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Determination of products derived from *trans*-resveratrol UV photoisomerisation by means of HPLC–APCI-MS

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Abstract

Resveratrol, a phenolic compound found in plant tissues, berries, and wine exists in *trans* and *cis* stereo isomeric forms. Naturally, due to differences in energetic state the *trans* form is the more common, but in wines the *cis* form is also present, resulting from photoisomerisation reactions. It is thought that from this reaction the only product arising is the *cis*-resveratrol. After UV irradiation of the *trans*-resveratrol solution for 10 min a third component appears with a growing concentration till 1 h of irradiation. From the MS/MS results we can assert that this compound is a product derived from oxidation, where the double bond at the centre of the resveratrol molecule, changes into a triple bond, forming a diphenylacetylene derivative (3,4′,5-trihydroxy-diphenylacetylene).

Keywords: LC MS/MS; Stilbene; Resveratrol; Photoisomerisation; 3,4′,5-Trihydroxy-diphenylacetylene

1. Introduction

Resveratrol, which is also known as 3,4′,5-trihydroxystilbene is a naturally occurring phytoalexin, produced by angiosperms, in response to injury, microbial infection, or UV radiation. A considerable amount of resveratrol can be found in for example grape skin, wine, brandy, vinegar, peanuts, lignified tissues, soy, etc. [1–3].

As a phenolic compound with antioxidant potential resveratrol plays a crucial role in the prevention of some certain human diseases. Resveratrol has been shown to modulate the metabolism of lipids, inhibit the oxidation of low-density lipoproteins, reduce platelet aggregation, and has anti-inflammatory, anti-tumor, neuro- and cardioprotective effects [4–9]. In wine resveratrol is thought to account in large part for the so-called French-paradox. This expression denominates the finding that the rate of coronary heart disease mortality in residents living in certain parts of France is lower than it would be expected, despite a diet prone to such events. The phenomenon is attributed to phenolic components in wine, and mostly to resveratrol [10,11].

Resveratrol exists in *trans* and *cis* stereoisomeric forms, both isomers occur naturally as do their glucosides (Fig. 1). In vitro the *trans* form is more photo- and thermostable, the *cis* form is very unstable isomerising easily to the *trans* form [12]. Standard *trans*-resveratrol solutions also show photoisomerisation: when exposed to daylight, they undergo changes in the *trans* stereoisomer ratio as the amount of the *cis* isomer increases to the detriment of the *trans* forms amount [13]. The same reaction takes place when the solution is exposed to UV light. The longer the irradiation the higher the *cis*-resveratrol concentration, until about 2 h, where the ratio of the *cis* isomer reaches its maximum [14].

Methods developed for detection of resveratrol are mainly suitable for the analyses of the *trans* form, some are suitable for determination of both isomers. Most commonly used techniques are: high-performance liquid chromatography (HPLC with UV–vis and fluorescence detection), HPLC coupled with mass spectrometry (HPLC–MS), or with tandem MS (HPLC–MS/MS), with both APCI and ESI ion sources, capil-

