RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2010; **24**: 634–642
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4427

Liquid chromatographic/electrospray ionization mass spectrometric identification of the oxidation end-products of *trans*-resveratrol in aqueous solutions

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Received 11 November 2009; Revised 10 December 2009; Accepted 18 December 2009

***trans*-Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenolic compound that exhibits antioxidant properties. Our study aimed at studying the $HO^{\bullet}$-induced oxidation of resveratrol ($100\ \mu mol.L^{-1}$) in aerated aqueous solutions. Gamma radiolysis of water was used to generate $HO^{\bullet}/O_2^{-}$ free radicals ($I = 10\ Gy.min^{-1}$, dose = 400 Gy). Oxidation products were identified by direct infusion mass spectrometry and high-performance liquid chromatography/mass spectrometry. For each product, structural elucidation was based on simple mass spectra, fragmentation spectra and deuterium/hydrogen exchange spectra; the comparison with mass spectra of synthetic products provided valuable information allowing the complete identification of the oxidation products. Four products resulting from the direct attack of $HO^{\bullet}$ radicals towards resveratrol were identified respectively as piceatannol (*trans*-3,5,3′,4′-tetrahydroxystilbene), 3,5-dihydroxybenzoic acid, 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. Copyright © 2010 John Wiley & Sons, Ltd.**

trans-Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (Fig. 1) is a natural phytoalexin belonging to the stilbene family found in a wide variety of dietary sources including grapes, plums and peanuts.[1,2] This phenolic stilbene has been the focus of a number of studies investigating its beneficial effects on neurological, hepatic and cardiovascular systems.[3,4] A primary interest for research on resveratrol (RVT) has come from the paradoxical observation that a low incidence of cardiovascular diseases may coexist with intake of a high-fat diet and moderate consumption of 150–300 mL/day of red wine, a phenomenon known as the 'French paradox'.[4,5] This fact could be explained by the powerful antioxidant activity of the trans-RVT[1,6] because free-radical-mediated peroxidation of membrane lipids and oxidative damage of DNA are considered to be associated with a wide variety of chronic pathologies, such as atherosclerosis, cancer and aging.[7,8] Therefore, research interest in the antioxidative activity of RVT and derivatives has grown during the past ten years.[9,10]

Although some oxidation products have been identified *in vitro*,[11] the mechanisms by which trans-RVT displays its antioxidant properties have not totally been elucidated. To improve the knowledge on the RVT antioxidant mode of action, this work has focused on the direct antioxidant properties of RVT *in vitro* against $HO^{\bullet}$ free radicals generated in aerated aqueous solution by gamma radiolysis.

Gamma radiolysis is a powerful tool for the modelling of oxidative stress because it allows generation of Reactive Oxygen Species (ROS) in solution, with well-known radiolytic yields[12] (the radiolytic yield is the number of free radicals produced per unit of energy absorbed, i.e. per joule). This method has been used to initiate the one-electron oxidation (via $HO^{\bullet}$ free radicals) of bioorganic substrates.[13,14] Among the ROS generated by water radiolysis (i.e $HO^{\bullet}$, O_2^{-} and H_2O_2), the hydroxyl radical is the most reactive species. Indeed, it possesses a high oxidation potential [E'° ($HO^{\bullet}/H_2O$) = 2.3 V at pH 7.0][12] and reacts on numerous biomolecules with high rate constants (usually 10^9–$10^{10}\ L.mol^{-1}.s^{-1}$).[15]

Resveratrol has been reported to be an antioxidant, that is a reducing agent, by reacting with ROS,[16,17] but literature lacks mechanisms of formation of radical-induced RVT oxidation products. Hence this work aimed at studying the *in vitro* oxidation of RVT by $HO^{\bullet}$ free radicals produced by water radiolysis and at identifying the oxidation products by using high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS) based on electrospray ionization (ESI)-generated negative ions. It can be noted that the collisional behaviour of deprotonated RVT obtained under electrospray conditions was recently investigated.[18] It was proven that the deprotonation reaction, responsible for the formation of the $[M-H]^{-}$ ion of RVT under ESI conditions, was

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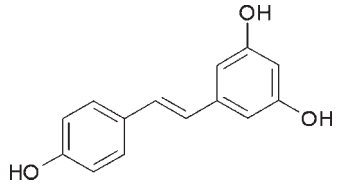

Figure 1. Chemical structure of *trans*-resveratrol.

assisted by the hydroxyl groups of the aromatic rings of RVT. In our study, the identification of these oxidation products was performed by MS^n experiments and deuterium labelling.

