OPTIMIZATION AND DEVELOPMENT OF THE RELIABLE HPLC-DAD METHOD FOR THE ANALYSIS OF RESVERATROL IN GRAPES

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Abstract

Secondary metabolites of grapevine, particularly stilbenes such as resveratrol, play a key role in plant defense mechanisms, making their reliable determination essential for a better understanding of the vine's natural resistance to pathogens and other stressors. In this study, a methodology based on solid-phase extraction (SPE) and high-performance liquid chromatography coupled with a diode-array detector (HPLC-DAD) was optimized, developed, and validated for the analysis of this compound in grape samples. The method demonstrated high accuracy and precision, along with the capability of detecting very low concentrations. Furthermore, the use of simple, widely available, and cost-effective techniques enhances the value of the applied methodology, making it well-suited for routine laboratory practices as well as future research on the grapevine immune system and the advancement of viticulture.

Introduction

From the earlier evidence of grape consumption in the Paleolithic era [1] to its current recognition as one of the most significant fruit species, the grapevine has undergone a long process of development and selection. Today, it accounts for more than 95% of the world's vineyard areas, and viticulture, beyond its economic importance, also plays a key role in preserving tradition, national identity, and cultural heritage [2]. The viticulture in the territory of today's Serbia dates back to the Roman Empire and gained strength during the Middle Ages. Despite later challenges, including the devastation caused by the appearance of phylloxera (*Phylloxera vastatrix* Planchon) and the consequences of the numerous wars, this agricultural sector in Serbia was successfully restored, adapted, and developed. Modern approaches to viticulture often combine tradition and innovation, with growing emphasis on sustainability and the vine's capacity to successfully cope with numerous challenges, including the pronounced effects of climate change [3] and increasing adaptability of pathogens and pests through various mechanisms. Under such conditions, the adaptive potential of the grapevine becomes particularly important, with secondary metabolites [4, 5]– especially stilbenes– playing a significant role. Resveratrol is one of the most important members of this chemical group, characterized by well-documented antimicrobial and antioxidant properties [6]. Although naturally present in grapevine tissues, its intensive biosynthesis is induced by diverse stress factors, among which the most notable are infections with phytopathogenic microorganisms [7], particularly fungi such as *Plasmopara viticola*, *Uncinula necator*, and *Botrytis cinerea*. In this context, this compound limits infection spread by establishing an initial barrier that slows down pathogen development [7, 8]. Consequently, resveratrol represents a crucial part of the natural defense system and remains the focus of extensive research directed at enhancing vine resistance through the plant's natural potential and identifying novel strategies in plant protection.

To further examine the grapevine’s defensive potential, it is necessary to first isolate the target compound from the plant material and accurately determine its content. This requires the careful selection and optimization of an appropriate extraction method. For the extraction of resveratrol from grapes, both conventional and unconventional approaches are used, including maceration,

Soxhlet extraction [9], solid-phase extraction (SPE) [10], ultrasound-assisted extraction, QuEChERS, enzymatic, and supercritical fluid extraction [11] [12]. The further choice of analytical technique, and ultimately the specific analytical method, depends on the properties of resveratrol, which is primarily a photosensitive and thermolabile compound [13]. To ensure the accuracy, precision, and reproducibility of the analysis results, it is crucial to minimize resveratrol degradation, both before and during the analysis. Therefore, high-performance liquid chromatography (HPLC) with various types of detectors is most commonly used due to its affordability, convenience, and widespread applicability [14]. Considering the above, this research aims to optimize, develop, and validate an efficient extraction and analytical method for the determination of resveratrol, as this process constitutes a crucial step towards advancing scientific knowledge and promoting the development of sustainable viticulture.

Materials and methods

Analytical standard of *trans*-resveratrol (98.37%) from Dr. Ehrenstorfer (Germany). The *trans*-isomer was included in this research due to its commercial availability, greater biological activity, and higher presence in grapes, compared to the *cis*-isomer. Acetonitrile, methanol (HPLC grade), ethanol (99%), and acetic acid (CH_3COOH) (reagent grade) were purchased from Fisher Scientific (SAD). Ultrapure water was purchased from J. T. Baker (Netherlands), while formic acid (HCOOH) and ethyl acetate were from Merck KGaA (Germany).

Standard solution was prepared by dissolving the analytical standard in a mixture of methanol and water (50:50 v/v) with 0.1% HCOOH (v/v), whereby the concentration was 1072.3 µg/ml. By its dilution, the series of working solutions was prepared in concentrations of 0.05–107.23 µg/mL. The working solutions were kept in the dark, at a temperature of 4˚C, until the analysis.

For the determination of *trans*-resveratrol in grapes, high-performance liquid chromatography with a diode array detector (HPLC-DAD) was applied. The whole process was optimized by testing different sample preparations and solvent combinations as mobile phases.

