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Detection of the polyphenolic components in *Ribes nigrum* L.

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Abstract

Background. The blackcurrant (*Ribes nigrum* L.) is a species of native currant which contains a lot of polyphenolic antioxidants which is used medicinally and has a fundamental role in the maintenance health.

Materials, methods and objective. Ultraviolet–visible spectrophotometry and ultraviolet range high performance liquid chromatography (HPLC) were used to characterize the polyphenolic content of common *Ribes nigrum* collected in the western part of the Banat Region in Romania.

Results. UV–visible spectrophotometry was a reliable tool for identifying the phenolic compounds class. Polyphenols calibration curves from the methanolic extracts showed a good linearity (r^2>0.984) within test ranges and generated a well–designed absorption band with a local maximum at 273.2 nm band, which can be attributed to thr electronic transition of the n–π* type. Chromatographic separation and analysis of the methanol extract was useful for the structural epigallocatechin (EGC) and epigallocatechin–3–gallate (EGCG) characterization of primary antioxidant compounds.

Conclusions. The new, slightly modified, chromatographic system can serve for the development of a quantitative assessment methodology of epigallocatechin and epigallocatechin–3–gallate compounds, as well as for the comparative characterisation and standardisation of the dominant polyphenolic components in *Ribes nigrum* using EGC and EGCG standards.

Key words

UV–visible spectrophotometry, HPLC, polyphenolic components

INTRODUCTION

Plants have the ability to synthesize chemical compounds (active principles), their active properties being correlated with the biochemical mechanisms of human metabolism. Polyphenol is the generic name of the compounds with several hydroxyl phenols in a single molecule [1, 2].

Polyphenolic compounds are pigments and antioxidants produced by plants through photosynthesis. *R. nigrum* contains important amounts of almost all known phenolic structure compound classes and stores complex mixtures of polyphenolic structure compounds that are accessible [3], easy to extract [4], with numerous properties, such as antioxidant, antibacterial, disinfectant, deodorising, etc. [5]. The dominant polyphenolic components in *R. nigrum* play a beneficial role in the maintenance of metabolic balance and of the health state of the living body [6]. The action of the substances from *R. nigrum* plants is a neutralising one and it is due mainly to the polyphenols [7]. These compounds play an active part in: detoxification, blocking germs [8] and destroying the bacterial membrane, and protection at the cellular level against the negative effects of free radicals [9, 10]. The oxidation process of these phenols results in compounds containing galloil rings [18], of which polyphenolic antioxidants are the most frequent type of molecule in this category [11, 12], being identified in over 4,000 components. Polyphenols can be classified as hydrolysable tannins and phenylpropanoids and then subdivided into lignans, flavonoids and condensed tannins [13]. Tannins (from a strictly biological point of view) are part of the plant's defence system against bacteria, viruses, etc. [14]. Tannins can affect nutrient cycling by hindering decomposition rates, complexing proteins, inducing toxicity to microbial populations and inhibiting enzyme activities (inhibiting action upon 5–lipoxygenase), and also the angiotensin conversion enzyme and activates hialuronidase [15, 2], and glycosyltransferase induces toxicity in microorganisms involved in carcinogenesis [16]. Monomers and dimers have qualities specific to vitamin P. Ellagic tannins, epicatechol gallate and epigallocatechol gallate act on immune mechanisms by enhancing phagocytosis, while EGCG and ECG are compounds that can hinder the formation of free radicals and protect DNA from damage provoked by reactive oxygen species [17]. *R. nigrum* contains a lot of polyphenolic antioxidants, such as flavonol glycosides, anthocyanidines, proanthocyanidines (condensed tannins), and phenolic acids [18]. The main active biological compounds in blackcurrant with a proven antioxidant activity [19] are the catechins: EGCG, EGC, ECG, and CE, found mainly in the fruit from where they can be removed with relatively high yields [20]. EGCG, EGC, ECG, EC and GCG are the major catechins in *R. nigrum*, but EGCG is the major component of the polyphenolic fraction representing about 10–50% of the total catechins in blackcurrant [21]. The *R. nigrum* wide chemical composition variety and strong antioxidant properties continue to attract the interest of researchers in the field of phytochemistry and pharmacogenesis [22].