EXPERIMENTAL

Chemicals and reagents

trans-Resveratrol (trans-RVT) (ref. 70675) was purchased from Cayman Chemical Company (Spi-Bio, Montigny-le-Bretonneux, France). All aqueous solutions of trans-RVT ($M = 228\ g.mol^{-1}$) were prepared at a concentration of $100\ \mu mol.L^{-1}$ (pH 7.0) with ultra-pure water (resistivity 18.2 MΩ, Maxima Ultra Pure Water, ELGA), using sonication for 2 h. Aqueous solutions of trans-RVT are stable for at least 1 week when protected from light to avoid chemical modifications and isomerization into *cis*-resveratrol, as checked by UV-visible spectrophotometry.[19] Piceatannol (ref. AGP-1015-50) was from ABCys (Paris, France). 3,5-Dihydroxybenzoic acid (ref. D110000), 4-hydroxybenzoic acid (ref. 240141), 3,5-dihydroxybenzaldehyde (ref. 144088) and 4-hydroxybenzaldehyde (ref. 368113) were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Generation of $HO^{\bullet}$ free radicals by gamma radiolysis of water

Radiolysis corresponds to the chemical transformations of a solvent because of the absorption of ionizing radiation, which produces a homogeneous solution of free radicals within a few nanoseconds. Radiolytically generated free radicals are independent of the nature and concentration of the dissolved compound as long as its concentration remains lower than or equal to $10^{-2}\ mol.L^{-1}$.[12]

Gamma irradiations were performed with an IBL 637 irradiator (CIS Biointernational, Gif-sur-Yvette, France) using a ^{137}Cs γ-ray source whose activity was ~222 TBq (6000 Ci). The main advantage of gamma radiolysis is that it allows the modulation of the cumulated amount of ROS produced by increasing or decreasing the radiation dose (expressed in Gy; $1\ Gy = 1\ J.kg^{-1}$), i.e. by selecting the time during which the sample is exposed to the γ-ray source: the longer the exposure, the higher the radiation dose. It can be noted that for diluted solutions, no direct interaction of γ-radiation with the molecule under focus occurs and the radiolytic effect is only due to the radical species produced by water radiolysis. The dosimetry was determined by the method of Fricke and Morse[20] and the dose rate was found to be $10\ Gy.min^{-1}$ in our experiments. A radiation dose of 400 Gy was delivered to the aqueous solutions of trans-RVT.

In the presence of oxygen, i.e. for aerated solutions, water radiolysis leads to the generation of both hydroxyl and superoxide free radicals ($O_2^{-\bullet}$ coming from the scavenging of e^-_{aq} and $H^{\bullet}$ by O_2), with respective yields of 2.8×10^{-7} and $3.4 \times 10^{-7}\ mol.J^{-1}$.[11] It is well known that hydroxyl radicals are highly reactive species,[15] whereas superoxide radicals are poorly oxidizing or reducing species.[21] Jia *et al*. have shown that RVT is only able to scavenge superoxide free radicals *in vitro* for concentrations much higher than $100\ \mu mol.L^{-1}$.[22] As a consequence, under our conditions, hydroxyl radicals are the only radical species initiating the oxidation process. Volumes of 1.5 mL of a non-irradiated solution were systematically taken as a control for each experiment. Prior to each set of experiments, glassware was carefully washed with TFD4 soap (Franklab, France), rinsed with ultra-pure water (resistivity 18.2 MΩ, Maxima Ultra Pure Water, ELGA) and finally heated at 400°C for 4 h to avoid any pollution by organic compounds.

Mass spectrometric analyses

Mass spectrometry was performed on an ion-trap mass spectrometer (LCQ Advantage, ThermoFinnigan, Les Ulis, France) equipped with an ESI source. The capillary temperature was held at 250°C and the relative sheath and auxiliary gas flow rates were set at 20 and 5, respectively (sheath gas, 0–100 units corresponds to $0–1.5\ L.min^{-1}$; auxiliary gas, 0–60 units corresponds to $0–18\ L.min^{-1}$). Other parameters, such as lens or capillary voltages, were tuned systematically to obtain the best signal intensities for each ion of interest. All experiments were performed in the negative-ion mode, and each spectrum was typically an average of 20 acquired scans. For tandem mass spectrometry (MS/MS) experiments, a typical isolation width of 1 Da was used.

For direct infusion analysis, irradiated aqueous solutions of trans-RVT were diluted in acetonitrile (1:1, v/v) prior to being infused continuously into the ESI source with an SGE 250 μL syringe at a flow rate of $12.5\ \mu L.min^{-1}$. To study deuterium exchange on trans-RVT and its oxidation end-products, 3 mL of irradiated and non-irradiated trans-RVT aqueous solutions were lyophilized to dryness and rediluted into the labelled medium D_2O/acetonitrile (1:1, v/v). Deuterium oxide has minimum isotopic purities of 99.96% and was from Aldrich Chemicals (Milwaukee, WI, USA).

In addition, some experiments were conducted by coupling a high-performance liquid chromatograph (Surveyor, Thermoquest, Les Ulis, France) to the mass spectrometer. As much of 3 mL of irradiated and non-irradiated RVT aqueous solutions were lyophilized to dryness and rediluted into 200 μL of H_2O/acetonitrile (20:80, v/v). Chromatographic conditions were as follows: 20 μL of sample were injected onto the column (Kromasil C18 250 × 2.1 mm, 5 μm, A.I.T. Chromato, France), whose temperature was held at 30°C. Gradient elution was carried out with H_2O (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of $150\ \mu L.min^{-1}$. The mobile phase gradient was programmed with the following time course: 20% mobile phase B at 0 min, linear increase to 80% mobile phase B at 20 min, 20% mobile phase B at 21 min and held 5 min. The mass spectrometer was used as a detector, working in the full-scan mode between 50 and 500 Da and in a dependent scan mode, allowing the fragmentation of selected precursor ions (typical isolation width of 1 Da and collision energy set at 35%, units as given by the manufacturer).

