-viniferin and pterostilben. Adapted from [83] with permission by the authors. Copyright: Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/)

Applications based on mass spectrometry detection kept pace with modern chromatographic separation or with improved extraction procedures, for a rapid and sensitive analysis. Following this trend, 41 stilbenes (from which 23 new ones) were determined in the same run in a 2013 study by researchers at the University of British Columbia, Canada. The new discoveries were possible by coupling the fast separation by UHPLC with ESI-Q-TOF MS detection [21].

In another example, resveratrol and several secondary metabolites were identified by RP-HPLC and μLC–ESI ion trap MS/MS following selective solid-phase extraction (SPE) with reusable molecularly imprinted polymers (MIPs). The coupling between selective pre-concentration using MIPs and the sensitivity of MS detection allowed achieving a detection limit of 8.87×10^{-3} mg/L for trans-resveratrol [46] (Fig. 40.4).

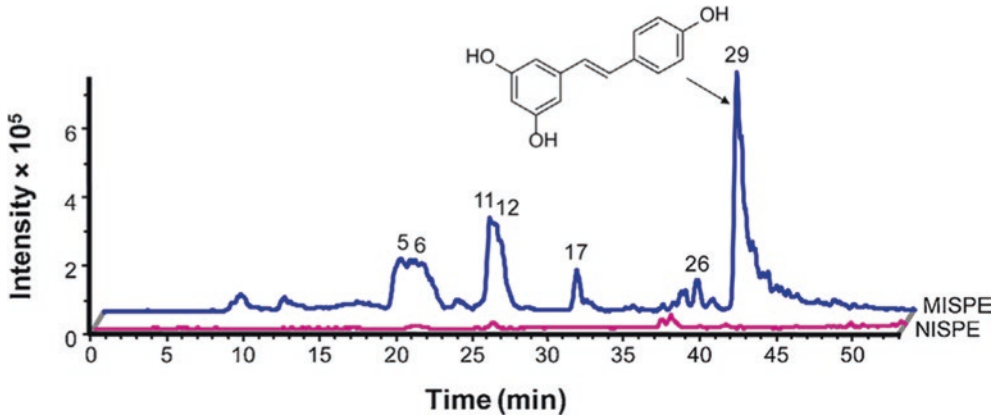

Fig. 40.4 Base peak chromatograms (BPC) of a Pinot noir red wine sample after treatment using either an (E)-resveratrol templated MISPE (back chromatogram) cartridge or the corresponding NISPE (front chromatogram) cartridge. The samples were analyzed in the m/z range from 100 to 1200, using LC–ESI-MS/MS in the negative ionization mode. MISPE: molecularly imprinted polymer solid-phase extraction. NISPE: non-imprinted polymer solid-phase extraction. Reprinted from [46]. Copyright (2013), with permission from Elsevier

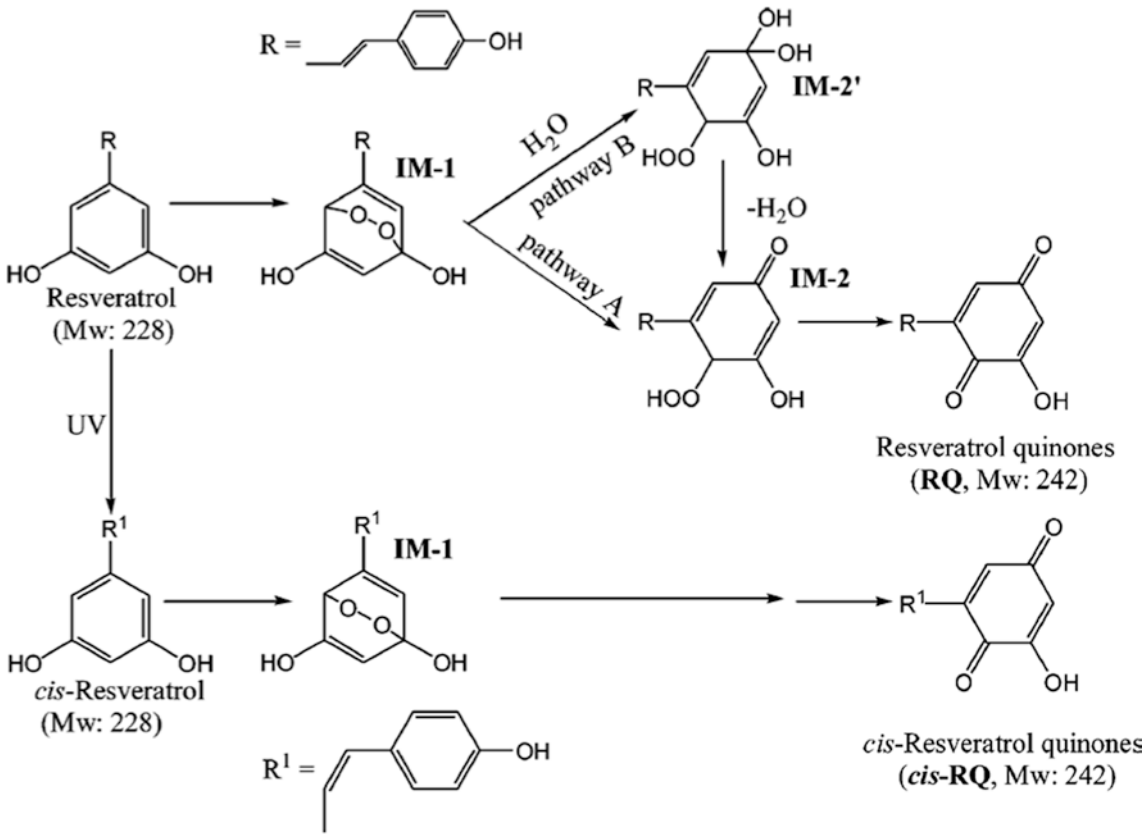

Fig. 40.5 Proposed mechanism for resveratrol against $^1O_2$. Reprinted with permission from [84]. Copyright (2010) American Chemical Society

The application of mass spectrometry in mechanistic studies of resveratrol against radical oxygen species can give insight into the pathways involved. Based on HPLC-ESI-MS data [84], a mechanism has been proposed for the interaction of resveratrol with $^1O_2$. The authors have shown that an endoperoxide intermediate is formed, followed by a hydrolyzed intermediate and quinine as final product (Fig. 40.5). This suggests that resveratrol can be useful as a drug for treating $^1O_2$-mediated diseases.