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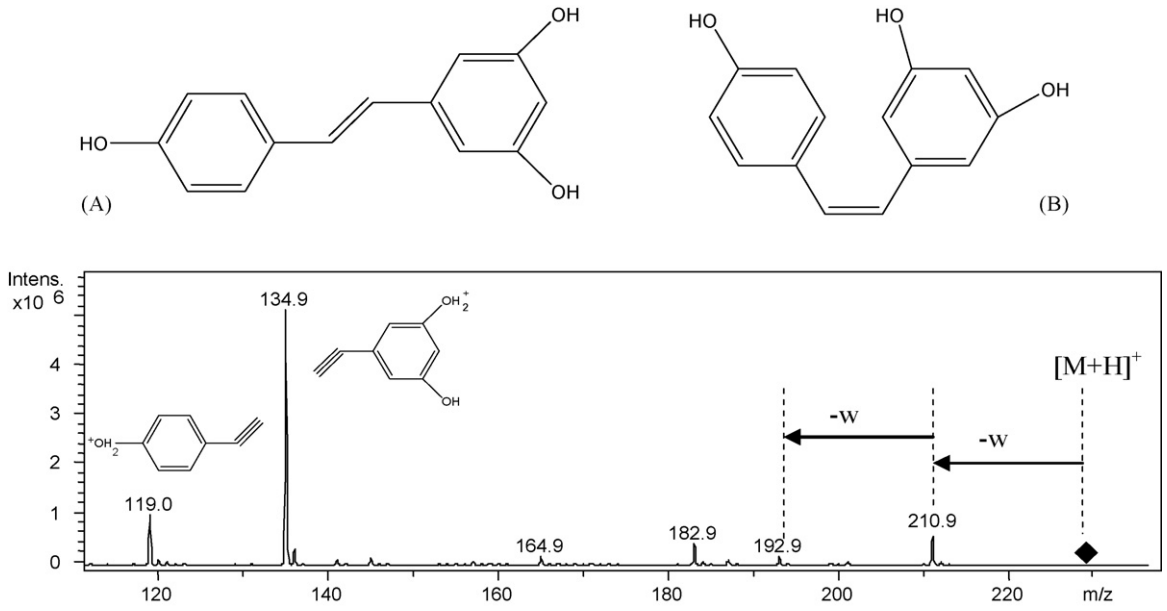

Fig. 1. Chemical structure of (A) *trans*-resveratrol and (B) *cis*-resveratrol and their APCI + MS/MS fragmentation.

lary electrophoresis (CE), or gas chromatography, coupled with MS (GC–MS) [15–21].

The aim of our study was to investigate the isomerisation of *trans*-resveratrol to the *cis* form and to determine other metabolites formed by the process using a HPLC–MS/MS assay.

2. Experimental

2.1. Materials and reagents

Trans-resveratrol (99%) was purchased from Sigma–Aldrich (Budapest, Hungary), ethanol from Reanal Finechemical Co. (Budapest, Hungary), methanol (HPLC-grade) from Scharlau Chemie S.A. (Barcelona, Spain). All other chemicals were of analytical grade. Freshly bidistilled water was used in solvents, and for preparation of aqueous solutions.

2.2. UV irradiation of the standard solution

The solid *trans*-resveratrol standard (0.5 mg) was dissolved in 1 cm^3 of ethanol in order to provide complete dissolution. It was then filled up to 10 cm^3 with 15/85 (v/v) ethanol water mixture. This solution was UV irradiated using a Cole Palmer 9815 UV lamp (Cole Palmer Instrument Co., Vernon Hills, IL, USA) at 365 nm. After 10, 30, 60, 120 and 300 min 200 μl of samples were taken out from the irradiated solution. The samples were kept in dark at 4 °C until the measurement.

2.3. HPLC analysis

The HPLC system used consisted of a Dionex P680 gradient pump (Dionex Corp., Sunnyvale, CA, USA), a helium degassing system, a Rheodyne 8125 injector valve with a 20 μl loop (Rheodyne Europe GmbH, Bensheim, Germany), and a Dionex 340D UV–vis diode array detector (Dionex Corp., Sunnyvale, CA, USA). A 250 mm × 4.6 mm column was filled with homemade 6 μ particle size 120 Å C_{18} reversed phase material [22]. A Chromeleon data management software (Version 6.60 SP3 Build 1485, Dionex Corp., Sunnyvale, CA, USA) was used to control the equipment and for data evaluation. A multistep gradient method was applied as described previously [23]. The eluate was monitored at ambient temperature at two different wavelengths, 285 and 306 nm, respectively, where *cis* and *trans* isomers have their absorbance maxima.