DOI: 10.1002/rcm

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RESULTS AND DISCUSSION

MS and HPLC/MS study of oxidized resveratrol

The first approach of this work was to study an aerated aqueous solution of RVT (100 μmol.L^{-1}), irradiated at 400 Gy, then diluted in H_2O/acetonitrile (1:1, v/v), and infused continuously into the ESI source. Figure 2 presents the full-scan mass spectrum obtained from 50 to 500 Da. Five ions of interest were present in this spectrum: the $[M–H]^-$ deprotonated RVT detected at *m/z* 227.2 and four others at *m/z* 121.1, 137.1, 153.1 and 243.2. The latter probably resulted from the addition of an oxygen atom on RVT because its mass differed by +16 Da (227 + 16 = 243). There was no such simple evidence for the other ions. These results were taken as a base for the identification of the oxidation products by high-performance liquid chromatography/mass spectrometry (HPLC/MS). To ensure that oxidation products were effectively generated during water radiolysis of RVT, and not into the ESI source, several aqueous solutions of RVT, irradiated at 400 Gy (+ non-irradiated control), were analyzed by HPLC/MS. Figure 3 presents trace chromatograms for the ions detected at *m/z* 227 (RVT), 243, 153, 137 and 121, for an aqueous solution of RVT (100 μmol.L^{-1}) non-irradiated (Fig. 3(a)) and irradiated at 400 Gy (Fig. 3(b)). For non-oxidized RVT (*m/z* 227), the intensity of the peak detected at approximately 13 min strongly decreased at 400 Gy, showing the consumption (i.e., oxidation) of RVT during water radiolysis. The other products were not present in the non-irradiated solution of RVT and were detected at 10.67, 4.56, 6.83 and 9.77 min, respectively, for *m/z* 243, 153, 137 and 121 when the solution was irradiated at 400 Gy. Thus, those products were generated during water radiolysis. In addition, and to be sure that all the potential oxidation products were detected, trace chromatograms were generated for every mass from 50 to 500 Da, in steps of 1 Da: no other peak was detected.

Reactive oxygen species, especially hydroxyl radicals, can initiate three types of oxidizing reactions: abstraction of one hydrogen atom, abstraction of one electron (charge transfer) or addition to double bonds or aromatic rings. The chemical structure of RVT presents many positions potentially sensitive to the attack of hydroxyl radicals: two benzene rings along with a conjugated aliphatic double bond, electron doublets of the three oxygen atoms and hydrogen atoms on aliphatic and benzenic carbons. The chemical structures of the RVT oxidation products were thus difficult to predict. Nevertheless, based on the mass of the ion detected at *m/z* 243.2 (i.e. 227 + 16), the first assumption is that water radiolysis of RVT could lead to piceatannol (M = 244.1 g.mol^{-1}, Fig. 4(a)), that is known to be a metabolite of trans-RVT *in vivo*.[23,24] The oxidation products detected at *m/z* 153, 137 and 121 could result from an oxidative fragmentation of RVT during water radiolysis, leading to the formation of 3,5-dihydroxybenzoic acid (M = 154.1 g.mol^{-1}, Fig. 4(b)), 4-hydroxybenzoic acid or 3,5-dihydroxybenzaldehyde (M = 138.1 g.mol^{-1}, Figs. 4(c) and 4(d)) and 4-hydroxybenzaldehyde (M = 122.1 g.mol^{-1}, Fig. 4(e)), respectively. Based on these hypotheses, we analyzed synthetic piceatannol, 3,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde by HPLC/MS. The retention times of these products are shown in Fig. 3(c). By comparing these retention times with those of the products generated during water radiolysis, we can conclude that 4-hydroxybenzoic acid

