Ethanol Concentration Effect on the Extraction of Phenolic Compounds from *Ribes nigrum* Assessed by Spectrophotometric and HPLC-DAD Methods

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The purpose of this study was to assess the phenolic compounds extraction from black currants (Ribes nigrum) by analyzing the effect of ethanol concentration (40-80 % v/v) on the polyphenols obtained. Total phenolic content (TPC) of the extracts, expressed as gallic acid equivalents (GAE), was investigated by the Folin-Ciocalteau method and the total monomeric anthocyanin content (TA), calculated as cyanidin 3-rutinoside equivalents (Cyd-3-rut), by pH-differential method. In order to determine the radical scavenging activities (RSA) of the extracts, DPPH scavenging assay was performed. The spectrophotometric analysis of the 70 % aqueous ethanol extract presented the highest values for TPC (3136.6 mg GAE/100 g), TA (182.4 mg Cyd-3-rut /100 g) and RSA (94.7 %). The quantification of the quercetin-3-rutinoside and the quercetin was carried out with a rapid method of reverse phase high-performance liquid chromatography (RP-HPLC). The obtained results through HPLC analysis confirmed the noticed tends using spectrophotometric analysis.

*Keywords: total phenolic content, DPPH, cyanidin 3-rutinoside, HP*LC, black currants

Phenolics have health-promoting benefits; they are of current interest due to their important biological and pharmacological properties, especially the antioxidant, anti-inflammatory, antimutagenic and anticarcinogenic activities. Polyphenols are especially important antioxidants due to their high redox potentials allowing them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [1]. Plant phenolics include simple phenols, phenolic acids (both benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins [2]. Strongly coloured berries contain high levels of polyphenols such as anthocyanins, flavonol glycosides and hydroxy-cinnamic acids [3]. Black currant berries are excellent sources of bioactive components such as anthocyanins (cyanidin 3-rutinoside, delphinidin 3-rutinoside), flavonols (quercetin 3-rutinoside, myricetin 3-rutinoside), procyanidins and phenolic acids (3-caffeoylquinic acid). Current studies have shown that anthocyanins display a wide range of biological activities including antioxidant [4], antimicrobial and anti-carcinogenic activities [5], improvement of vision [6], induction of apoptosis [7], and neuroprotective effects [8].

Recently, ultrasound was effectively and rapidly applied to extract polyphenolics from plant material [9]. The low frequency of ultrasound (in the kHz range) has the advantage of reduced extraction times and enhanced extraction yield. The efficient extraction of polyphenolics by ultrasound is attributed to the cavitational phenomenon, through the microscopic bubbles produced by alternating low- and high - pressure waves generated by ultrasonic sound. Bubbles filled with solvent vapours are produced at low pressure, compressed and finally implode at high pressure, leading to a powerful shock wave and enhanced mixing in the solvent extraction system [10]. High-power ultrasound improves solvent extraction from plant material mainly due to its mechanical effects [11]. These effects via cavitational collapse of bubbles enhance mass transfer rate and solvent penetration into cellular materials. In addition, the disruption or damage of biological cell walls by ultrasound results in facilitated release of the intracellular contents.

The aim of this study was to investigate the effects of ethanol concentration on the ultrasound extraction of the phenolic compounds from black currants. The binary mixture of solvents (ethanol: water) was chosen due to its non-toxic properties and for limited number of works about the extraction of polyphenols from black currants in these systems. The extracts were investigated for total phenolic content, total monomeric anthocyanin content and antioxidant activity. Furthermore, individual flavonoids (catechin, epicatechin, quercetin-3-rutinoside and quercetin) were determined using the HPLC developed method.

Experimental part

Materials and methods

Folin-Ciocalteau phenol reagent, DPPH (1,1-diphenyl-2-picryl-hydrazyl), methanol, ethanol, were provided by Merck. Polyphenols standards: gallic acid and catechin, epicatechin, quercetin-3-rutinoside (rutin), quercetin were supplied by Sigma-Aldrich. All the other reagents used in the experiments were of analytical grade. Black currants (*Ribes nigrum)* were obtained from Romania local market. The sample was dried, then ground (Fritsch Pulverisette 14, at 6000 RPM), sifted for homogenization through a sieve of 0.5 μm and stored at -20 °C to avoid compound degradation.