2.4. HPLC coupled to APCI + mass spectrometry

The HPLC system was connected to a Bruker HCT Esquire (Bruker Daltonics, Bremen, Germany) MS instrument through a microsplitter valve (Upchurch Scientific, Oak Harbor, WA, USA), the flow rate was 1.5 $cm^3\ min^{-1}$ with a splitting ratio of 7 over 3. The mass spectrometer equipped with APCI, the ion source was operated in positive mode. Nitrogen was used as drying gas at 250 °C, with a flow rate of 5 $dm^3\ min^{-1}$ the pressure of the nebulizer was set at 30 psi. We used the smart parameter setting (SPS) with target masses of 227.2 and 229.2 *m/z*. The scanning mass to charge range was 50–2000 *m/z* with a scanning speed of 8100 *m/z* per second. Maximum accumulation time was 200 ms. For the MS/MS analysis, manual MS^n was used for fragmentation of 227.2 and 229.2 precursor ions, in positive mode with capillary exit set at 108.2 V, maximum accumulation time at 200 ns; and mass range scanned from 50 to 1000 *m/z*. For control of the instrument, the Esquire Controll Version 5.3 Build 11, and for data evaluation the Data Analysis Version 3.3 Build 146 software was used, both obtained from Bruker Daltonics.

2.5. Molecular modeling and calculation of electronic spectrum

Hypechem 7.03 for Windows (Hypercube Inc., FL, USA) was used for calculations. We applied the ZINDO/S semiempirical method. The parameters of the calculations used configuration interaction and the singly excited method.

3. Results and discussion

3.1. HPLC with UV diode array detection

A reverse phase HPLC system coupled with an MS was assembled for the eluation and detection of products derived from *trans*-resveratrol UV photoisomerisation. Five parallel experiments were prepared and each sample was injected three times. In the control sample, which was a 0.05 mg/cm^3 concentration *trans*-resveratrol solution, kept in darkness till the measurement, only a single peak was detected, which eluted at 21.9 min (compound A in Fig. 2). After 10 min of UV irradiation at 365 nm a second peak appeared in the chromatogram, eluting at 23.58 min, representing the *cis* stereoisomeric form (compound B in Fig. 2). This second peak was followed by another, low-intensity peak at 23.85 min. In the samples irradiated for 0.5 and 1 h the height of this unknown peak (compound C in Fig. 2.) grew significantly showing in correlation with other authors reports [24], that the *cis*-resveratrol molecule is not the only product originating from the isomerisation reaction. The height of this peak reached its maximum in the 1 h irradiated sample as peak height did not grew significantly after 2 h. In the experiment where 5 h of UV irradiation was applied, we could not detect any further products. The only noticeable change was the decreasing height of all the three peaks. UV–vis spectra of the three peaks have also been collected, and it was found that