40.3.2 GC-MS for the Analysis of Stilbenes

Stilbenoids have been analyzed also by coupling gas chromatography with mass spectrometry (GC-MS). A chemical derivatization step has to precede analysis by gas chromatography in order to transform the non-volatile stilbenes into volatile, thermostable compounds. Various extraction methods for stilbenes have been developed to facilitate their sensitive detection by GC-MS such as: solid-phase extraction (SPE) [85] or solid-phase microextraction (SPME) [85], dispersive liquid–liquid microextraction (DLLME) [5], stir bar sorbtive extraction [86, 87]; directly suspended droplet microextraction (DSDME) [88] etc.

Such an example is the detection of five polyphenols (*trans*- and *cis*-resveratrol, piceatannol, catechin and epicatechin) in wine and grapes using a SPME-GC coupled to a quadrupole mass selective spectrometer equipped with an inert ion source that operates in electron-impact (EI) mode at 70 eV [89]. The derivatization reaction used in the study is silylation, the most commonly used for GC analysis of polyphenols. An alternative derivatization approach relies on acetylation [72].

A dispersive liquid–liquid microextraction (DLLME) technique has been proposed for the first time in a 2012 study for the determination of three hydroxylated stilbenes (trans-pterostilbene, resveratrol and piceatannol) in wine samples. By coupling this with GC-EI-MS analysis it was possible to quantify the above stilbenes in the range from 0.6 and 5 ng/mL [5]. More recently, in 2016, a similar approach that employed GC-EI-HRMS ("HRMS", high resolution mass spectrometry) was used to quantify semi-volatile compounds, including cis-/trans-resveratrol, trans-pterostilbene for 25 sample wines from diverse geographic areas in Spain [73]. Quantitative and selective detection in the ng/mL range was achieved by combining sample extraction by SPE, with concentration by DLLME and detection by GC-EI QTOF-MS. Notably, the study described a powerful strategy for the postrun identification of other stilbenes that were not considered during method development, e.g. dihydro-resveratrol in Temperanillo wine. Based on the TIC chromatograms, several peaks deemed compatible with the molecular ion of dihydro-resveratrol were identified with the "find by formula" function. By rendering the molecular ion in the EI, and comparing the m/z ratios of the fragment ions with the known ones for dihydroresveratrol, a cluster of ions compatible with the formula of dihydroresveratrol was identified at 23.63 min in the EIC chromatogram. Positive identification of this stilbene was based on mass accuracy (within ±5 mDa), isotopic pattern and the distance between ions in the cluster in the EIC chromatogram (Fig. 40.6).