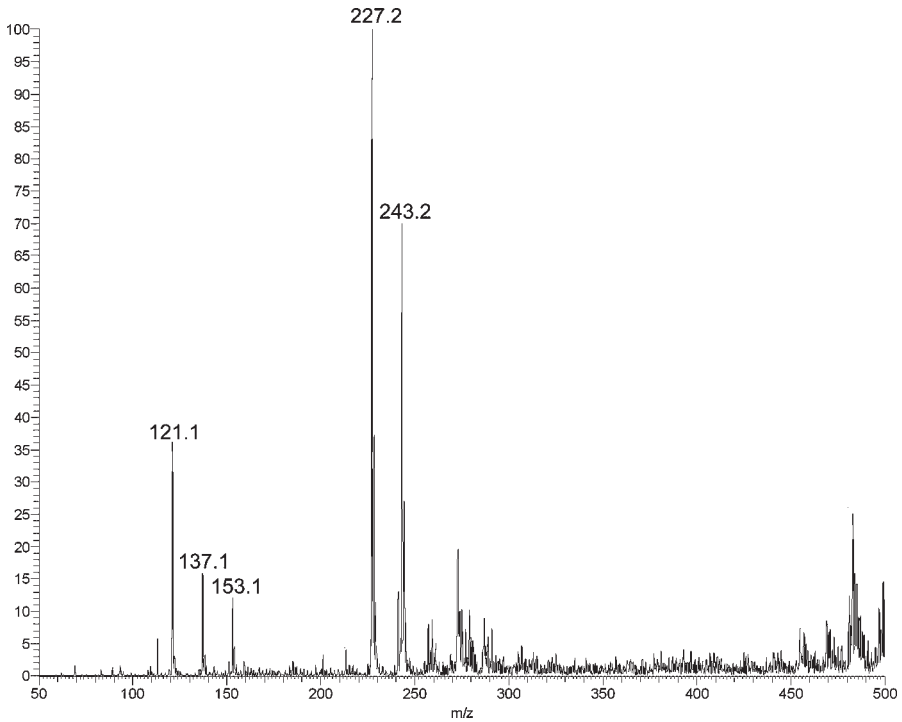

Figure 2. Full-scan mass spectra of an aqueous solution of trans-RVT (100 μmol.L^{-1}) oxidized at 400 Gy under aerated conditions.

DOI: 10.1002/rcm

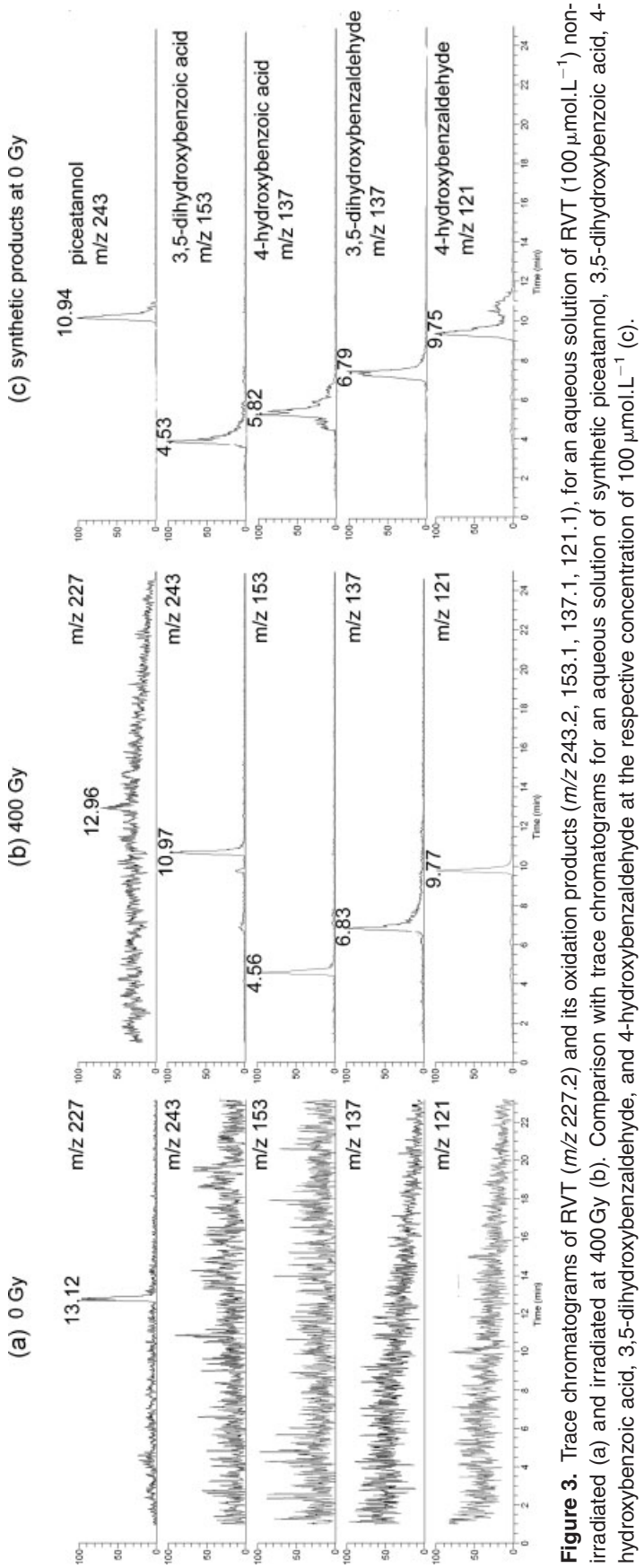

Figure 3. Trace chromatograms of RVT (*m/z* 227.2) and its oxidation products (*m/z* 243.2, 153.1, 137.1, 121.1), for an aqueous solution of RVT (100 μmol.L^{-1}) non-irradiated (a) and irradiated at 400 Gy (b). Comparison with trace chromatograms for an aqueous solution of synthetic piceatannol, 3,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 3,5-dihydroxybenzaldehyde, and 4-hydroxybenzaldehyde at the respective concentration of 100 μmol.L^{-1} (c).