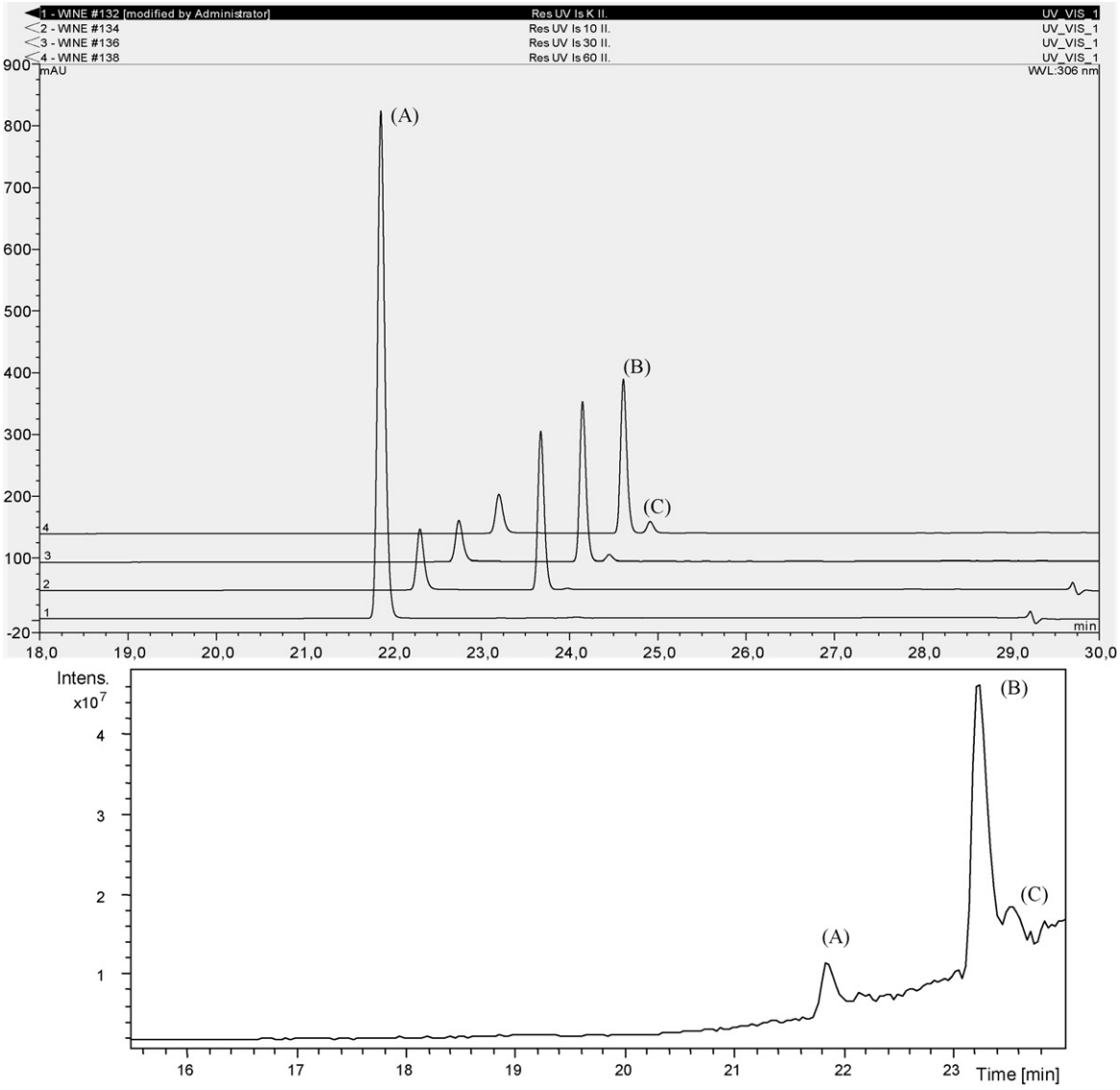

Fig. 2. Total ion current of the 1 h sample and UV chromatograms of the (1) control and the UV irradiated samples, (2) after 10 min, (3) after 30 min and (4) after 60 min. (A) *trans*-resveratrol, (B) *cis*-resveratrol and (C) the unknown substance.