Sample preparation

The ultrasound-assisted extraction (UAE) was carried out in an ultrasonic device (Bioblock Scientific) with a frequency of 100 kHz, equipped with a digital timer and a

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temperature controller. UAE is one of the simplest extraction technique used to recover the polyphenols from different matrices in shorter times than other extraction systems [12]. Dried black currants (1 g) were mixed with 10 mL of extraction solution and sonicated for 30 min at room temperature. Each extract was centrifuged at 4000 rpm for 20 min. The residue was re-extracted by repeating the procedure mentioned above. The two supernatants were combined, filtered, and stored in the dark at 4^0C to avoid possible loss of phenolic compounds due to oxidation and other degrading reactions. The temperature in the ultrasonic bath during solvent extraction was maintained at room temperature (23-25^{0}C) because possible degradation of polyphenolics takes place at increased temperatures. The solvent to the material ratio was chosen according to previous work [13,14] and to avoid waste of solvent and bulky handing in the subsequent processes.

Spectrophotometric analysis

Total polyphenolic content (TPC)

Spectrophotometric measurements were carried out with a Specord M400, Carl Zeiss Yena spectrophotometer using a 1 cm quartz cell. The concentration of total phenolics was measured by the method described by [15] modified by [16]. One milliliter of sample or standard solutions of gallic acid was added to a 25 mL volumetric flask containing 9 mL of distilled water. One milliliter of Folin & Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7% Na_2CO_3 solution was added with vigorously mixed. The solution was then immediately diluted to volume with ddH_2O and mixed thoroughly. After incubation for 90 min the absorbance versus prepared blank was read at 750 nm. Total phenolic contents of black currant extracts were expressed as mg gallic acid equivalents (GAE).

DPPH radical scavenging assay

DPPH scavenging activity was determined using a method of [17] modified by [18]. One hundred μM DPPH was dissolved in methanol. The black currant extracts, 0.1 mL, were added to 2.9 mL of the methanolic DPPH solution. The mixture was shaken vigorously and allowed to stand in the dark for 30 min. The decrease in absorbance of the resulting solution was monitored at 517 nm at 30 min. A control solution consisted of 0.1 mL of extraction solution and 2.9 mL of DPPH solution was prepared. The radical stock solution was prepared fresh daily. The antioxidant activity percentage was calculated with the following equation:

$$\%, RSA = \left(\frac{A_{control} - A_{sample}}{A_{control}} \right) \cdot 100 \qquad (1)$$

$A_{control}$ and A_{sample} are the absorbances of control and sample prepared as mentioned above.

Total monomeric anthocyanin content (TA)

Cyanidin-3-rutinoside (Cyd-3-rut) is a pigment in the black currants found to be the most thermally stable anthocyanin [19]. Total monomeric anthocyanin was estimated by a *p*H-differential method [20]. Two dilutions of black currant extracts were prepared, one with potassium chloride buffer (*p*H 1.0), and the other with sodium acetate buffer (*p*H 4.5). Absorbances were measured simultaneously at 510 nm and 700 nm after 30 min of incubation at room temperature. The content of total anthocyanins was expressed in mg of cyanidin-3-rutinoside equivalents using a molar extinction coefficient (ε) of cyanidin-3-rutinoside of 7000 L mol^{-1} cm $^{-1}$ [21] and molar weight (MW) (595.2 g mol $^{-1}$).

HPLC-DAD analysis

Chromatographic analysis was performed on an Agilent 1100 system equipped with a diode array detector (DAD), connected to a ChemStation software. The separation was performed with a reverse phase Kromasil 100-5C18 column (150 mm ×4.6 mm, 5μm). A gradient system was used: eluent A was water with 0.03 % trifluoroacetic acid (TFA) and eluent B was methanol. The flow rate was 1 mL/min. and the injection volume was 10 μL. The elution conditions applied were: 0-3 min. 20% B, 3-7 min., linear gradient from 20 to 40 % B, 7-10 min. linear gradient from 40 to 60 % B, and 10-15 min. 60% B isocratic. Simultaneous monitoring was set at 280 nm for catechin and epicatechin, 350 nm for quercetin-3-rutinoside and 370 nm for quercetin.

A stock solution containing flavonoid standards was prepared and diluted with methanol to an appropriate concentration in the range of 1-50 mg/L for catechin, epicatechin and quercetin and 1-100 mg/L for quercetin-3-rutinoside. At least five different concentrations of analytes were injected in triplicate.