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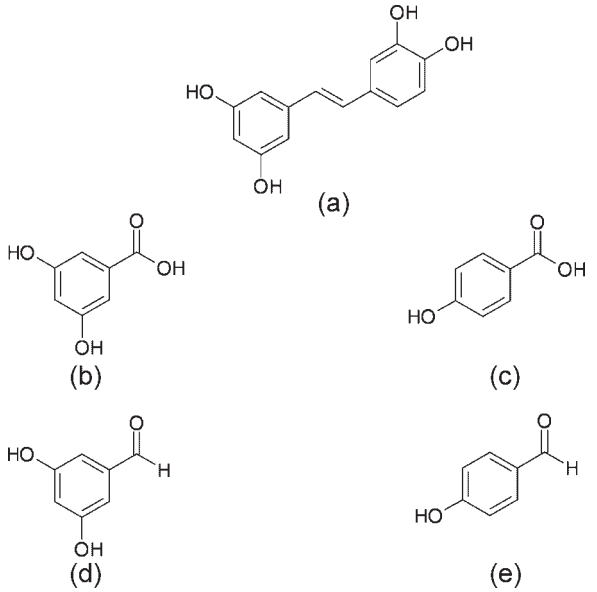

Figure 4. Chemical structures of the oxidation products of RVT: (a) piceatannol, (b) 3,5-dihydroxybenzoic acid, (c) 4-hydroxybenzoic acid, (d) 3,5-dihydroxybenzaldehyde, and (e) 4-hydroxybenzaldehyde.

was probably not an oxidation product of RVT, whereas piceatannol, 3,5-dihydroxybenzoic acid, 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde could be oxidation products of RVT *in vitro* under our experimental conditions. The identification of the four latter products was completed by recording mass spectra with or without deuterium exchange.

Identification of the oxidation product detected at *m/z* 243

This product could result from the formal incorporation of one oxygen atom, leading to the generation of a new hydroxyl group present in piceatannol. Thus, the CID spectrum of a solution of RVT (100 μmol.L^{-1}) irradiated at 400 Gy was compared to that of an aqueous solution of authentic piceatannol 100 μmol.L^{-1} (non-oxidized) (Fig. 5 vs. Supplementary Fig. s1, see Supporting information). As expected, the major product ions from piceatannol (Supplementary Fig. s1, see Supporting information), i.e. *m/z* 225.1, 201.1, 199.2, 175.2, 173.1, 159.2 and 157.2, were detected from the solution of oxidized RVT (Fig. 5). In addition, all these product ions were previously detected by Stella *et al.*[18] for authentic piceatannol. Thus, this molecule is one of the oxidation products of RVT *in vitro*. The structure of piceatannol is shown in Fig. 4(a).

Identification of the oxidation product detected at *m/z* 153

The oxidation product detected at *m/z* 153.1 could result from a fragmentation of RVT during water radiolysis, leading to the loss of one part of the molecule accounting for 74 Da. This important loss could result from a breaking of the styrene double bond of RVT leading to the formation of 3,5-dihydroxybenzoic acid. This assumption was verified by comparing the spectra of the oxidation product and of 3,5-dihydroxybenzoic acid (Fig. 6). For both of them, the monodeprotonated ion was detected at *m/z* 153.1 and leads to the fragment ion detected at *m/z* 109.1 under CID conditions, the latter resulting from the loss of 44 Da. In the labelled medium D_2O/acetonitrile (Fig. 6, insets), both

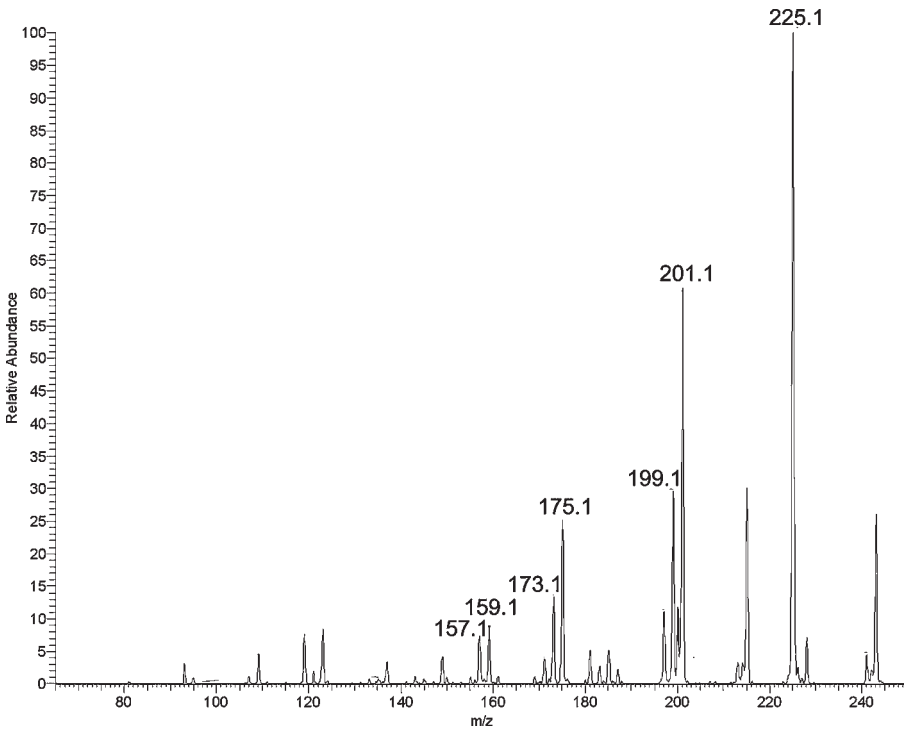

Figure 5. CID spectrum of the ion detected at *m/z* 243.2 for an aqueous solution of RVT (100 μmol.L^{-1}) oxidized at 400 Gy under aerated conditions, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed with direct infusion (collision energy = 40%).

