PAPER

OPTIMISATION OF THE EXTRACTION OF FLAVONOIDS FROM APPLES USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

The ultrasound-assisted extraction of flavonoids from apple samples was modelled using response surface methodology. A three-level-three-factor central composite design using the response surface methodology (RSM) was employed to optimise three extraction variables, including temperature, extraction time and ultrasonic power, for the achievement of the highest extraction yield of the flavonoids from lyophilised apple samples. The optimised extraction conditions were 44.61ºC, an extraction time of 26.90 min, and ultrasonic power 480 W. The experimental yield of flavonoids was 6.58 mg g-1 expressed as rutin equivalent, which was close to the predicted yield (6.69 mg g-1). Optimised extraction conditions were applied for the analysis of apple samples of six cultivars.

Keywords: apple, central composite design, flavonoids, HPLC, response surface methodology, ultrasonic extraction

1. INTRODUCTION

Apples play an important role in the human diet. They are one of the most consumed fruits in the whole world (WU *et al*., 2007, CEYMANN *et al*., 2012). Based on data from the year 2014, approximately 84.63 million tonnes of apples are grown per annum. Countries that grow the most apples are China (approximately 40.92 million tonnes per annum), the USA (approximately 5.19 million tonnes per annum) and Poland (3.20 million tonnes per annum) (FAO Statistical Database, 2017). Apples are widely used in the food industry to produce various products and drinks (juice, wine, cider); they are also used unprocessed (MARKS *et al*., 2007, PRICE *et al*., 1999).

Some of the most important biologically active substances in apples are phenolic compounds, which are attributed to natural antioxidants. Oxidative stress causes changes in cell metabolism related to DNA and protein damage as well as lipid peroxidation (COOKE et al., 2003; PIZZIMENTI *et al*., 2010). It can cause inflammatory processes, cardiac, vascular and other diseases (MADAMANCHI *et al*., 2010). Phenolic compounds neutralise reactive forms of oxygen and nitrogen (PANDEY and RIZVI, 2009) and therefore are valuable for the treatment and prophylaxis of various diseases. Qualitative and quantitative composition analyses of raw materials that accumulate phenolic compounds are important and relevant.

The selection of extraction conditions is an important analytical step in developing the qualitative and quantitative analysis methodologies of phenolic compounds in multi-component matrices. Our developed and validated method of flavonoid and phenolic acid determination in apples is published in the paper by LIAUDANSKAS *et al*. (2014). In developing this method, the extraction parameter selection was empirical. It is relevant to compare flavonoid extraction yield when the samples are extracted using empirical extraction conditions and when conditions are selected based on statistical modelling.

2. MATERIALS AND METHODS

2.1. Plant material

Apple samples of the Ligol cultivar were chosen for the extract optimisation analysis. The Ligol cultivar (winter cultivar, bred in Poland) is one of the main cultivars in commercial apple orchards in Lithuania. Optimised extraction conditions were applied for the analysis of the apple samples of different cultivars. The following apple cultivars were included in the comparable researches: Aldas (early winter cultivar, bred in Lithuania, recommended for ecological orchards), Auksis (early winter cultivar, bred in Lithuania,), Connel Red (winter cultivar, bred in USA), Ligol, Lodel (early winter cultivar, bred in Lithuania) and Rajka (early winter cultivar, bred in Czech Republic). The apple trees were grown in the experimental orchard (block 2, row 4, trees 21-40) of the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Babtai, Lithuania (55°60′ N, 23°48′ E). The altitude of Babtai town is 57 m above sea level. Trees were trained as a slender spindle, and pest and disease management was carried out according to the rules of the integrated plant protection. The experimental orchard was not irrigated. Tree fertilisation was performed according to soil and leaf analysis. In addition, nitrogen was applied before flowering at the rate of 80 kg ha^{-1}, and potassium was applied after harvest at the rate of 90 kg ha^{-1}. Soil conditions of the experimental orchard were the following: clay loam, pH - 7.3, humus - 2.8%, P_2O_5 - 255 mg kg^{-1} and K_2O - 230 mg kg^{-1}.