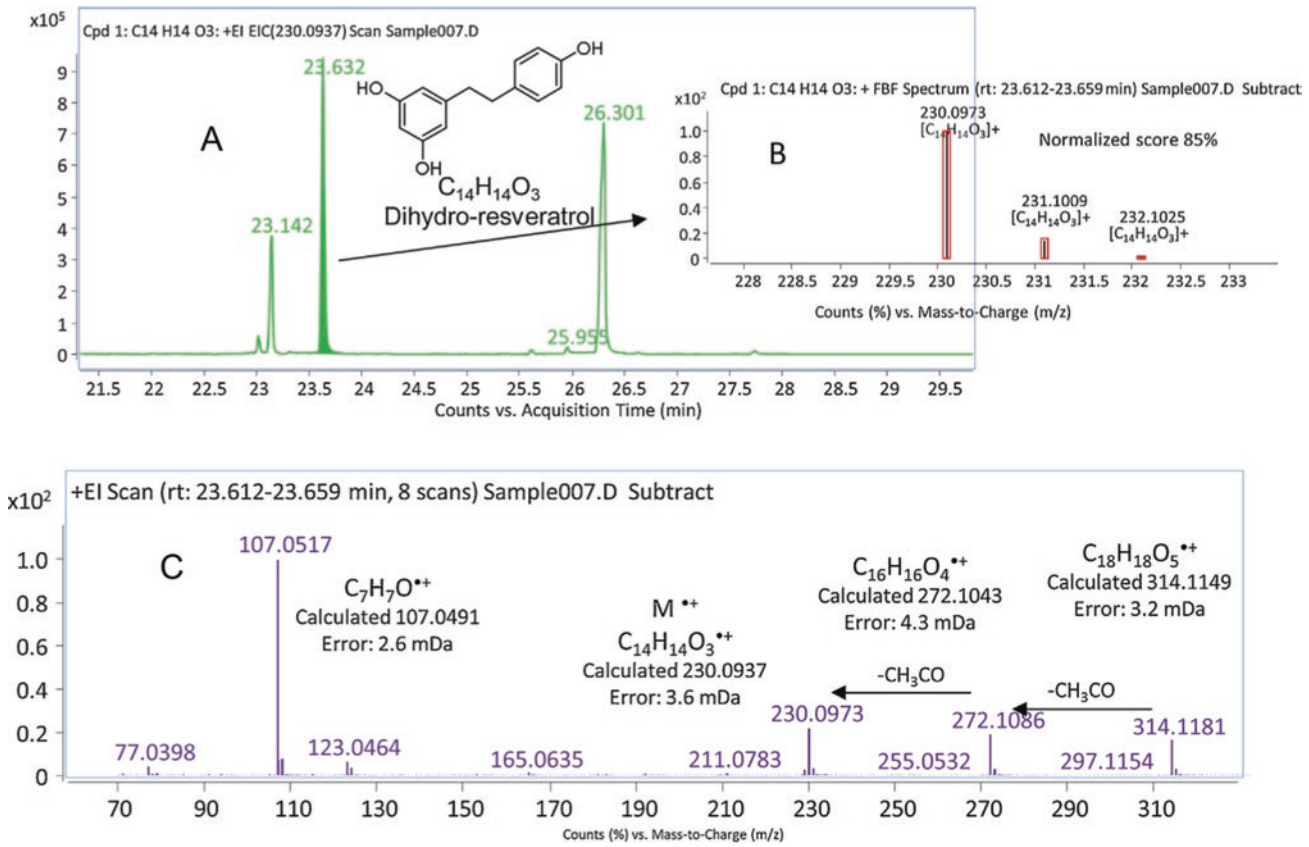

Fig. 40.6 Application of the "Find by Formula" approach for the detection of dihydro-resveratrol in a Temperanillo red wine: (**a**) EIC chromatogram for the molecular ion. (**b**) Matching the experimental spectra (black) with the predicted ones (red) for the molecular ion ($M^{\cdot+}$) of the candidate peak. (**c**) Experimental EI-HRMS spectrum of the candidate peak for dihydro-resveratrol showing mass errors under 5 mDa. Reproduced with permission from [73]. Copyright (2016) Elsevier

Resveratrol and its stilbene analogues were suggested to act as chemopreventive agents in colon cancer. Studies have been conducted both *in vitro* on colon cancer cell lines and *in vivo* on immune-deficient mice to check the efficiency of 24 stilbenes against this disease. An important part in this work was the GC-MS analysis of the serum from mice treated with the investigated compounds [90].

A method based on stir bar sorptive extraction coupled to gas chromatography–mass spectrometry by means of a thermal desorption unit (SBSE-TD-GC–MS) has been optimized for the determination of cis/trans isomers of resveratrol, piceatannol and oxyresveratrol in wines [87]. This study uses a GC coupled with a quadrupole mass selective spectrometer and an inert ion source. The compounds were quantified in the selected ion monitoring (SIM) mode in order to improve the sensitivity (Fig. 40.7). The major compound determined was *trans*-resveratrol, with concentrations in the range of 3–230 μg/L, depending on the type of wine.

40.3.3 Offline Analysis of Stilbenes by MS

In a few studies, although stilbenes were separated by HPLC, detection by mass spectrometry was done off-line. For example, a new resveratrol dimer (*cis*-ε-viniferin) was isolated and identified in an Algerian red wine using HPLC and MALDI-TOF MS [44]. Spectra were recorded in the positive-ion mode using the reflectron and with an accelerating voltage of 20 kV. One advantage of this technique was that it allowed the simultaneous detection of *trans*-ε-viniferin. Consequently, based on MS data is has been shown that both viniferin isomers exhibited marked cytotoxic activity breast human cancer cell lines, and antimutagenic activity [76]. *Trans*-/*cis*-ε-viniferin, astilbin, isoastilbin and the resveratrol tetramer (−)-hopeaphenol from Xinjiang grapes were isolated, purified and subjected to ESI-MS-QTOF [91]. Structure confirmation by MS allowed linking the stilbenes with their particular antioxi-