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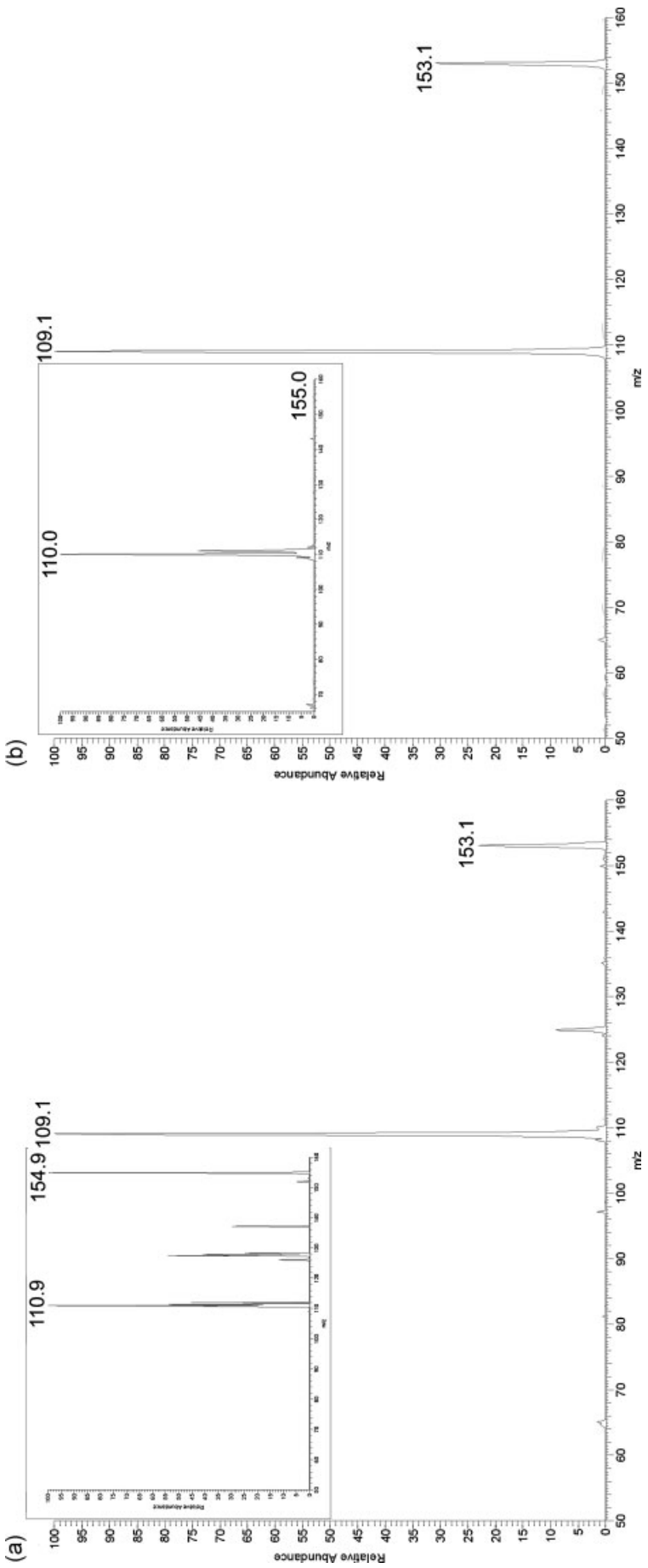

Figure 6. (a) CID spectrum of the ion detected at *m/z* 153.1 for an aqueous solution of RVT (100 μmol.L^{-1}) oxidized at 400 Gy under aerated conditions, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed in the negative mode (collision energy = 33%), inset: CID spectrum in the labelled medium D_2O/ACN (1:1, v/v); and (b) CID spectrum of the ion detected at *m/z* 153.1 for an aqueous solution of synthetic 3,5-dihydroxybenzoic acid at 100 μmol.L^{-1}, non-oxidized, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed in the negative mode (collision energy = 30%), inset: CID spectrum in the labelled medium D_2O/ACN (1:1, v/v).