2.2. Sample preparation

Apples were cut into slices of equal size (up to 1 cm in thickness), and the stalks and the seeds were removed. The apple slices were immediately frozen in a freezer (at -35°C) with air circulation. Apple samples were lyophilised with a ZIRBUS sublimator 3× 4×5/20 (ZIRBUS technology, Bad Grund, Germany) at a pressure of 0.01 mbar (condenser temperature, -85°C). The lyophilised apple slices were ground to a fine powder (about 100 μm) by using the knife mill Grindomix GM 200 (Retsch, Haan, Germany).
Loss on drying before analysis was determined by drying the apple lyophilisate in a laboratory drying oven to complete the evaporation of free water and volatile compounds (temperature 105°C) and by calculating the difference in raw material weight before and after drying (European Pharmacopoeia, 2010). The data were recalculated for the absolute dry lyophilisate weight.

2.3. Chemicals

All solvents, reagents, and standards used were of analytical grade. Acetonitrile, aluminium trichloride hexahydrate, hexamethylenetetramine and acetic acid were obtained from Sigma-Aldrich GmbH (Buchs, Switzerland), and ethanol from Stumbras AB (Kaunas, Lithuania). Hyperoside, rutin, quercitrin, phloridzin, procyanidin B1 and procyanidin B2, and chlorogenic acid standards were purchased from Extrasynthese (Genay, France); reynoutrin, (+)-catechin and (−)-epicatechin were purchased from Sigma-Aldrich GmbH (Buchs, Switzerland); and avicularin, procyanidin C1 and isoquercitrin were purchased from Chromadex (Santa Ana, USA). In the study, we used deionised water that the Crystal E HPLC (Adrona SIA, Riga, Latvia) water purification system produced.

2.4. Extraction

A total of 2.5 g of lyophilised apple powder (exact weight) was weighed, added to 30 mL of ethanol (70%, v/v) and extracted in a Sonorex Digital 10 P ultrasonic bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany). A total of 480 W is the maximum ultrasonic power that can be achieved by using the Sonorex Digital 10 P ultrasonic bath. The obtained extract was filtered through a paper filter, and the apple lyophilisate on the filter was washed twice with 10 mL of ethanol (70%, v/v) in a 50mL flask. Then, the extract was filtered through a PVDF syringe filter with a pore size of 0.22 μm (Carl Roth GmbH, Karlsruhe, Germany).

2.5. Determination of the total flavonoid content

The total flavonoid content in the extracts of lyophilised apple samples was determined by applying the technique that URBONAVIČIŪTĖ *et al*. (2006) described. It was calculated using the rutin calibration curve and was expressed as its equivalent (mg RE/g) for absolute dry weight (DW).

2.6. High-performance liquid chromatography

The qualitative and quantitative analyses of phenolic compounds were performed according to the previously validated and described high-performance liquid chromatography (HPLC) method (LIAUDANSKAS *et al*., 2014).

2.7. Experimental design and statistical analyses

Before the development of the study through the response surface methodology (RSM), the flavonoid yield from the apples of three extractants (ethanol, methanol, and acetone) of different concentrations were compared. It was determined that the highest yield of flavonoids after four hours of extraction was achieved by macerating apple samples with ethanol 70% (v/v); therefore, this extractant was chosen for further analyses.
Selection of extraction method: The efficacy levels of maceration and extraction in ultrasonic bath methods were compared, and it was determined that the yield was higher when sonification method was applied. The results for the extractant and extraction method selections are discussed more in depth in the paper by LIAUDANSKAS *et al*. (2014).
In general, such multiple parameters as liquid/solid ratio, temperature, time, solvent polarity and ultrasonic power influence the efficiency of the extraction of a compound, and these are the primary extraction parameters that many other authors have referred to (TIAN *et al*., 2013; RADOJKOVIC *et al*., 2012; CHEN *et al*., 2012) In this study, three factors (or independent variables) were selected: temperature (20-60°C), extraction time (5-95 min) and ultrasonic power (48-480 W) (Table 1).