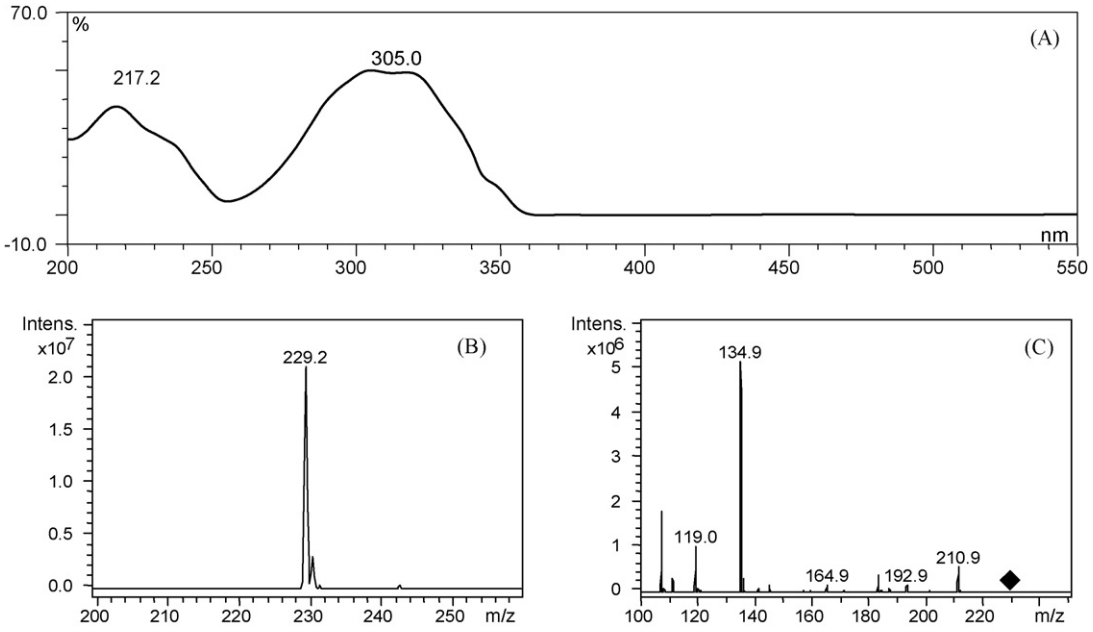

Fig. 3. (A) UV spectrum, (B) APCI + MS and (C) APCI + MS/MS of *trans*-resveratrol (control).

the UV–vis spectrum and absorbance maxima for *cis*- and *trans*-resveratrol are the same as described in literature [14], and for the third peak the absorption maximum at 302.4 nm is very close to the *trans*-resveratrol's absorbance maximum (Figs. 3–5).

3.2. APCI + MS and MS/MS detection

Mass spectrometry worked properly parallel the RP HPLC detection, the investigated compounds efficiently got transformed to protonated quasimolecular ions $[M+H]^+$. Mass spectrum of *trans*-resveratrol shows an intense peak at 229.2 *m/z*, followed by a peak of 230.2 and 231.2 *m/z* as isotopes of carbon (Fig. 3B). MS spectrum of *cis*-resveratrol proved to be exactly the same as the *trans* form (Fig. 4B). The MS spectrum of the third, unknown component differed in one way. Beside the 229.2 *m/z* mass, another peak emerged at 227.2 *m/z* also with a similar isotope pattern (Fig. 5B).

The MS/MS analysis of the three main peaks of *trans*-, *cis*-resveratrol and the unknown substance provided a similar fragmentation pattern as described by Montoro et al.

