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Rapid analysis of stilbenes and derivatives from downy mildew-infected grapevine leaves by liquid chromatography–atmospheric pressure photoionisation mass spectrometry

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Abstract

Resveratrol, *trans*-ε-viniferin and *trans*-δ-viniferin are the major stilbenes induced in downy mildew infected grapevine leaves. In addition, nine minor polyphenolic compounds, described as stilbenes derivatives, have been separated and detected among known stilbenes after a methanolic microextraction of small pieces (1–2 mg) from infected grapevine leaves with a rapid, qualitative and optimized HPLC method coupled to mass spectrometry using atmospheric pressure photoionisation (APPI–MS^n). The characterization of unknown stilbenic derivatives as six resveratrol dimers, two dimethylated resveratrol dimers and a resveratrol trimer are reported. Therefore, structures have been proposed for the dimethylated resveratrol dimers. Use of an easy sample treatment and the LC–APPI–MS^n method results in spectral data of these minor naturally occurring viniferin analogues.

Keywords: LC–APPI–MS; LC–ESI–MS; Stilbene; Viniferin; Downy mildew; Grapevine; *Vitis vinifera*

1. Introduction

Downy mildew is one of the worldwide destructive grapevine diseases caused by a biotroph oomycete, *Plasmopara viticola* [1]. Interaction between plants and their pathogens can be very specific and complex. Phytoalexins as antifungal chemicals are known for taking part in the plant defence strategies [2]. Langcake and Pryce [3,4] described the presence of non-flavonoid phenolics, stilbenes, as phytoalexins from grapevine leaves after biotic and abiotic stresses (Fig. 1). These stilbenes are resveratrol derivatives, which are represented by several chemical and structural isomers [5]. Experiments have shown that their toxicity was closely linked to their chemical structure [6]. Some of them can be highly toxic against *P. viticola* at low concentration [7]. Mass spectrometry is more and more highlighted in life sciences with increasing applications of atmospheric pressure ionisation (API) techniques for the identification of low molecular compounds or macromolecules, using electrospray ionisation (ESI), chemical ionisation (APCI) and photoionisation (APPI) [8,9]. The APPI interface developed by Robb et al. [10], creates photons emission from a gas discharge lamp, which allows a selective ionisation of analytes among the mobile phase. The addition of acetone or toluene, as doping agent, to the mobile phase enhances analytes ionisation, which improves their signal and sensitivity for some class of compounds, particularly low polar compounds [8,9].

This paper shows that some still unknown stilbenes are present in grapevine leaves contaminated by *P. viticola*. Their importance as natural fungicide implicated in the resistance of some grapevine cultivars to downy mildew can be estimated only if their chemical structure is determined. However, their identification using conventional ESI source is difficult because of their low concentration and low polarity. In that case, the use of a highly sensitive and low noise APPI mass spectrometric source can be very useful.

We described here a rapid and sensitive method of stilbene analysis where an APPI source delivers cleaner mass spectra allowing a more efficient mass determination of low concentration compounds. In addition, we know how important the sample preparation method in the identification of natural and organic products is [11]. The following analytical procedure has the advantage that a very small amount of plant material and a minimum of sample preparation are required.

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2. Experimental

2.1. Reagents

Pure *trans*-resveratrol, catechin and epicatechin standards have been provided by Sigma–Aldrich (Buchs, Switzerland). Pure *trans*-piceid was purified from *Polygonum cuspidatum* according to Waterhouse and Lamuela-Ranventos [12], *trans*-pterostilbene was synthesized as described by Pont and Pezet [13], *trans*-ε-viniferin and *trans*-δ-viniferin were purified from lignified grapevine cane and by enzymatic oxidation of resveratrol, respectively as described by Pezet et al. [5]. *Cis*-isomers were obtained after sunlight exposition of methanolic solutions of each *trans*-stilbenes and used as *cis*-standards directly in the HPLC analytical system. Every used organic solvents were HPLC grade.

2.2. Plant material

Cuttings from *Vitis vinifera* var. Chasselas have been provided by the vineyard of the Swiss Federal Agricultural Research Station of Changin (Agroscope-RAC, Switzerland).

2.3. Plant infection

Plant infection has been undertaken according to Gindro et al. [14]. Leaves were harvested for analysis 7 days after inoculation, when sporangiophores of *P. viticola* were visible at their underside.