Table 1. Factors and levels for RSM, and central composite experimental design with the independent variables.

Run	Coded and non-coded variable levels						Total flavonoid content, mg RE g^{-1}
	X_1	Temperature, °C	X_2	Extraction time, min	X_3	Ultrasonic power, W	
1	0	40	0	50	0	264	6.889
2	0	40	0	50	-1	48	6.58
3	0	40	-1	5	0	264	4.199
4	0	40	1	95	0	264	6.312
5	-1	20	-1	5	-1	48	3.154
6	1	60	0	50	0	264	6.315
7	0	40	0	50	0	264	7.087
8	1	60	1	95	-1	48	5.618
9	1	60	1	95	1	480	5.963
10	-1	20	1	95	-1	48	4.682
11	1	60	-1	5	-1	48	4.102
12	-1	20	-1	5	1	480	3.555
13	0	40	0	50	0	264	6.794
14	0	40	0	50	0	264	7.175
15	0	40	0	50	0	264	6.821
16	-1	20	0	50	0	264	4.966
17	0	40	0	50	0	264	6.982
18	-1	20	1	95	1	480	5.406
19	1	60	-1	5	1	480	4.665
20	0	40	0	50	1	480	7.465

A three-level-three-factor central composite design was employed to determine the optimal combination of flavonoid extraction variables from apple samples. Table 1 represents the coded and non-coded values of the experimental variables and 20 experimental points. Six replicates (1, 7, 13, 14, 15, 17) were used to evaluate the pure error. Experimental data showed that response variables were fitted to a quadratic polynomial model. The general form of the quadratic polynomial model is presented in Fig. 1, where Y is the dependent variable; β_0, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linearity, square and interaction respectively. X_i and X_j are the independent variables.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i$$

Figure 1. The general form of the quadratic polynomial model.

Design-Expert® 6.0.8 software (Stat-Ease Inc., Minneapolis, Minnesota, USA) was used to analyse the data, develop models and optimise the extraction conditions. The fitness of the quadratic polynomial model was inspected with the regression coefficient of R^2. The p-value was used to check the significance of the regression coefficient.
All of the experiments (except extraction optimisation researches) were carried out in triplicate. Means and standards errors were calculated with SPSS 20.0 software (Chicago, USA). A single factor analysis of variance (ANOVA) along with the post hoc Tukey's HSD test was employed for statistical analysis. Differences were considered to be significant at the $p<0.05$ level.

3. RESULTS AND DISCUSSIONS

3.1. Optimisation of extraction conditions of total flavonoids in apples

The design matrix and the corresponding results of RSM experiments to determine the effects of the three independent variables, including temperature (X_1), extraction time (X_2) and ultrasonic power (X_3), are shown in Table 2. Through multiple regression analysis of the experimental data, the model for predicted response Y could be expressed with the following quadratic polynomial equation (in the form of coded values), presented in Fig. 2.

$$Y\,(TFC) = 6.86 + 0.49X_1 + 0.83X_2 + 0.29X_3 - 1.08X_1^2 - 1.47X_2^2 + 0.30X_3^2 - 0.071X_1X_2 - 0.027X_1X_3 + 0.013X_2X_3$$

Figure 2. The model for the predicted response Y expressed by the quadratic polynomial equation (in the form of coded values).

Statistical testing of the model was performed in the form of analysis of variance (ANOVA). The ANOVA for the fitted quadratic polynomial model of extraction of polysaccharides is shown in Table 2.

Table 2. Analysis of variance for fitted quadratic model of extraction of phenolic compounds.