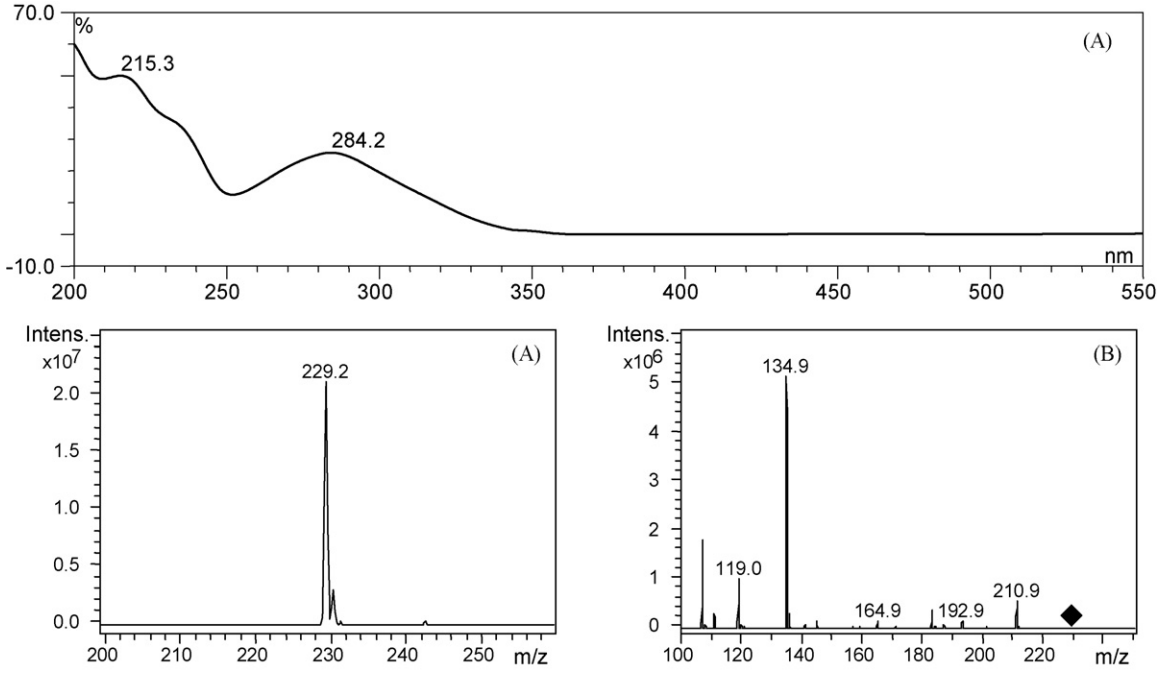

Fig. 4. (A) UV spectrum, (B) APCI + MS and (C) APCI + MS/MS of *cis*-resveratrol.

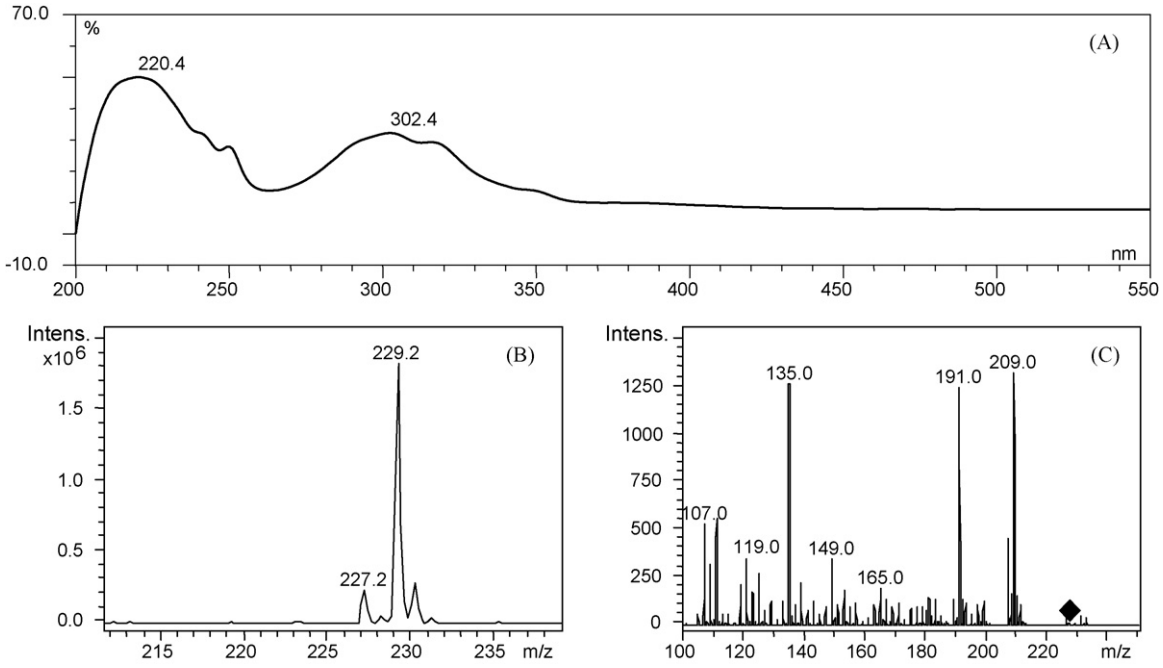

Fig. 5. (A) UV spectrum, (B) APCI + MS and (C) APCI + MS/MS of the unknown compound.