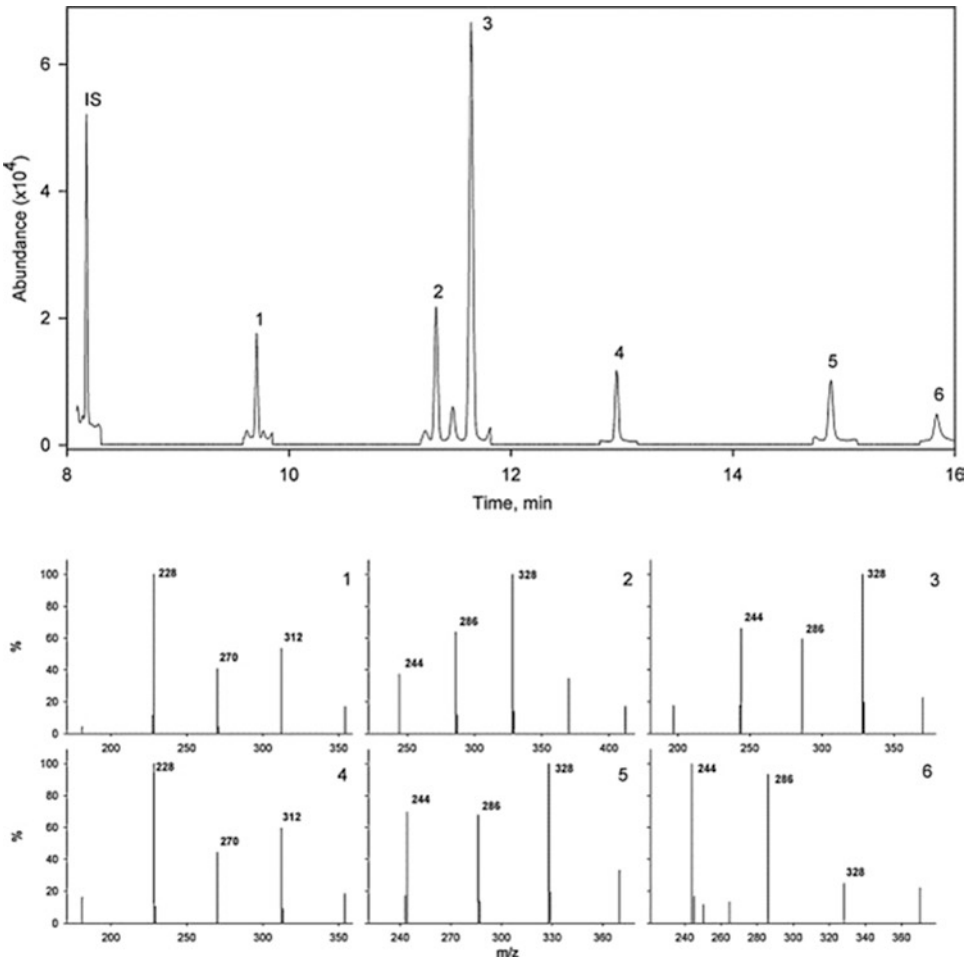

Fig. 40.7 (**a**) SBSE-TD-GC–MS chromatogram obtained for a spiked white wine fortified at 2 μg L^{-1} under SIM mode. Peaks correspond to: (1) cis-resveratrol, (2) cisoxyresveratrol, (3) cis-piceatannol, (4) trans-resveratrol, (5) trans-oxyresveratrol and (6) trans-piceatannol. (**b**) Mass spectra of each compound. Reprinted from [87] with permission from Elsevier

dant effects observed on human hepatoma cells. In the same study, it was shown via real-time quantitative PCR analysis that these compounds induced the down-regulation of oxidative stress genes at cellular level [91].

Last but not least, four stilbenoids, E-miyabenol C, E-cis-cis-miyabenol C, E-trans-cis-miyabenol C and E-cis-trans-miyabenol C, were purified from grapevine stalks by using a countercurrent-separation technique, namely centrifugal partition chromatography. Electrospray ionization ion trap mass spectrometry (ESI-IT-MS) was used for the detection of these molecules [92] proving to be a very powerful method for their fast detection and structure confirmation even from very small amounts of sample. These stilbenoids can find potential applications in the biomedical field as some studies indicated that they inhibit the formation of filaments by α-synuclein, a hallmark of Parkinson's disease [92].

40.3.4 Mass Spectrometry Analysis of Stilbenes Without Separation: Direct Infusion ESI-MS

In direct infusion mass spectrometry, the samples are analyzed without prior extraction or separation. This eliminates the bias due to sample pre-treatment and allows a fast screening of samples for the compounds of interest. Identification of these compounds is made by comparing their ESI-MS/MS fragmentation pattern with that of standard compounds from spectral libraries. A high number of substances from different chemical classes such as organic acids, inorganic acids and phenolic compounds can thus be identified in the same run in a matter of minutes [49]. Nonetheless, direct infusion mass spectrometry analysis has also a disadvantage, i.e. it cannot differentiate isomeric and isobaric compounds.

The MS spectra of commercial tannin and of several types of red wine (FN, PN, CS, NM), from the same vineyard and harvest (2012) collected by ESI-MS direct infusion in positive mode are shown in Fig. 40.8 (m/z range 100–850). Also included is the profile acquired for oenological tannin used in the same vineyard for tannin correction of wine in some winemaking procedures. The ESI-MS and ESI-MS/MS were acquired using a QTOF Micro mass spectrometer in positive mode and a micro ESI source with the capillary voltage at 3200 V, at a flow rate of 5 μL/min, according to published procedures [93–95].

As observed from Fig. 40.8, the wines analyzed have common peaks either in all wines and tannin, such as the peak with m/z of 381.27, peaks common to a particular wine and tannin (i.e. peaks with m/z of 397.25 and 719.42, found in tannin and in FN wine or peak with m/z of 274.25 found in tannin and PN wine), peaks found in some wines, but not in others or in tannins (i.e. peak with m/z of 809.17 found in PN and NM and peak with m/z of 513.07, found in PN and CS; none of these peaks were observed in tannin), or peaks specific to one type of wine (i.e. peak with m/z of 459.12, specific to tannins or peak with m/z of 493.12, specific to CS wine).

The peaks with the highest intensity that were either common or specific to tannin or to wines were selected for fragmentation and are currently investigated for identification of their identity (and structures). MS/MS fragmentation the peaks observed in the MS in tannin and various wines produced a series of spectra shown in Figs. 40.9 and 40.10.

All these MS spectra of tannins and various wines were recorded under ESI positive ionization; under slightly acidic conditions (red wines are acidic, with a pH ~3.5). However, the full composition of a particular wine (and tannin) is usually not revealed under a particular set of experimental conditions. For example, analyzing the same wines under very acidic conditions (i.e. in acetonitrile (ACN) containing 0.1% formic acid (FA)) can increase the number of molecules that one can identify. Furthermore, analyzing the same samples by ESI under negative ionization at neutral or alkaline conditions will surely lead to identification of additional peaks that correspond to molecules which are part of and perhaps specific to some particular wines. Examples of MS spectra of wines analyzed in ACN or in ACN containing 0.1% FA are shown in Fig. 40.11 (FN wine) and Fig. 40.12 (PN wine). In FN wine (Fig. 40.11), the peaks with m/z of 397.24, or 719.42 are specific to FN analyzed in ACN with no FA, while the peaks with m/z of 331.02, 535.02 or 639.04 are specific to FN analyzed in ACN with 0.1% FA. Common peaks were also observed (i.e. peaks with m/z of 453.16 or 493.02). In PN wine (Fig. 40.12), peaks specific to PN analyzed in ACN without FA (m/z of 116.06, 513.08 or 809.19) or peaks specific to PN analyzed in AC with 0.1% FA (m/z of 365.08 or 599.04), as well as peaks common to both conditions (m/z of 381.05 or 493.12) were also observed.

The MS spectra are a snapshot capturing unique characteristics of each type of wine and can be used by applying chemometrics for data interpretation in order to differentiate wine varieties. The high throughput of direct infusion ESI-MS allowed, among others, to follow the evolution of samples of must and wines obtained from different grape varieties before and after fermentation by acquiring specific fingerprints and identifying marker ions for wine and must. Principal Component Analysis was used to group samples according to these markers [96, 97]. As some authors noted, ESI-MS in negative mode can provide more analytical information compared to the positive mode as it leads to lower amounts of salt adducts and to a higher number and variety of ions [98].