The aim of the presented study was to develop an HPLC assay for the detection and quantification of biologically natural phenolic products. Methods are described for the general analysis of total ultraviolet (UV) absorbing constituents of *R. nigrum*. The interpretation of the chromatographic eluate and the significance and general utility of this new analytical method are discussed.

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MATERIALS AND METHODS

Vegetal Material. The blackcurrant fruits were collected in July 2010 from hillsides and hedgerows in Surduc Forest, located in western part of the Banat Region in Romania. The taxonomic identification of the plants was carried out by the ***Botany discipline***. A voucher specimen has been deposited in the Herbarium. For the preliminary phyto–chemical analysis etheric, alcoholic, aqueous solution were obtained through successive, selective extractions using solvents of different polarities, to determine the compound classes contained using specific colour reactions [23]. According to preliminary analyses, the amount studied contains 30 mg of flavonoids. The methanol and hexane used to extract and to elute were of HPLC grade (Sigma Chemicals). The standard compound used to make the calibration curves were purchased from Merck.

Extraction of Components. The extraction was carried out using an amount of 15.0 g of pre-conditioned R. nigrum. The original material was washed three times for 10 minutes, with 30 mL hexane (suitable for HPLC) each time, to remove pigments and non-polar compounds. Following filtration, the vegetal material was dried in a nitrogen current and subjected to Soxhlet extraction with 250 mL of methanol (99.85%) for one hour [24]. To prevent polyphenolic compounds oxidation the extractor was filled with nitrogen during extraction. The extract obtained was diluted in methanol to obtain the proper concentration for the spectrophotometry, and for the chromatographic column eluate at detector level in the case of HPLC [19].

Optical absorption spectrum in the ultraviolet range. **A**bsorption of the methanol layer was measured in 1 cm cuvettes, at a wave length of 273.2 nm, using methanol as blank. The *R. nigrum* methanol extract was assessed quantitatively using a UV–VIS spectrophotometer T60U, PG Instruments Limited, UV WINÒversion 5.05; detection was undertaken at 273.2 nm. The conditions used for the measurement were: a spectral slit width of 1 mm and scan speed of 90 nm/min. The calibration curves obtained using standard compound confirmed the systems linearity over the whole testing range [22].

HPLC analytic separation. HPLC analysis was carried out with a 25 × 0.46 cm inverse stationary phase Nucleosil column (C_{18}). A Jasco HPLC–1575 Series Chromatograph equipped with Intelligent Pump, UV Detector Jasco–1575 and auto sampler Jasco AS 1555 was used. The column used was of stainless steel C_{18}, 250 × 4.6 mm, 5 Micron, Lichrosphere operating at room temperature. The elution was carried out isocratically at a flow rate of 1.0 mL/min. using a mixture of 70% methanol and 30% aqueous solution containing 0.5% formic acid to 5.00 (±0.2) as the mobile phase. The detector was set at 273.2 nm. The responses of peak area were recorded and integrated using Brown Chromatographic Software. A double fascicle spectrophotometer operating in the 190–900 nm spectral range was used as a detector for HPLC. The extract and the reference solvent were introduced in cuvettes with an optic route of 0.5 cm. The response factors obtained supported the hypothesis concerning the identity of the components separated from the methanol extract of R. nigrum.

RESULTS

R. nigrum fruits (raw material) were collected and pre-conditioned through drying and chopping to separate polyphenolic compounds. Collecting and preservation of the fruits was in accordance with provisions of the Pharmacopoeia [24]. The extraction process was carried out taking into account two major aspects: testing the compatibility of the different phenolic fractions with different extraction agents, and optimising the technological parameters of the extraction process (mass ratio extraction agent: vegetal material, extraction temperature, number of extraction steps, yield).