[25] (Figs. 3C, 4C and 5C). MS/MS spectrum of *trans*-resveratrol showed a fragment at 210.9 *m/z* representing the loss of one hydroxyl group as water, another fragment at 192.9 *m/z* representing the loss of two water units. The fragments at 134.9 and 119.0 *m/z* contain the phenolic ring of the molecule with a short carbon chain and a triple bond (Fig. 1B) [25]. A similar fragmentation pattern was found for *cis*-resveratrol. The fragmentation of the third peak however provided fragments at 209.0, 191.0 and at 135.0 and 119.0 *m/z*, respectively.

3.3. Structure identification of the unknown compound

The 2 Da difference between 227.2 and 229.2 *m/z* in the MS spectrum can originate from the loss of two hydrogen atoms suggesting that the unknown substance is an oxidation product. Oxidation might eventuate at the hydroxyl groups providing a keto group or at the double bond in the centre of the molecule providing a triple bond. Third possibility is a photocyclization reaction to a phenanthrene derivative, which is a well-known reaction although it is only potential from the *cis* form of stil-

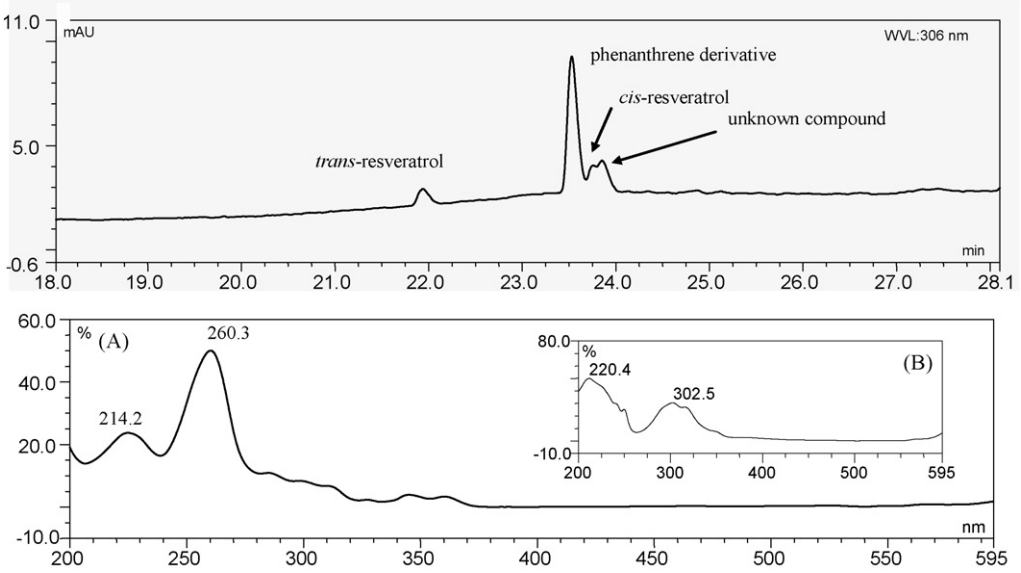

Fig. 6. Chromatogram of the irradiated *cis*-resveratrol enriched solution with the UV–vis spectra of (A) 2,5′,7-trihydroxy-phenanthrene and (B) the unknown compound.

benes [26]. All three molecules would have the same 227.2 Da molecular mass as quasimolecular ions.