More towards biomedical applications, direct infusion ESI coupled with an ion trap mass spectrometer has been

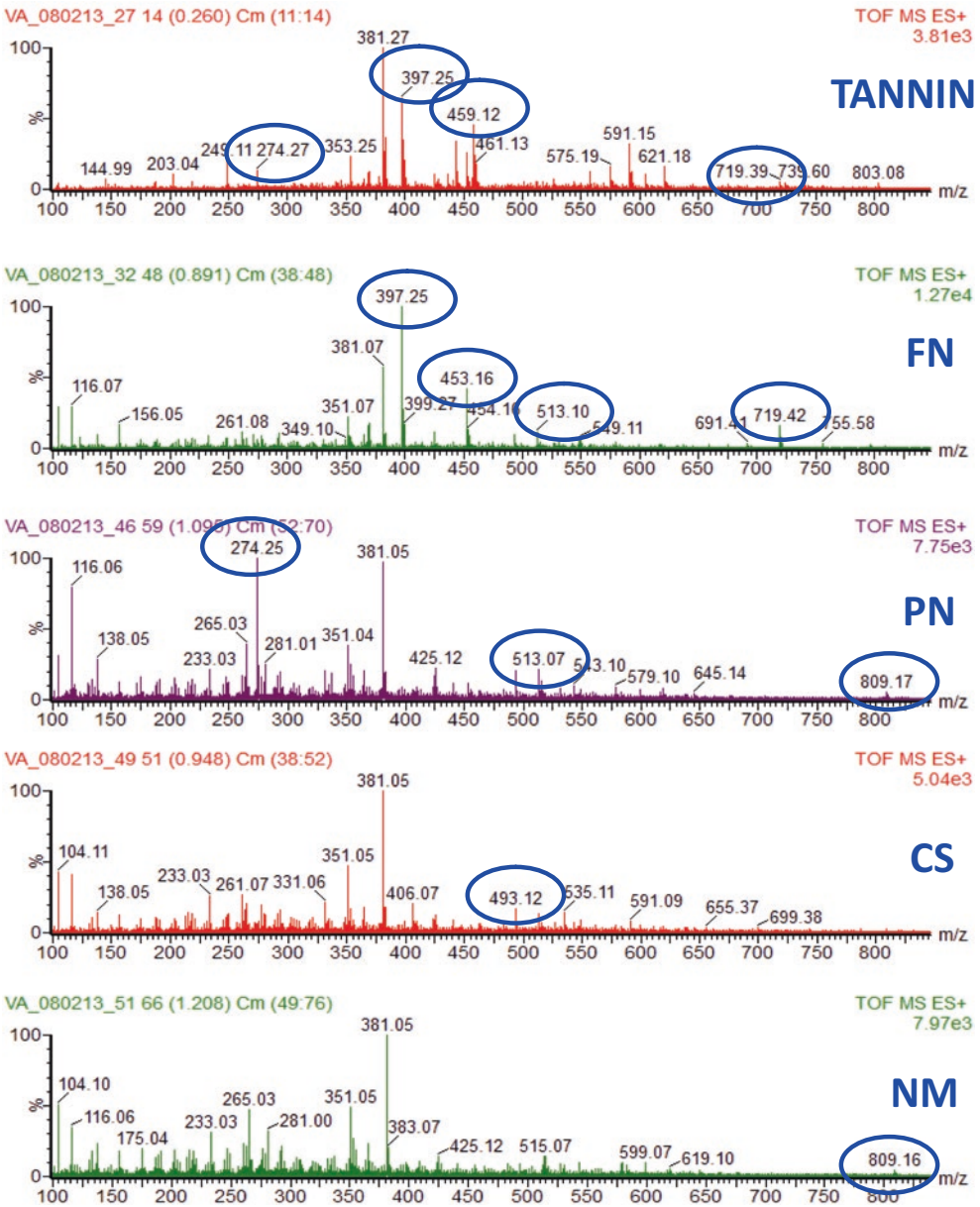

Fig. 40.8 ESI-MS analysis (direct infusion) of tannin and various wines (FN, PN, CS, NM). Shown are the peaks with the m/z ranging from 100–850. Circled are the peaks either common to all wines or specific to some wines and tannin or specific to one particular wine. These peaks are discussed in the text

used to study the interaction between e-viniferin glucoside (VG), a resveratrol-derived dimer, and amyloid β-peptides responsible for triggering neuronal degeneration [99]. Using this technique it was possible to observe a non-covalent complex between VG and Aβ. The formation of this complex leads to decreased cytotoxicity of Aβ against PC12 cells lines *in vitro*.

The increasing evolution of analytical instrumentation providing higher mass resolution, robustness and accuracy has raised the ESI-MS technique from the rank of "fingerprinting" tool to one of metabolite profiling [100]. ESI-MS combined with single or hybrid quadrupole, time-of-flight, ion trap, Orbitrap and FT-ICR-MS technology are time consuming analytical approaches that offer the possibility to