Optical absorption spectrum in the ultraviolet range. The ultraviolet range covers radiations with wave lengths λ varying between 100–380 nm. Electronic spectra are the most complex spectra, electronic transitions also involving transitions between rotation and vibration levels, spectra having the character of a band. The absorption of light radiations by the molecules occurs according to the Lambert–Beer law observed in diluted solutions with no intermolecular interaction. Polyphenols calibration curves showed a good linearity (r^2>0.984) within test ranges.

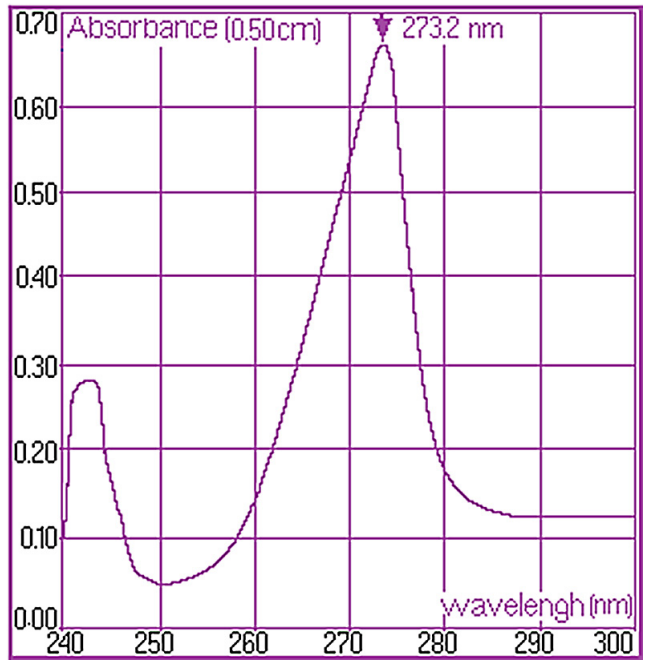

Figure 1. Optical absorption spectrum in the ultraviolet range for the methanol extracts of *R. nigrum*

Based on the absorption spectrum, we decided to monitor the chromatographic eluate at 273.2 nm.

HPLC analysis of polyphenolic compounds from the methanol extract of R. nigrum fruits. Usually, spectrophotometric methods provide simple and fast screening methods to quantify classes of phenolic compounds in plant samples.

However, due to the complexity of the plant phenolics and different reactivity of phenols toward assay reagents, a broad spectrum of methods was used for assay of the constituents, leading to differing and often non-comparable results. Additionally, the methods are quite prone to interferences,

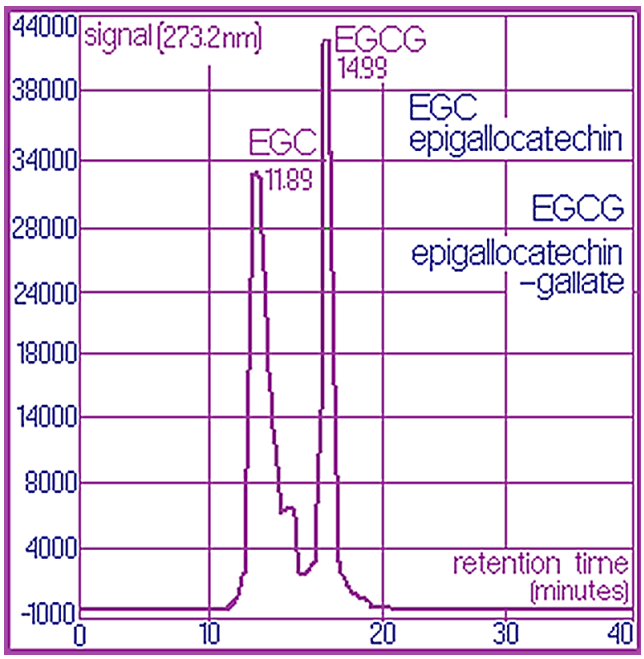

Figure 2. Chromatogram of the methanol extracts from *Ribes nigrum* L

consequently, often resulting in over- or underestimation of the contents. HPLC combined with the instrumental assay are specified for the profiling and quantification of phenolic compounds. Taking into account the spectrophotometric measurement limitations, i.e. the fact that the extract obtained is, in fact, a mixture not separated from bioactive compounds, chromatographic analysis was also considered mandatory. As a consequence, an HPLC analysis of the same methanol extract from *R. nigrum* was conducted, aimed at separating and analysing the compounds.