Results and discussions

Spectrophotometric analysis (TPC, RSA and TA of black currant extracts)

In this study, the effect of binary mixtures of solvents ethanol: water on the polyphenols extraction from the black currants was investigated. The total phenolic contents in different extracts were determined from the regression equation of the calibration curve, thus, TPC varied from 1558.0 to 3136.6 mg GAE / 100 g of black currant powder. Figure 1A shows the effect of ethanol concentration on the phenolic content of black currant extracts. The hydro-alcoholic mixture was employed in an attempt to extract as many compounds as possible. This is based on the ability of alcohol solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium to low-polarity constituents [22]. The 70 % aqueous ethanol was the most adequate concentration for polyphenols extraction, in good agreement with the literature [23]. Soluble phenolic compound are generally extracted using water, methanol, ethanol or acetone. The presence of attached sugars tends to render the phenolic compounds more water soluble, and combination of the above solvents with water represent thus better solvents for glycosides. In contrast, less polar aglycones, such as isoflavones, flavanones, and highly methoxylated flavones and flavonols tend to be more soluble in non-aqueous solvents [24].

The antioxidant activity of black currant extracts was determined using DPPH free radical scavenging assay. The RSA values of the extracts are presented in Figure 1B. The highest antioxidant activity (94.7 %) was observed for the extract in 70 % : 30 % ethanol: water. These results correspond to earlier reported by Viskelis [25].

Anthocyanins are polyphenolic pigments responsible for most of the color diversity found in plants [26]. Anthocyanin pigments undergo reversible structural transformations with a change in *p*H manifested by different absorbance spectra. The oxonium form predominates at *p*H 1.0 while the hemiketal (colorless) form at *p*H 4.5. The *p*H-differential method is based on this reaction and allows accurate and rapid measurements of the total amount of anthocyanins, even in the presence of polymerized degraded pigment and other interfering compounds [27]. Since Cyd-3-rut is the most abundant anthocyanin in black currants [28] the extinction coefficient of Cyd-3-rut standard was used to measure the total anthocyanins. The concentration of total monomeric anthocyanin in black currant extracts ranged