The oxidation of the hydroxyl groups to keto groups can be precluded as the UV–vis spectrum of the unknown compound did not notably differ from the spectrum of the *trans*-resveratrol. The only significant difference is a shoulder peak at 249.7 nm. The results of the MS/MS experiments also excluded this possibility (Fig. 5C). The two fragments loosing the water units (Fig. 1) at 209.0 and 191.0 *m/z* (2 Da difference) can only be observed if the two hydrogen is missing from the centre of the molecule, as water loss is not possible from the keto groups. It is also noticeable that the fragments at 135.0 and 119.0 *m/z* are present in all the three compounds MS/MS spectra. This is due to the formation of a triple bond during the fragmentation of the standard resveratrol molecule [25].

In literature a phenanthrene derivative from *cis*-stilbenes is described as a potential product of UV-photoisomerisation reaction [26] where the *cis*-stilbene product undergoes a photocyclization reaction to phenanthrene. To test this possibility an experiment was performed where *cis*-resveratrol was enriched in a previous isomerisation mixture and was irradiated at 312 nm. After 2 h of irradiation beside of the three already existing components a new component appeared which eluted at 23.53 min not overlapping with the peak of the unknown substance (Fig. 6). To ensure that this new peak is the assumed phenanthrene derivative semiempirical quantumchemical calculations were carried out using the Hyperchem software. The peak maxima in the calculated UV–vis spectrum of the 2,5′,7-trihydroxy-phenanthrene proved to have a significant correlation with the maxima of the observed spectrum. It also notably differed from the UV–vis spectrum of the unknown compound (Fig. 6A and B and Table 1). At this point MS/MS experiments would not provide convincing results as the observed fragmentation pattern is conceivable from both structures.

The mass spectrometric and UV–vis spectral results indicate that the third unknown compound is a triphenyl-acetylene derivative of *trans*-resveratrol where the double bond at the centre of the molecule, changes into a triple bond forming 3,4′,5-trihydroxy-diphenylacetylene (TDPA) (Fig. 7). For further confirmation of this molecular structure semiempirical quantumchemical calculations were performed using the Hyperchem software. The calculated spectrum of TDPA and the observed spectrum of TDPA (the absorbance maxima) are near to each other (mostly within 1.5 nm) demonstrating the probability of the proposed structure (Table 1).

Table 1
UV absorbance data (absorbance maxima in nm) of *trans*-resveratrol, 3,4′,5-trihydroxy-diphenylacetylene and 2,5′,7-trihydroxy-phenanthrene

Observed	Calculated
Trans-resveratrol	
217.2	219.6
305	300.01
TDPA	
220.04	215.99
249.7	251.12
302.4	301.2
2,5′,7-Trihydroxy-phenanthrene	
214.2	213.0
260.3	263.6

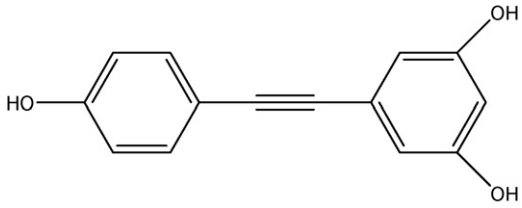

Fig. 7. Chemical structure of the oxydated product (3,4′,5-trihydroxy-diphenylacetylene).

4. Conclusion

UV-photoisomerisation reaction of *trans*-resveratrol solutions at 365 nm UV light gives rise to two products. Beside of *cis*-resveratrol a small portion of a further product will also be synthesized by the irradiation. HPLC coupled with MS and MS/MS, UV spectroscopy and semiempirical quantumchemical calculations were used for the determination of the chemical structure of the new isomerisation product. It was found that the new product is an oxidative derivative of *trans*-resveratrol with a triple bond at the centre of the molecule: 3,4′,5-trihydroxy-diphenylacetylene.

Under different reaction conditions the main reaction product *cis*-resveratrol can react further to build a phenanthrene derivative (2,5′,7-trihydroxy-phenanthrene) as described in literature. In conclusion it can be professed that the widely used photoisomerisation reaction can only be used for quantitative production of *cis*-resveratrol under very well controlled experimental conditions.

Acknowledgement

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