DISCUSSION

It was found that the *R. nigrum* methanol extract from the fruit generated a well–designed absorption band with a local maximum at 273.2 nm, a band that can be attributed to an electronic transition of the n–π^* type, i.e. a transition in which phenol oxygen electrons are largely involved. Figure 1 shows the optical absorption spectrum in the ultraviolet range for the methanol extract of *R. nigrum* fruits.

Another absorption maximum much below 242 nm probably involves transitions that are less specific to the phenol OH group. The transitions between the rotation energetic states are excited by lower energy radiations corresponding to the remote IR range and microwaves. Pure rotation spectra have low analytic implications and, therefore, they will not be debated further, In addition, the spectral range at wavelengths below 242 nm is susceptible to interferences with other components of secondary interest for the present study. Figure 2 shows the chromatogram obtained under the working conditions described above. The two chromatographic peaks located at 11.89 min. and at 14.99 min. correspond to the dominant components of the methanol extract from *R. nigrum*, peaks identified as EGC and EGCG, respectively. The method used is based on an HPLC technique published in literature, but slightly modified. The main modification of the initial method was replacement of the mobile phase with non-volatile components with a completely volatile one, the eluent used in the presented research consisted of a mixture of 70% methanol and 30% aqueous solution containing 0.5% formic acid (formic acid has a low UV absorbance). Confirming data published in literature [25, 7], identification was made of the higher mobility component (lower retention time) as EGC, and the component with higher retention time as EGCG. Retention values cited in literature were obtained in conditions similar to those adopted in the presented study (column C_{18}, eluent acetonitrile–water 60:40, aqueous phase containing 0.1% phosphoric acid, flow rate 1.0 mL/min; UV detection was made at 273.2 nm).

In the presented study, a sample mixture with known identity of the components was analysed [20]. Retention times close to the values in this study support the hypothesis concerning the identity of the components separated from the methanol extract from *R. nigrum*. The relative recoveries for the methanol extract from *R. nigrum* were established by using the external standard methodology, by comparison with the standard chromatogram. Every real sample was analysed three times, with four replicates each time. The results of optimization and validation acceptably assays had reliable, accurate, sensitive and ideal recoveries that were convenient and effective for phenolic compounds. HPLC efficiency is incentive by the resolution of standards and the ability to design the response of the detector during calibration, and by the sensitivity, accuracy, and precision, frequency of false positives and negatives during assays. The developed assay was successfully applied to fingerprint assay of extracts from *R. nigrum* as well as quantify the relevant markers Phenolic compounds present in tissue samples under optimum parameters. This method can be applied to analyze the phenolic compounds in the extract from *R. nigrum* and other plants. The method developed here and the testing of the methodology of chromatographic separation of polyphenolic components from the methanol extract from *R. nigrum* are an important part of the study (different matrices).

To better valorise these resources, i.e. to better recover the polyphenolic aromatic structure compounds, it is necessary to establish a relatively simple extraction technology that does not need numerous handlings and chemical changes that could result in a partial or total loss of biological activity.

CONCLUSIONS

Chromatography is an advantageous method for *R. nigrum* components analysis. In addition, the development of the specific and selective methods of detection opens a new perspective to the valorisation of this technique in phyto–chemical analyses in general, and in the characterisation of the plants containing polyphenols, in particular. The HPLC assay was established in terms of linear response range, limit of detection, limit of quantification and precision.

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