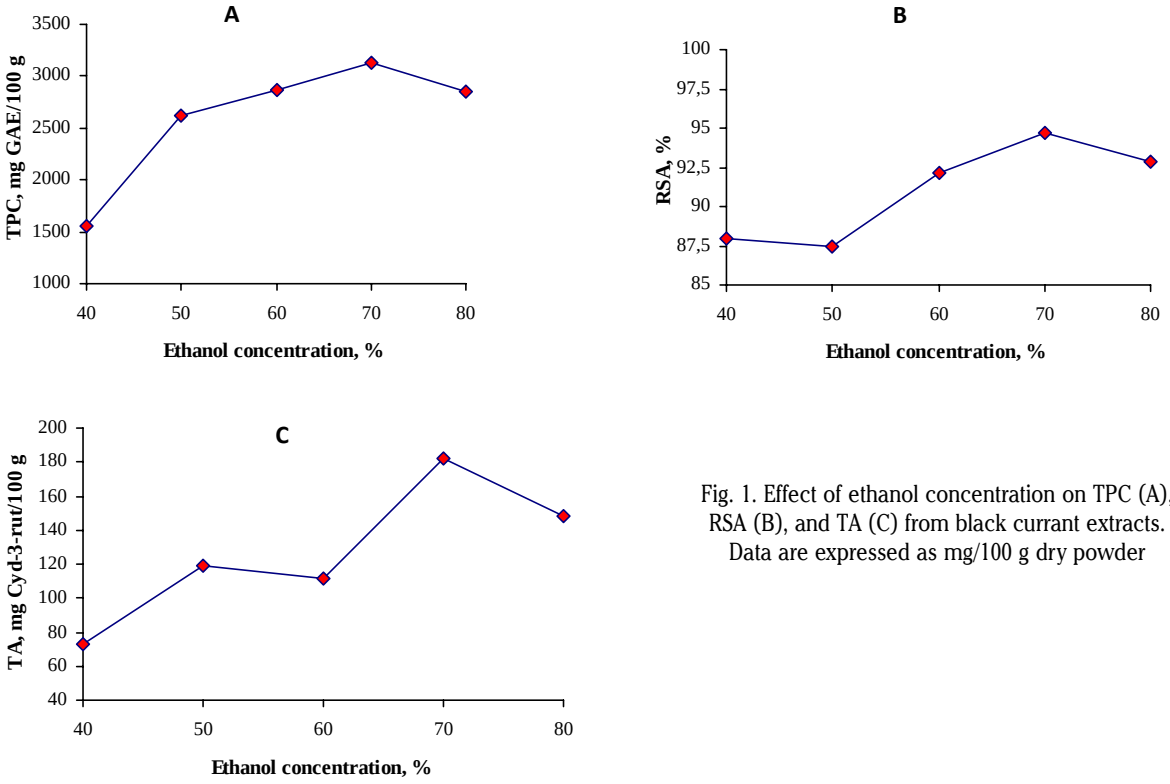

Fig. 1. Effect of ethanol concentration on TPC (A), RSA (B), and TA (C) from black currant extracts. Data are expressed as mg/100 g dry powder

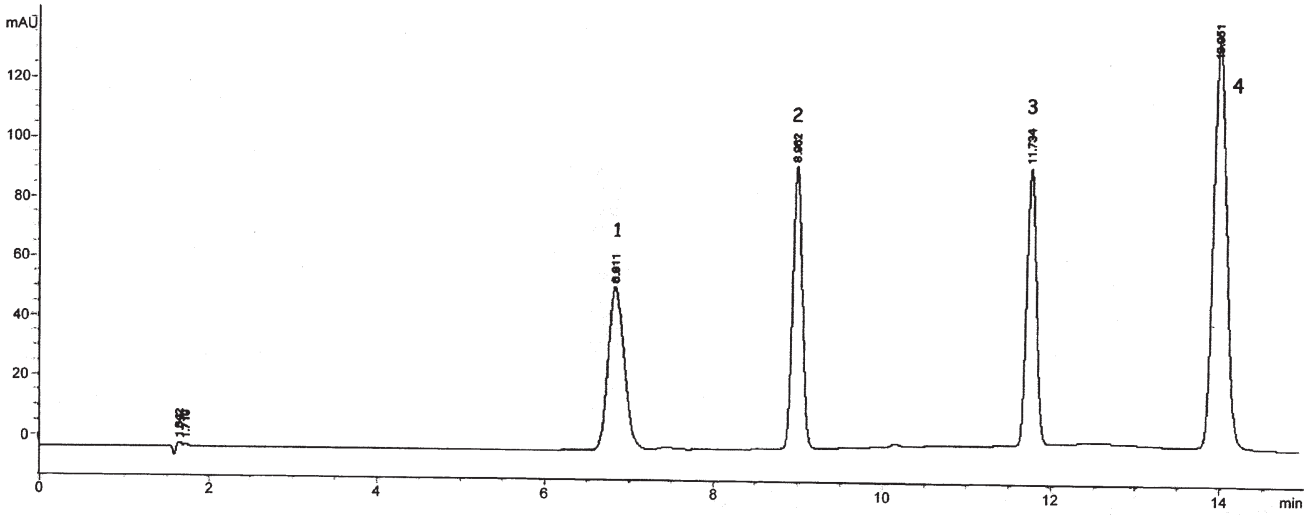

Fig. 2. Chromatogram of the mixture of flavonoid standards: 1= catechin (tr= 6.81 min, λ= 280 nm); 2= epicatechin (tr= 8.96 min, λ= 280 nm); 3= quercetin-3-rutinoside (tr= 11.73 min, λ= 350 nm); 4= quercetin (tr= 13.95 min, λ= 370 nm)

Table 1
LINEARITY RESULTS , LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

Compound	λ (nm)	Equation	r^2	LOD (mg/L)	LOQ (mg/L)
Catechin	280	Y = 14.4263x + 1.3968	0.9999	0.58	1.77
Epicatechin		Y = 14.4475x + 4.9587	0.9999	0.56	1.68
Rutin (quercetin-3-rutinoside)	350	Y = 31.9016x - 11.6837	0.9996	1.24	3.74
Quercetin	370	Y = 81.2615x - 11.1438	0.9999	0.48	1.47

from 73 to 182 mg/100 g (fig.1C) being consistent with other research [29]. The highest values for all spectrophotometric analysis were at a concentration of 70 % ethanol.

HPLC-DAD analysis

A gradient elution RP-HPLC method was developed for the analysis of four phenolic compounds in aqueous ethanol extracts. Different combinations of the methanol and water with TFA, distinct flow rates and column temperatures were tested, also. Thus, a solvent system consisting of 0.03% TFA in water and methanol, a flow rate of 1 mL/min. at 25 ^{0}C was chosen because it allows separation of compound with good resolution. Using a reverse phase Kromasil column the retention times for catechin, epicatechin, quercetin-3-rutinoside and quercetin were presented in figure 2. Total time of analysis was less than 15 min.