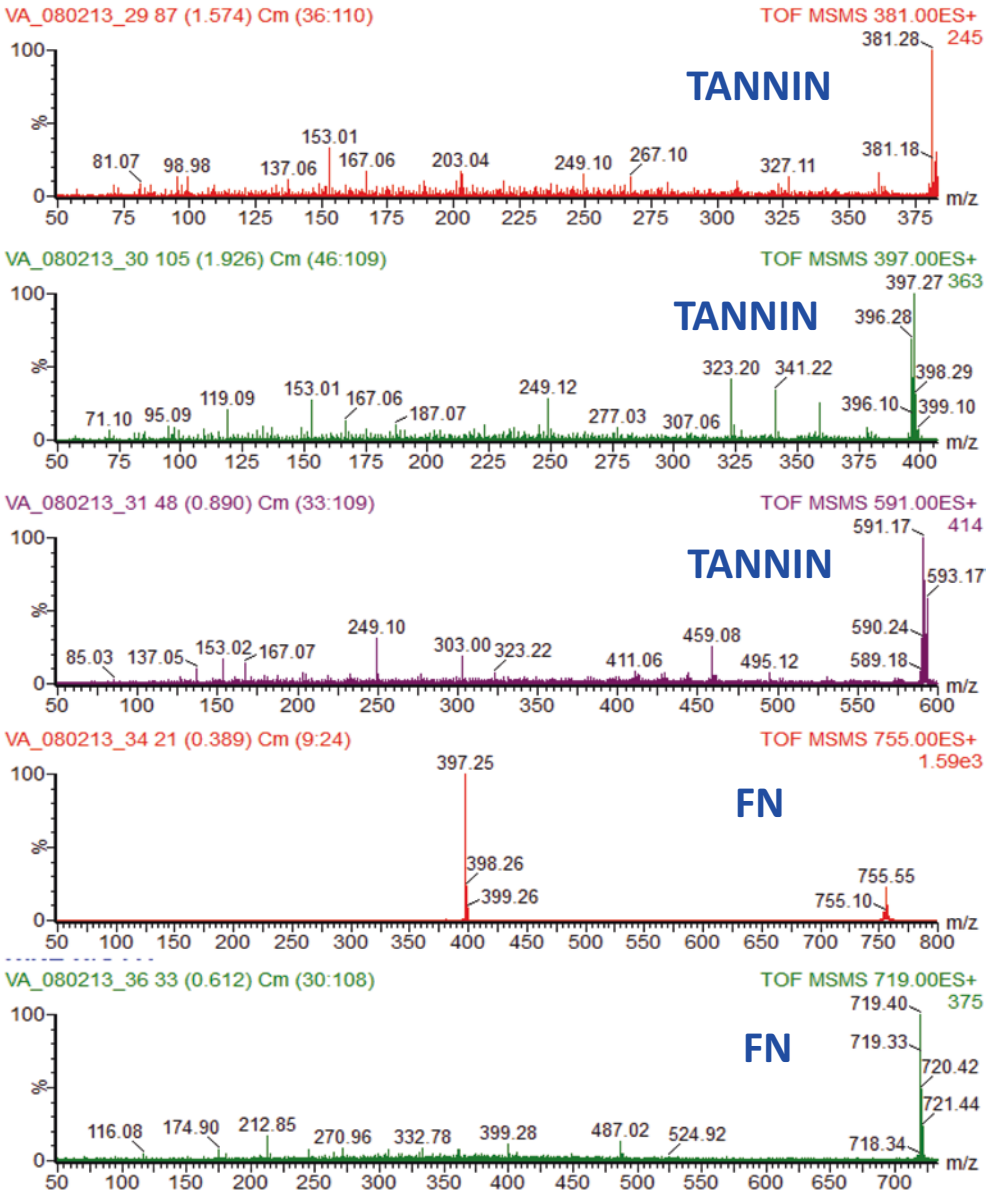

Fig. 40.9 ESI-MS/MS analysis (direct infusion) of precursor ions detected in ESI-MS in tannin (m/z of 381.28, 397.27, and 591.17) and FN wine (m/z of 755.55, 719.00). The collision energy was optimized for each precursor and varied from 5–50 V

obtain a complete metabolome analysis in complex samples [101]. For example, the ion cyclotron resonance-Fourier transform mass spectrometry (ICR-FT/MS) has been used to develop a non-targeted method for metabolite profiling in oenology [102] or to differentiate grapes and corresponding wines from distinct vineyards, according to complex chemical fingerprints [103].

A whole range of applications of direct infusion mass spectrometry have been described in the last years, from disease phenotyping to food metabolomics. While high-throughput approaches for wine metabolomics gained constant interest among researchers in the past 5 years [104–107], the analysis of stilbenes by direct infusion mass spectrometry was not a particular area of interest.