Source	Sum of squares	Degree of freedom	p-value
Model	32.32	9	<0.0001 Significant
Residual	0.60	10	
Lack of fit	0.49	5	0.0698 Not significant
Pure error	0.11	5	
Cor. total	32.92	19	

R^2 = 0.9818; R^2_{adj} = 0.9674; C.V. = 4.27%; adequate precision = 26.232.

The results of the analysis of variance for the fitted quadratic model of extraction of phenolic compounds are presented in Table 2. They indicated a high degree of correlation between the observed and predicted values. The lack of fit test determines whether a selected model is adequate for explaining the experimental data or whether another model should be reselected. The value of the lack of fit test indicated that the fitting model was adequate. An adequate precision is a measure of the signal-to-noise ratio, which when greater than 4 is considered to be adequate (CANETTIERI *et al*., 2007). In addition, the value of adequate precision demonstrates an adequate signal. At the same time, a relatively low value of the coefficient of variation indicates a better precision and reliability of the experimental values. Therefore, the model is adequate for prediction in the range of experimental variables.
Residual analysis of the response surface design was performed. A normal probability plot was applied to the residuals. The data points fell along a straight line, indicating that they were distributed normally. In addition, residual runs for analysis were used. It was determined that the order of observations did not influence the results. Residuals versus predicted responses were plotted. The data points fell on both sides of the zero line, so no pattern could be concluded. The relationship between the actual and predicted values was evaluated. The data points fell along a straight line indicating close similarity between the two data points and the adequacy of the model.

Table 3. Regression coefficients estimate and their significance test for quadratic model.

Source	Sum of squares	Degree of freedom	p-Value
X_1	2.40	1	<0.0001
X_2	6.90	1	<0.0001
X_3	0.85	1	0.0037
X_1^2	3.23	1	<0.0001
X_2^2	5.94	1	<0.0001
X_3^2	0.24	1	0.0717
X_1X_2	0.040	1	0.4337
X_1X_3	5.886×10^{-3}	1	0.7605
X_2X_3	1.378×10^{-3}	1	0.8825

The significance of each coefficient measured using the p-value is listed in Table 3. A smaller p-value means the corresponding variables are more significant. The p-value of the model is less than 0.0001, which indicates that the model is significant and can be used to optimise the extraction variables. The three independent variables (X_1, X_2, X_3) and two quadratic terms (X_1^2 and X_2^2) significantly affect the extraction yield of flavonoids. The interaction among temperature (X_1), extraction time (X_2) and ultrasonic power (X_3) did not affect the extraction yield of flavonoids significantly.

The three-dimensional response surface is the graphical representation of the regression equation and is very useful for judging the relationship between independent and dependent variables. Different shapes of the contour plots indicate whether the mutual interactions among the variables are significant or not. A circular contour plot means the interactions between the corresponding variables are negligible, whereas an elliptical contour suggests that the interactions between the corresponding variables are significant (MURALIDHAR *et al.*, 2001). The three-dimensional representation of the response surfaces generated via the model is shown in Fig. 3–5. With these three variables, when two variables are depicted in three-dimensional surface plots, the third variable is fixed at the zero level.

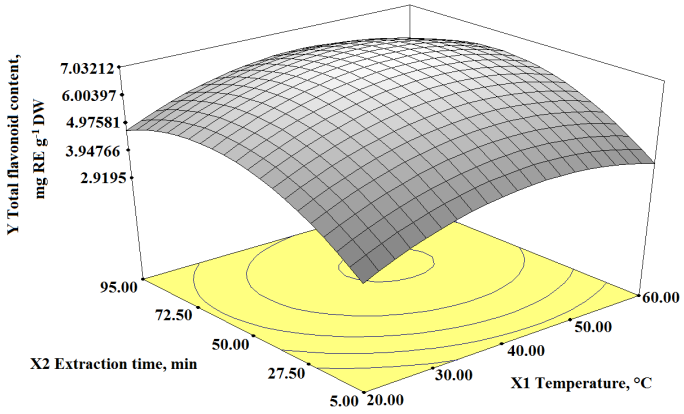

Figure 3. Response surface plot showing the effect of temperature (X_1) and extraction time (X_2). X_3 (Ultrasonic power) = 264 W.