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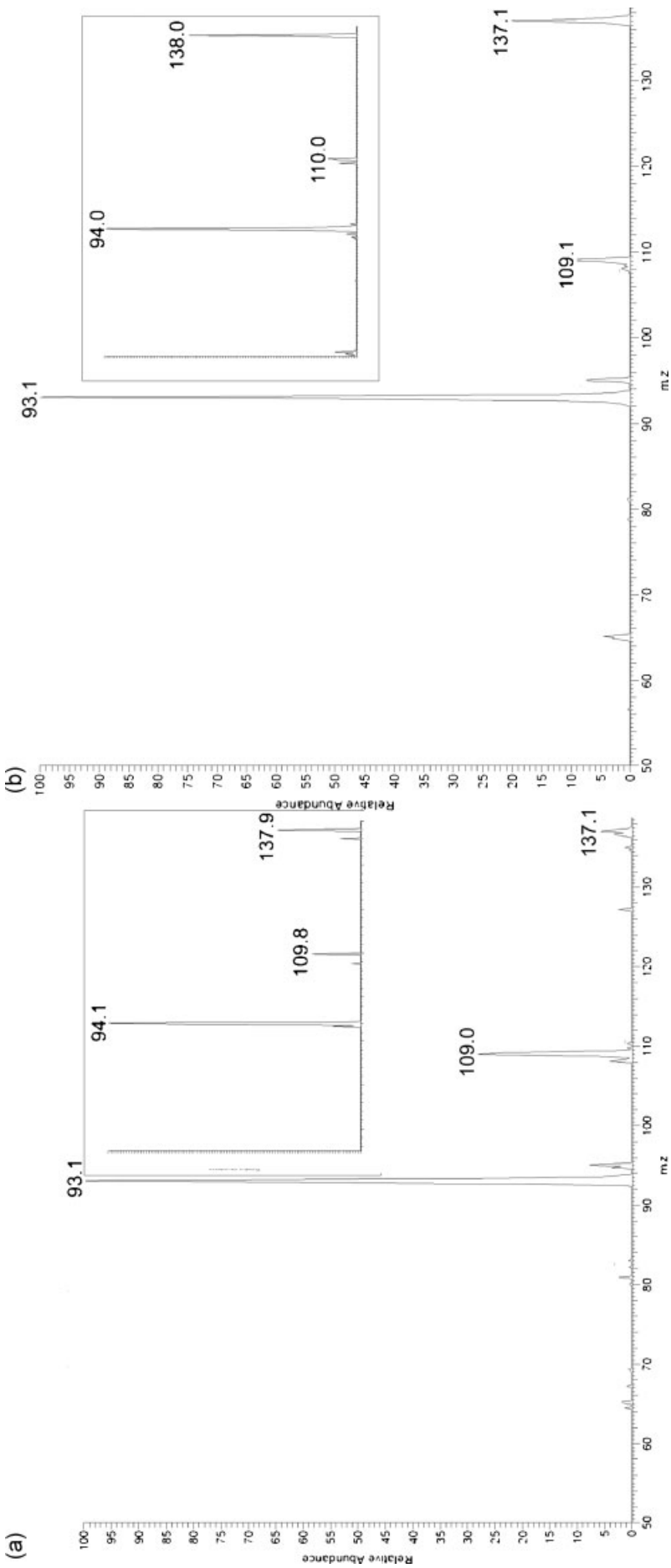

Figure 7. (a) CID spectrum of the ion detected at *m/z* 137.1 for an aqueous solution of RVT (100 μmol.L^{-1}) oxidized at 400 Gy under aerated conditions, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed in the negative mode (collision energy = 33%), inset: CID spectrum in the labelled medium D_2O/ACN (1:1, v/v); and (b) CID spectrum of the ion detected at *m/z* 137.1 for an aqueous solution of synthetic 3,5-dihydroxybenzaldehyde at 100 μmol.L^{-1}, non-oxidized, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed in the negative mode (collision energy = 34%), inset: CID spectrum in the labelled medium D_2O/ACN (1:1, v/v).

DOI: 10.1002/rcm

the hydroxylic (one of the two potentials) and the acidic protons could exchange with deuterium. The monocharged ion was thus detected at *m/z* 155. For synthetic 3,5-dihydroxybenzoic acid (Fig. 6(b), inset), its fragmentation led to a doublet ion at *m/z* 110/111, generated by the loss of 45/44 Da along with H/D scrambling occurring on the benzene ring and involving one of the hydroxyl groups, as observed by Stella *et al*.[18] This value probably corresponds to the loss of the neutral molecule C_2H_3DO (45 Da) from the benzene ring (fragmentation by rearrangement involving two carbons, one of the hydroxyl groups, and H transfers), eventually after H/D exchange between one deuterium of the hydroxyl group and hydrogen in position C4 of the benzene ring (loss of C_2H_4O, 44 Da), with assistance of the carboxylic acid group. The same pattern, including H/D scrambling, seemed to be observed for the ion at *m/z* 155.1 from a solution of oxidized RVT (Fig. 6(a), inset), thus confirming the identification of this oxidation product. This experimental evidence tended to demonstrate that 3,5-dihydroxybenzoic acid was one of the oxidation products of RVT under our experimental conditions. The structure of 3,5-dihydroxybenzoic acid is shown in Fig. 4(b).