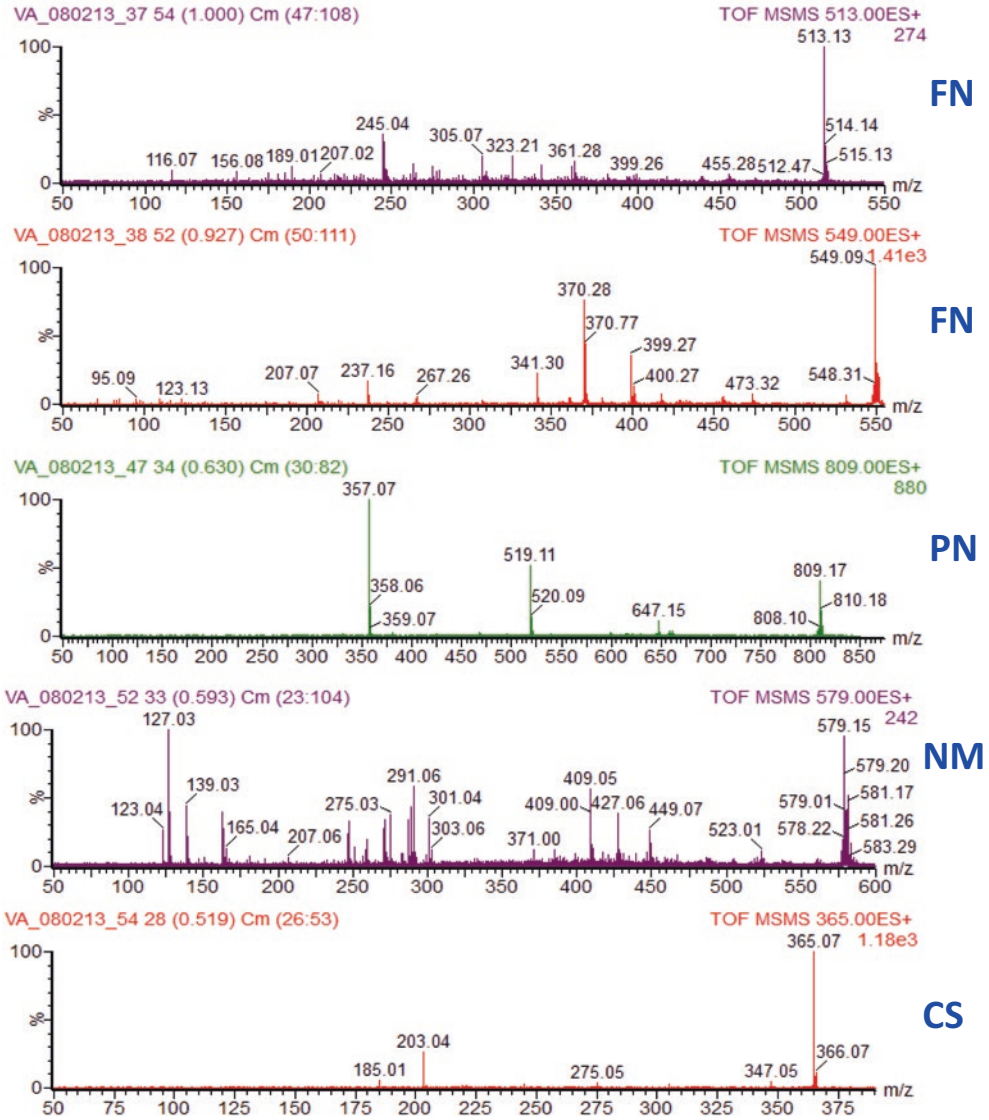

Fig. 40.10 ESI-MS/MS analysis (direct infusion) of precursor ions detected in ESI-MS in FN wine (m/z of 513.13 and 549.09), PN wine (m/z of 809.00), NM wine (m/z of 579.00) and CS wine (m/z of 365.00). The collision energy was optimized for each precursor and varied from 5–50 V

40.4 Conclusions

Resveratrol and related stilbenes represent a class of compounds with extraordinary potential for the biomedical field, due to their aging-delaying effects and their potential against cardiovascular diseases, diabetes and cancer. New chemical compounds from this class and even more importantly, their interactions in the human body remain yet to be discovered. Mass spectrometry proved to be of critical importance for the study of stilbenes that are important in the biomedical field from the simple screening of samples in search of those containing the desired stilbenes up to complex investigations aiming to unravel new chemical structures and understanding their interactions. In the past 5 years metabolomics emerged as a powerful approach for profiling grapes and wines, heavily relying on MS for compound identification and quantitative detection, although the detection of the specific chemical class of stilbenes was not of a particular interest. There is a