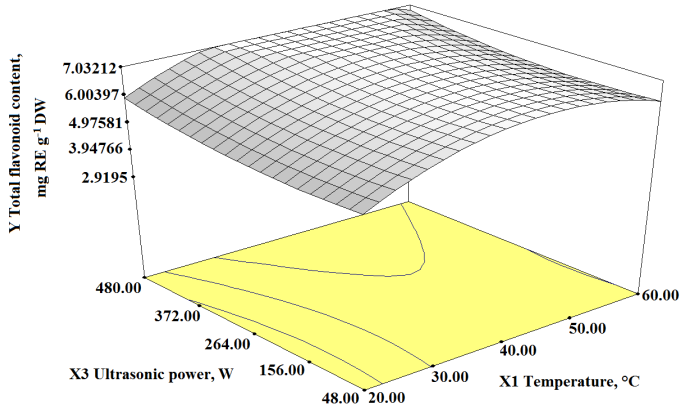

Figure 4. Response surface plot showing the effect of temperature (X_1) and ultrasonic power (X_3). X_2 (Extraction time) = 50 min.

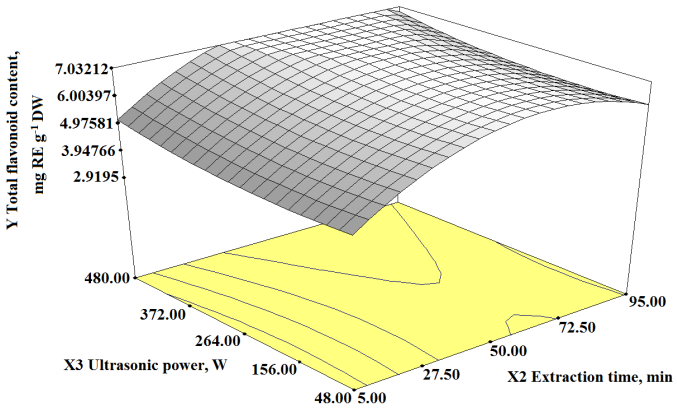

Figure 5. Response surface plot showing the effect of extraction time (X_1) and ultrasonic power (X_3). X_2 (Temperature) = 40°C.

It is found in Figs. 3-5 that all of the three response surfaces are convex in shape, which indicates that the ranges of variables were chosen properly. As shown in Fig. 3, the yield of extraction increases when extraction time and temperature are increased. Based on the chosen model, the projected highest amount of phenolic compounds (6.69 mg g^{-1}) is achieved when samples are extracted at 44.61°C for 26.90 min. According to the applied model, a further increase of extraction time and temperature decreases the extraction yield.

Flavonoid extraction yield dependency on temperature and ultrasonic power is presented in Fig. 4. The highest extraction yield is achieved when apple samples are extracted at highest ultrasound power (480 W), at 44.61°C. Extraction time and ultrasound power influence on flavonoid extraction yield is presented in Fig. 5. The highest yield is achieved when the ultrasound power is maximal (480 W), and extraction time is 50 min.

3.2. Optimization of extraction parameters and validation of the model

Through these three-dimensional plots, the suitability of the model equation for predicting the optimal response values was tested using the selected optimal conditions. The results (Table 3) showed that the optimized conditions were ultrasonic temperature of 44.61°C, extraction time of 26.90 min, and ultrasonic power 480 W. Under these conditions, the predicted extraction yield of flavonoids was 6.69 mg g^{-1}. However, considering the operability in actual production, the optimal conditions can be modified as follows: temperature of 45°C, extraction time of 27 min, and ultrasonic power 480 W. Under the modified conditions, the experimental yield of flavonoids was 6.58 mg RE g^{-1} (n = 3), which was close to the predicted value. Extraction yield was 4.1 mg RE g^{-1} when extraction conditions were selected empirically (LIAUDANSKAS *et al.*, 2014). When extraction conditions were optimized by statistical modelling method the yield was 37.69% higher than the yield when extraction conditions were selected empirically.