Identification of the oxidation product detected at *m/z* 137

Similarly to the peak observed at *m/z* 153.1, the peak at 137.1 could result from an oxidative breaking of the double bond of trans-RVT, leading to the formation of 3,5-dihydroxybenzaldehyde as oxidation product. CID spectra of synthetic 3,5-dihydroxybenzaldehyde (Fig. 7(b)) and of a solution of oxidized trans-RVT (Fig. 7(a)) were similar and showed two major product ions at *m/z* 109.1 and 93.1, resulting from losses of 28 (CO) and 44 Da, respectively, from the precursor ion at *m/z* 137.1. CID spectra obtained in the labelled medium D_2O/ACN (1:1, v/v) (Figs. 7(a) and 7(b), insets) are also similar and showed the presence of one exchangeable site on this molecule. Losses of 28 and 44 Da were observed in these conditions, producing ions at *m/z* 109.8 and 94.1, respectively. No H/D scrambling was observed for this product: first, the ion at *m/z* 109.8 could be generated by the loss of carbon monoxide, which does not contain any exchangeable hydrogen; second, H/D scrambling previously observed for one of the oxidation products of RVT (dihydroxybenzoic acid, *m/z* 153), and occurring along with the loss of 44 Da, probably needs the assistance of one carboxylic acid group that is not present in this molecule. The structure of 3,5-dihydroxybenzaldehyde is shown in Fig. 4(d).

Identification of *m/z* 121

As previously suggested, the last peak detected at *m/z* 121.1 could be the monodeprotonated ion of 4-hydroxybenzaldehyde. Once again, CID spectra of this ion from a solution of oxidized RVT and a solution of synthetic 4-hydroxybenzaldehyde were very similar (Figs. 8 and Supplementary Fig. s2, see Supporting information). The precursor ion (*m/z* 121.1) mainly lost a molecule of carbon monoxide during fragmentation to lead to the product ion at *m/z* 93.2. Spectra were difficult to acquire and their bad quality could be due to the high stability of deprotonated 4-hydroxybenzaldehyde because of the charge delocalization by resonance. In addition, deprotonated 4-hydroxybenzaldehyde does not contain any exchangeable hydrogen atoms, thus an exper-

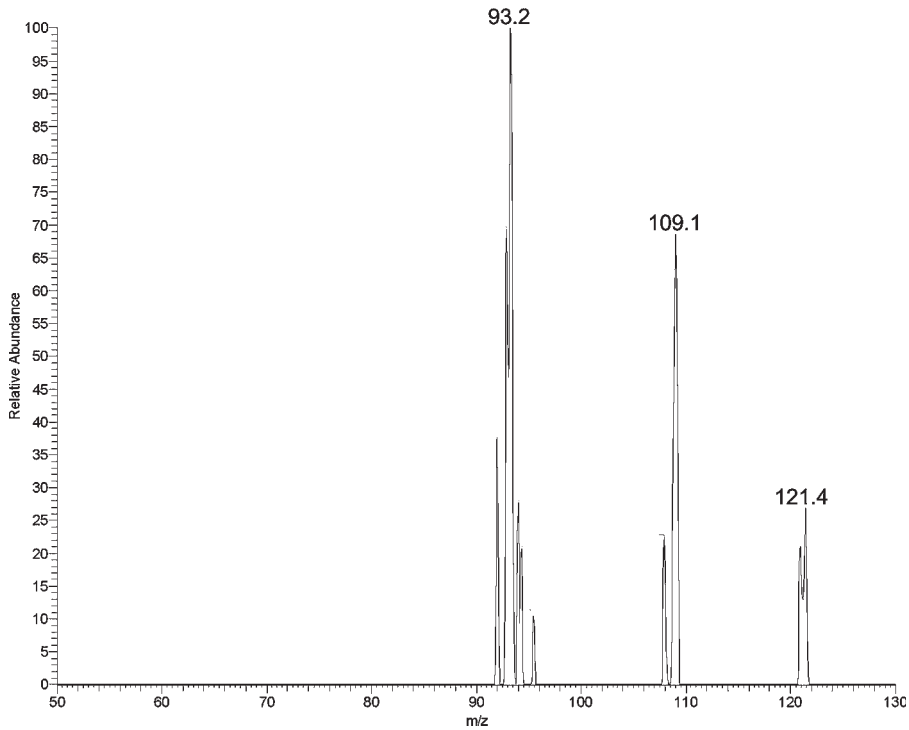

Figure 8. CID spectrum of the ion detected at *m/z* 121.1 for an aqueous solution of RVT (100 μmol.L^{-1}) oxidized at 400 Gy under aerated conditions, diluted in H_2O/acetonitrile (1:1, v/v) and analyzed in the negative mode (collision energy = 35%).

DOI: 10.1002/rcm

iment for a H/D exchange study was not performed. The structure of 4-hydroxybenzaldehyde is shown in Fig. 4(e).

CONCLUSIONS

Resveratrol was oxidized by hydroxyl free radicals, the most reactive species involved in oxidative stress phenomena *in vivo*. RVT acts as an antioxidant, i.e. a reducing agent that can be oxidized, leading to various products. The oxidation products of RVT were characterized by HPLC/MS as piceatannol, 3,5-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde. For each product, simple mass spectra, CID spectra, studies of H/D exchange and comparison with standard mass spectra were in good agreement with chemical structures. 3,5-Dihydroxybenzoic acid, 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde could be formed by the oxidative breaking of the styrene double bond of RVT, whereas piceatannol could result from a hydroxylation of one benzene ring of RVT.

Studies at several radiation doses and kinetic studies should be carried out in order to establish a scheme of reaction mechanism. These experimental results could open the way to the development of new drugs that could potentially have the same properties as RVT in terms of increasing the antioxidant status.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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DOI: 10.1002/rcm