The optimal conditions were selected according to the best resolution, after which validation of the chromatographic method was carried out by defining the parameters–linearity, precision, repeatability, accuracy, and limits of detection (LOD) and quantification (LOQ) [15].

All procedures were carried out to determine the *trans*-resveratrol content in grapes precisely.

Results and discussion

Considering the available literature data and our previous experience, this research employed SPE extraction, which enables the selective extraction of stilbenes with minimal losses and degradation. This method was optimized to reliably isolate analytes. The chosen preparation method was based on grinding frozen berries in the presence of the cold solvent, which proved to be the fastest and least invasive approach, while preserving the content of resveratrol. This procedure made it possible to obtain small fractions and a homogeneous consistency, which significantly increased the extraction efficacy. Compared to other procedures such as drying, dehydration at elevated temperatures, and lyophilization, the applied method showed an advantage in preserving thermolabile compounds and avoiding additional technological limitations due to high sugar content. During the optimization of the extraction, different solvents such as ethyl acetate, ethanol, methanol, and acetonitrile were tested, showing different efficiencies in terms of yield and reproducibility. Methanol proved to be superior to its purpose, while the addition of acetonitrile at the final step of extraction improved it. Their combination, therefore, represented an optimal solution.

Also, within this research, the HPLC-DAD method was chosen, considering that it provides reliable quantification of resveratrol and has been widely confirmed for the analysis of stilbenes in grapes [16].

For the development of the method, the first step was to optimize the chromatographic conditions to achieve the best resolution, sensitivity, and accuracy in the determination of *trans*-resveratrol in grapes. Different combinations of mobile phases were examined, such as acetonitrile and 1% CH_3COOH; acetonitrile and water, acetonitrile and 10% methanol (1% CH_3COOH), methanol and water (1% CH_3COOH), as well as acetonitrile and 0.1% HCOOH. The most favorable separation was achieved using the last mobile phase in gradient mode, while isocratic analysis did not show good peak separation in the matrix. The combination that proved most effective for this analysis consisted of a mobile phase of acetonitrile (B) and 0.1% HCOOH in water (A), with a column temperature of 35 ˚C, a flow rate of 0.32 mL/min, and detection at 306 nm (Figure 1). The gradient elution program was set as follows: B (%) 10 at 0 min, 10 at 4 min, 50 at 18 min, 80 at 18.2 min, 10 at 20.2 min, and 10 at 26 min. The chosen combination enabled clear separation of stilbene peaks and reduced interferences from the sample matrix.

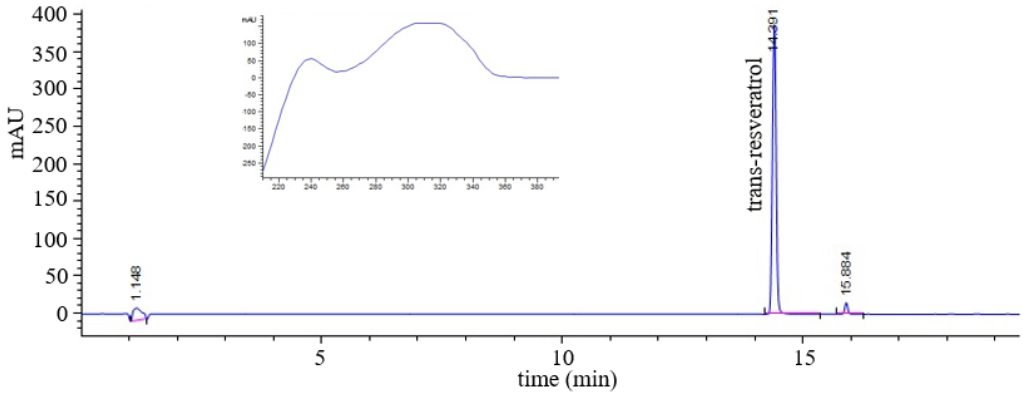

Figure 1. Chromatogram of *trans*-resveratrol (1.07 µg/ml) and its spectrum at a wavelength of 306 nm

For the method validation, linearity was evaluated at seven concentration levels (0.05–107.23 µg/mL), with an obtained R^2 value of 0.9989, confirming excellent linearity of detector response, in accordance with ICH guidelines [18]. The precision of the method was confirmed by a low value of RSD% (0.19%), while the limits of detection (LOD) and quantification (LOQ) were 0.0053 and 0.0160 µg/mL, indicating a high sensitivity of the method. Recovery values were in the range of 99.21–106.25%, further confirming the satisfactory extraction yield and the suitability of the method for quantitative analysis in grape samples.

Conclusion

The optimized, developed, and validated HPLC-DAD method, with the optimized SPE procedure, proved to be a reliable and efficient combination for the determination of *trans*-resveratrol, with the capability of detecting this compound at very low concentrations. The added value of the applied chromatographic and extraction methods is reflected in their widespread use in laboratories, availability, and economic acceptability, making them suitable for routine laboratory applications.

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