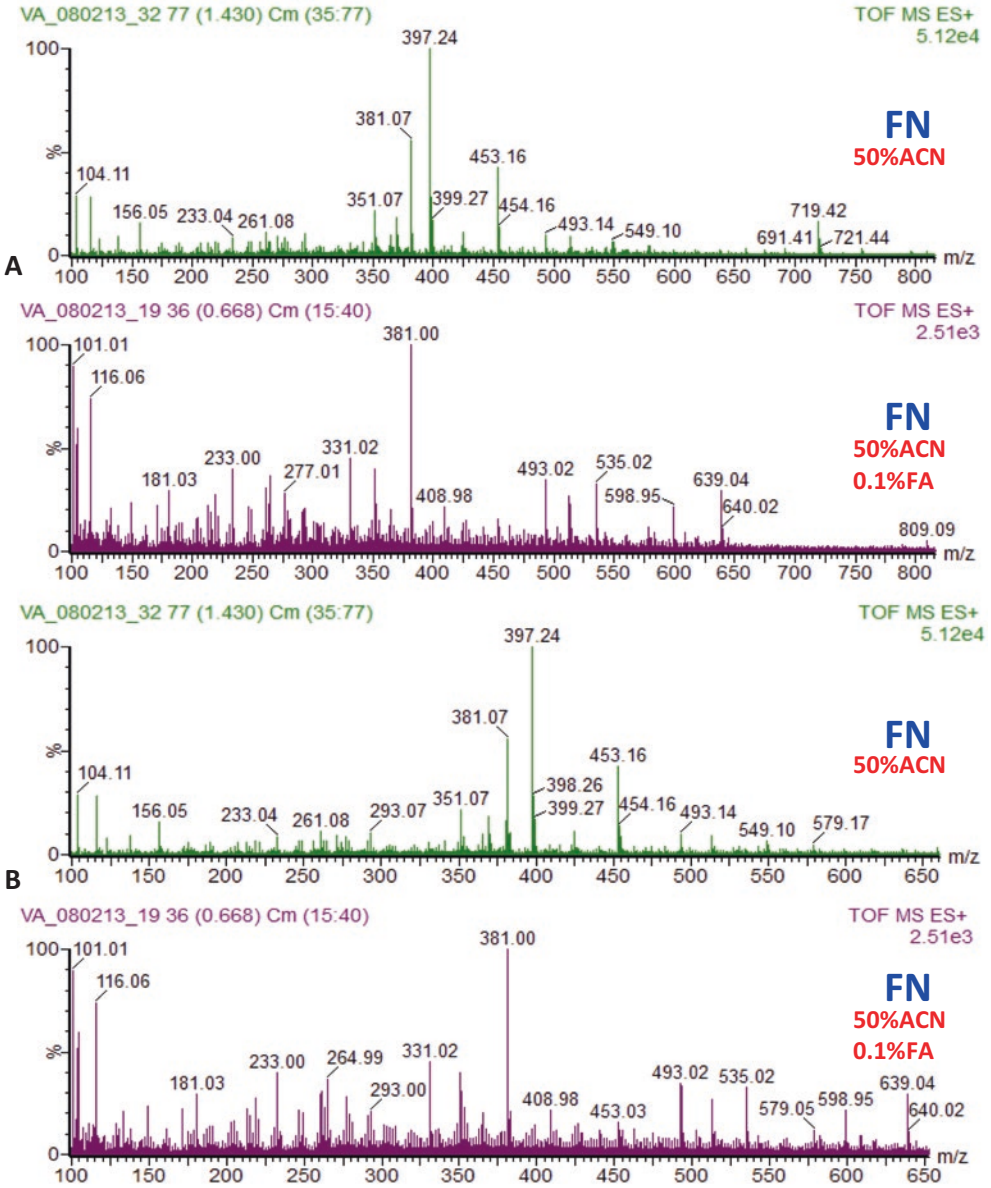

Fig. 40.11 ESI-MS analysis (direct infusion) of FN wine analyzed in ACN and ACN with 0.1% FA. The m/z range is 100–850 (**a**) and 100–650 (**b**)

huge potential to be explored to gain insight into the unique characteristics brought by variety, terroir, production year etc. and undoubtedly, as databases are continuously updated and with the advances in instrumentation, much more knowledge on stilbenes and their detection by MS will be generated in the years to come. Not last, MSI proved very powerful for mapping the spatial distribution of stilbenes in grapevine leaves, when correlated with other imaging techniques in order to understand the mechanism behind phytoalexins production by the plant. Such understanding would be invaluable for the selective and quantitative purification of stilbenes from natural sources.

Clinical trials featuring resveratrol and more recently pterostilbene brought also progress towards the development of novel drugs based on stilbenes, fueling the interest in the identification of such novel compounds, as well as their selective and sensitive quantitative detection in biological samples facilitated by MS.

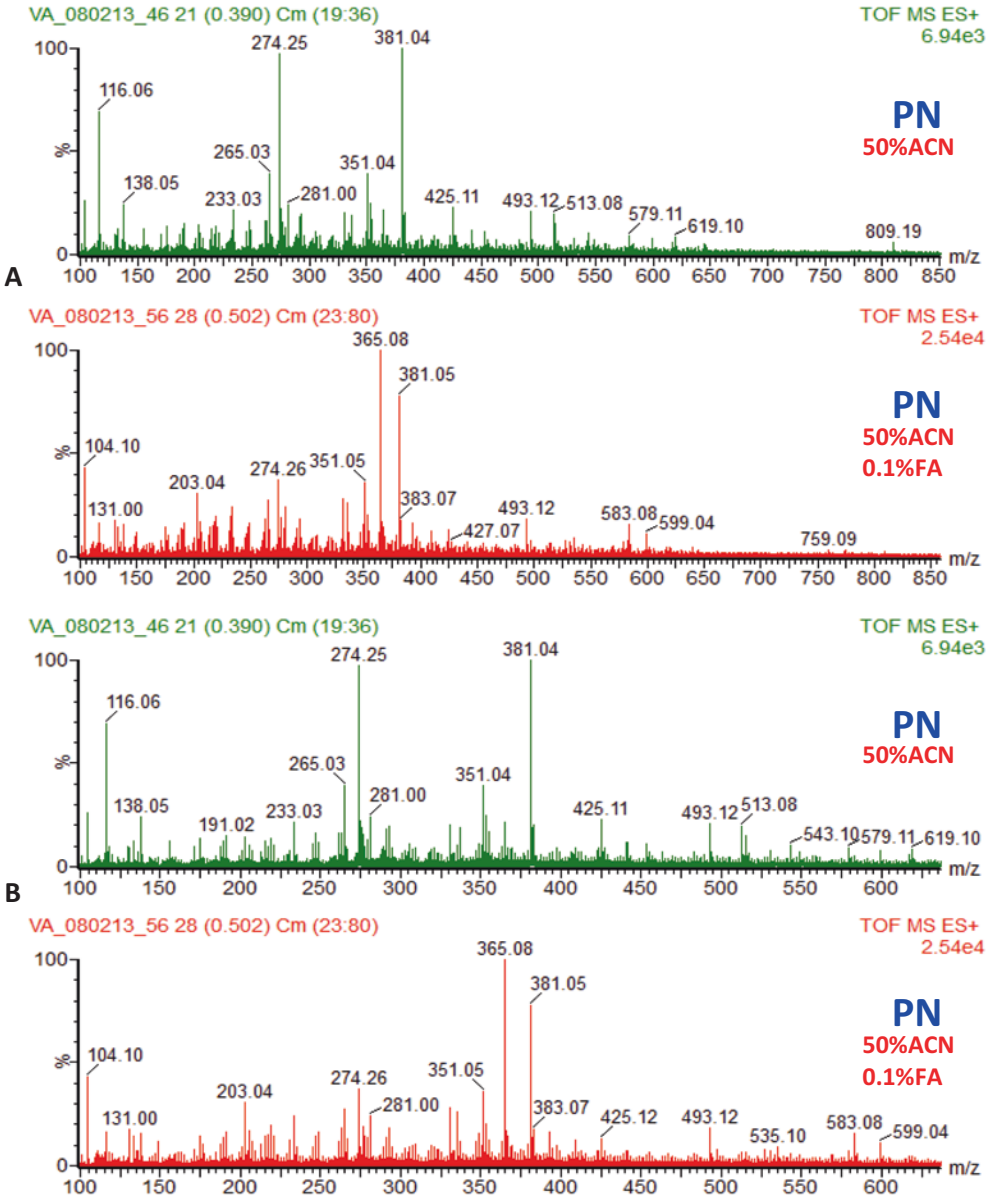

Fig. 40.12 ESI-MS analysis (direct infusion) of PN wine analyzed in ACN and ACN with 0.1% FA. The m/z range is 100–850 (**a**) and 100–650 (**b**)

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