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Computer-Assisted Approach for the Development of RP-HPLC Methods for the Separation and Quantification of Bioactive Plant Secondary Metabolites

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1. Introduction

A variety of plant secondary metabolites have a remarkable position as bioactive components in medicinal plants and have evidenced to exhibit numerous biological activities and several health benefits against chronic and degenerative human diseases. Moreover, secondary metabolites occurring in edible plants form an integral part of human diet, contributing to the sensory properties of plant-based aliments and to their beneficial effects on human health.

Numerous are the instrumental analytical separation techniques that are employed to identify and quantify the bioactive compounds occurring in medicinal plants and plant-derived food products. Among them, high performance liquid chromatography, mostly in reversed phase separation mode (RP-HPLC) and generally coupled to mass spectrometry (MS), is the technique of choice for the identification and quantification of plant secondary metabolites. The optimization of HPLC methods is generally carried out by conventional trial-and-error approaches, requiring the screening of a variety of experimental conditions, which include column temperature, pH, composition of the mobile phases, as well as shape and duration of the gradient elution program (1).

This communication describes the development of computer-assisted RP-HPLC methods for the separation, identification and quantification of phenolic compounds, which are a large class of plant secondary metabolites comprising a great number of heterogeneous structures that range from simple molecules to highly polymerized compounds.The study has been conducted by a Design of Experiments (DoE) approach that allow the simultaneous optimization of gradient time (t_G), column temperature (T) and binary eluent composition on the basis of retention times and peak areas of the analytes of interest, obtained in twelve different experiments. These experiments consist in the linear gradient separations of the investigated compounds performed at two different gradient times and column temperatures, using either the aqueous component of the mobile phase at three different pH values or a combination of two organic solvents at three different volume ratios as the gradient former.The RP-HPLC methods developed by the computer-assisted approach described in this paper were used for the separation and quantification of phenolic compounds occurring in fruits of Olea europaea, extra virgin olive oil (EVOO) and olive mill waste water (OMWW). .

2. Materials and methods

HPLC experiments were performed on a Shimadzu (Milan, Italy) LC-10AVP system equipped with two solvent delivery pumps, photodiode array detector,and a Rheodyne manual injector valve, using a Polaris C18-A column (150 × 2.0 mm i.d., 5 μm) with a C18 (30 × 2 mm i.d., 5 μm) guard cartridge, both from Agilent (Milan, Italy). Method development and modeling were performed by the DryLab® 4 optimization software (Molnár-Institute, Berlin, Germany). Standard phenolic compounds were purchased either from Sigma-Aldrich (Milan, Italy) or from Extrasynthes (Genay Cedex, France). All other chemicals were from Carlo Erba Reagents (Cornaredo, Milan, Italy). Olive fruits, EVOO and OMWW of different botanical origin were collected in Algeria by INRAA.

3. Results

We have evaluated the influence of pH and mobile phase composition, column temperature and gradient elution program on the retention behaviour in RP-HPLC of selected standard phenolic compounds in order to obtain their "baseline separation", while minimizing the analysis time. Analogous experiments were also carried out using samples extracted from EVOO, OMWW and olive fruits. Appropriate selection of optimal experimental conditions for RP-HPLC separations of the selected analytes were carried out using the software modeling program DryLab® 4, which allowed to study their retention properties in the process of method optimization. Using this software, the simultaneous optimization of pH of the aqueous component of the mobile phase (pH), gradient time (t_G) and column temperature (T) required 12 experiments, which are illustrated in the design of experiments depicted in panel A of ***Figure 1***.

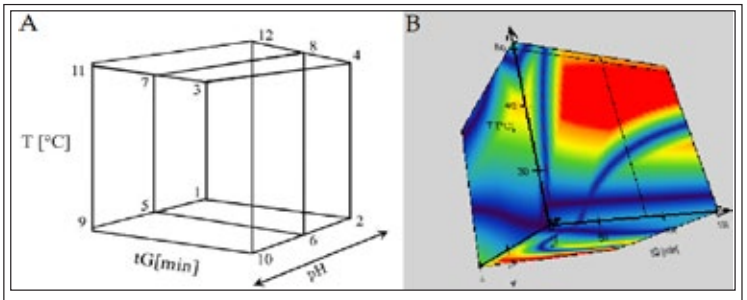

Figure 1 *Design of experiments (DoE) used in this study (panel A) to obtain a thee-dimensional resolution map (panel B) showing the influence of t_G, pH, and T on the resolution of phenolic compounds occurring in EVOO*

The experiments 1,3,5,7, 9, and 11 were carried out with a linear steep gradient (20 min), and experiments 2, 4, 6, 8, 10, and 12 with a linear flat gradient (60 min), both from 2 to 62% (v/v) acetonitrile in water containing formic acid at three different pH values. The pH of the aqueous component of the mobile phase employed in experiment 1, 2; 3, and 4 was pH 2.1, in experiments 5, 6, 7, and 8 it was pH 2.5, and in experiments 9, 10, 11, and 12 it was pH 2.9. The temperature of the column was either 25°C (experiments 1, 2, 5, 6, 9, and 10) or 50°C (experiments 3, 4, 7, 8, 11, and 12).

The results of this limited number of experiments, elaborated by DryLab®4 software, allowed to construct the 3-D resolution maps depicted in panel B of ***Figure 1***, which was used to optimize column temperature, pH of the mobile phase, and duration and shape of the elution gradient used for the RP-HPLC separation of phenolic compounds occurring in olive fruits, EVOO, and OMWW. In our study, 3-D resolution maps were constructed using either mixtures of standard phenolic compounds or samples of these compounds extracted from EVOO, OMWW and olive fruits. Excellent correlation between simulated and experimental separations of phenolic compounds were obtained.

Further investigations were carried out to examine the possibility of replacing acetonitrile with methanol or with a mixture of these solvents as the gradient former used for RP-HPLC analysis of phenolic compounds occurring in the investigated samples. The simultaneous optimization of gradient time (t_G), pH, and ternary composition (tc) of the mobile phase required to carry out a total of 18 experiments. The additional six experiments resulted from having performed the separation of the selected phenolic compounds using, at two different gradient time, the gradient former of three different composition: methanol, acetonitrile, and 50% (v/v) mixture of both organic solvents. Optimal separation of standard and extracted phenolic compounds were obtained with the gradient former consisting of 60% (v/v) methanol: acetonitrile, containing 0.1% (v/v) formic acid.

4. Conclusions

The optimization of RP-HPLC separations of plant secondary metabolites can be quickly and easily carried out using a modelling software, such as DryLab®4, which predicts the chromatographic behavior of the analytes on the basis of a limited number of experiments. Such approach allows the development of robust and reliable RP-HPLC methods and reduces the consumption and waste of armful and expensive organic solvents and, therefore, is beneficial for both the environment and the economy.

5. Acknowledgements

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References

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