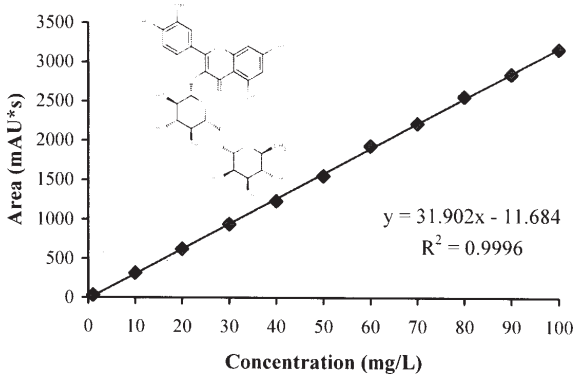

Fig. 3. Calibration curve of quercetin-3-rutinoside

Concentration (mg/L)	Compound			
	catechin	epicatechin	quercetin	quercetin-3-rutinoside
	RSD, %			
10	0.28	0.08	0.15	0.24
30	0.13	0.03	0.09	0.10
50	0.73	0.05	0.38	0.13

Table 2
WITHIN-DAY PRECISION DATA AT DIFFERENT CONCENTRATION LEVELS

Ethanol concentration, %	Flavonoid concentration, mg/L		Total flavonoids mg/Kg
	quercetin-3-rutinoside	quercetin	
40	21.20	0.81	192.00
50	20.13	1.26	289.08
60	19.71	1.28	276.48
70	19.48	1.27	**299.41**
80	18.86	1.27	276.22

Table 3
TOTAL CONTENT OF QUERCETIN-3-RUTINOSIDE AND QUERCETIN DETERMINED BY HPLC METHOD

Linearity

The external standard method was the technique used for quantitation. Peak areas from HPLC chromatogram were plotted against the known concentrations of stock solutions of varying concentrations. Table 1 presents the equation of the regression line, correlation coefficient (r^2) for each compound. Excellent linearity was obtained for catechin, epicatechin quercetin and rutin respectively.

Limit of detection and quantification

The calculations for the limits of detection (LOD) were based on the standard deviation of y-intercepts of the regression lines (σ) and the slope (S) using the following equation $LOD=3.3\sigma/S$. Limits of quantification (LOQ) were calculated by the equation $LOQ=10\sigma/S$ [30]. The detection and quantification limits are presented in table 1.

Precision

Repeatability was studied by calculating the relative standard deviation (RSD) in %, for 3 determinations of the following concentrations: 10, 30 and 50 mg/L working standard solutions, performed on the same day and under the same experimental conditions. The RSDs values of the peak area, were found to be < 1 %, as presented in table 2.

Quantification of quercetin-3-rutinoside and quercetin was carried out by the integration of the peak using the external standard method and the results being presented in table 3. The catechin and epicatechin concentrations in all extracts were below the detection limit (table1). The calculated amounts of determined flavonoids were given in mg/Kg of dry black currant powder.

The highest content of quercetin-3-rutinoside and quercetin was found for 70 % aqueous ethanol extract, which confirmed the noticed tends using spectrophotometric analysis.

Conclusions

The results showed that black currants (*Ribes nigrum)* contained considerable amount of polyphenols and ethanol concentration had significant effects on the extraction rate of these aromatic secondary metabolites of plants. The spectrophotometric analysis of the 70 % aqueous ethanol extract presented the highest values for TPC (3136.6 mg GAE/100 g), TA (182.4 mg Cyd-3-rut /100 g).

Black currants had strong antioxidative activity, tested by free radical scavenging activity, being influenced by the extracting solvent thus, RSA values have increased from 87.9 % in 40% ethanol to 94.7 % in 70 % ethanol and then slow decrease to 92.8 % in 80% ethanol.

The HPLC developed method was applied to the separation of some flavonoids found in black currant extracts. The proposed method is simple, sensitive, rapid and could be applied for monitoring quercetin-3-rutinoside and quercetin from black currant extracts.

Black currants represent a source of interesting natural bioactive compounds. The results obtained may show black currants as a source of well known antioxidant compound such as gallic acid, cyanidin-3-rutinoside, quercetin-3-rutinoside and quercetin.

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