2.4. UV treatment

Leaves were collected from healthy cuttings, and their undersides were exposed for 10 min to UV-C radiations (Philips TUV 30W, 92 μW cm-2 at 253 and 7 nm, at 13 cm from leaves) and then maintained for 26h in the dark in a wet chamber and removed for analysis.

2.5. Micro extraction of stilbenes

The method of stilbenes micro extraction is described by Pezet et al. [5], with slight modifications: one small leaf piece (fresh weight between 1 and 3 mg) was placed in 50 μL of methanol in tightly closed Eppendorf tubes, extracted during 10 min at 60 °C under constant agitation then placed in an ice bath. Samples of methanol solutions (10–30 μL) were directly injected in the HPLC analytical system.

2.6. HPLC of stilbenes

Qualitative analysis of stilbenes has been carried out with a binary pump (Agilent 1100, G1312A), a solvent degasser (Agilent 1100, G1379A), an autosampler (Agilent 1100, G1313A), and a diode array detector (280 and 307 nm, Agilent

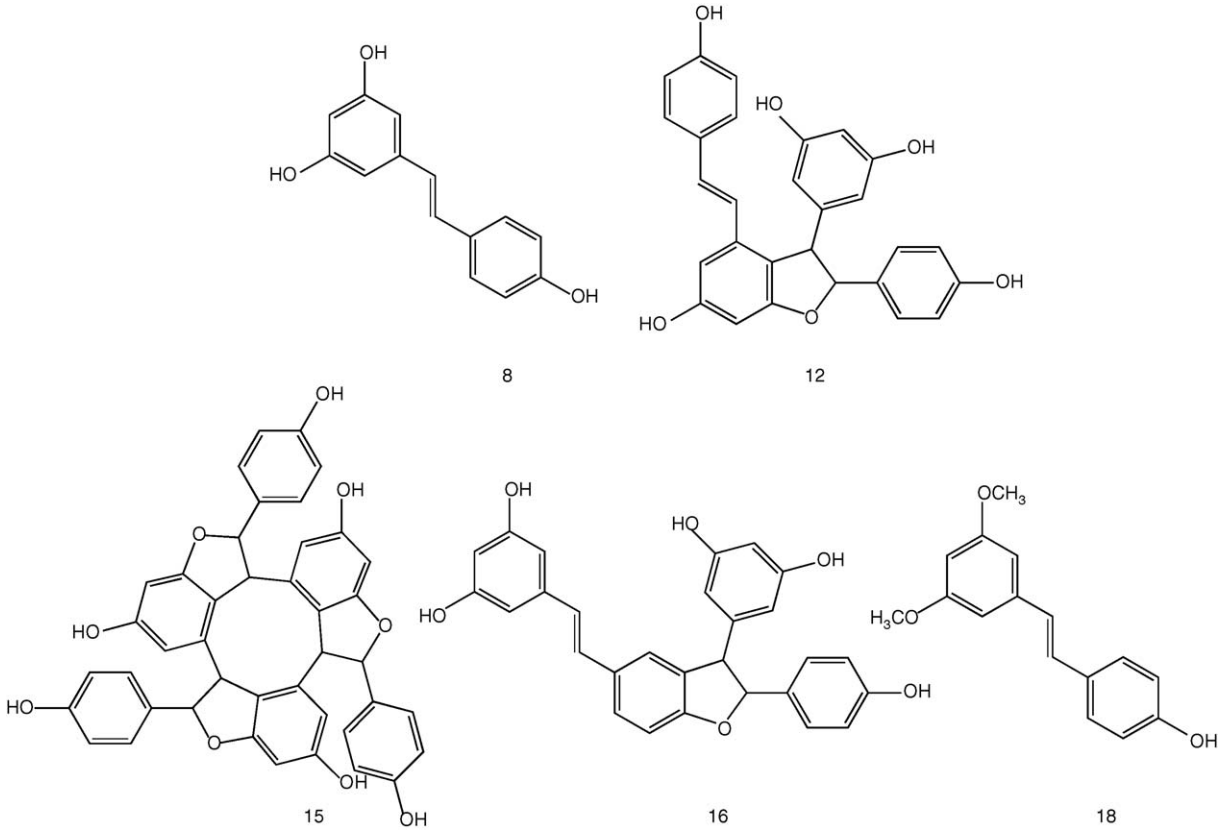

Fig. 1. Known structures of: *trans*-resveratrol (**8**); *trans*-ε-viniferin (**12**); α-viniferin (**15**); *trans*-δ-viniferin (**16**); and *trans*-pterostilbene (**18**).