3.3. Analysis of ethanol extracts of apple samples of different cultivars

The chemical composition in fruits of different apple cultivars may vary significantly (CEYMANN *et al*., 2012; ŁATA *et al*., 2005; WOJDYŁO *et al*., 2008), therefore it is very important to determine the qualitative and quantitative composition of individual phenolic compounds in apples that are grown under Lithuanian climatic conditions. Optimized extraction conditions were applied for the HPLC analysis of ethanol extracts of apple samples of six cultivars grown in Lithuania: Aldas, Auksis, Connel Red, Ligol, Lodel and Rajka using previously developed and validated HPLC method (LIAUDANSKAS *et al*., 2014). These phenolic compounds of various groups were identified and quantified in analysed extracts: procyanidin B1, (+)-catechin, chlorogenic acid, procyanidin B2, (−)-epicatechin, procyanidin C1, rutin, hyperoside, isoquercitrin, reynoutrin, avicularin, quercitrin and phloridzin (synonym phlorizin). Apple sample chromatogram (cultivar Lodel) is presented in Fig. 6. Total amount of phenolic compound in analysed apple sample extracts varied from 2.521 mg g^{-1} (cultivar Connel Red) to 6.430 mg g^{-1} (cultivar Aldas).

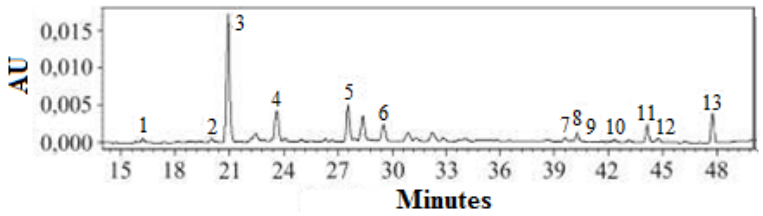

Figure 6. Chromatogram of the ethanol extract of apple sample (λ=280 nm, cultivar Lodel).
1 - Procyanidin B1, 2 - (+)-catechin, 3 - chlorogenic acid, 4 - procyanidin B2, 5 - (–)-epicatechin, 6 - procyanidin C1, 7 - rutin, 8 - hyperoside, 9 - isoquercitrin, 10 - reynoutrin, 11 - avicularin, 12 - quercitrin, 13 - phloridzin.

The highest total amount of quercetin glycoside 0.811 mg g^{-1} was determined in apple samples of cultivar Aldas. It was 1.93 times higher than the lowest total amount of quercetin glycoside (0.420 mg g^{-1}), determined in apple samples of cultivar Auksis. Quantitative composition of quercetin glycoside group compounds determined in apple samples is presented in Table 4.
Hyperoside was a predominant quercetin glycoside group compound in apple samples of cultivars Aldas, Auksis, Connel Red, Ligol and Lodel. It amounted to 25.14-35.16% of the total amount of identified and quantified quercetin glycoside group compounds. VAN DER SLUIS *et al*. indicated similar tendencies of a hyperoside quantitative composition variation. Hyperoside amounted to 23-33% of the total identified quercetin glycosides in samples of cultivars that these authors analysed (VAN DER SLUIS *et al*., 2001). The composition of apple samples of cultivar Rajka stood out among the analysed cultivars, as the predominant compound in this cultivar was quercitrin. Its amount was 1.66 times higher than the amount of hyperoside determined in the apple samples of this cultivar. Quercetin glycoside rutin-hyperoside-isoquercitrin triplet (where the predominant compound is always hyperoside, and levels of rutin are the lowest) was characteristic in apple sample extracts of all analysed cultivars. This pattern was also established in studies of other scientists (MARKS *et al*., 2007; PRICE *et al*., 1999; SCHIEBER *et al*., 2001). The ratio of rutin-hyperoside-isoquercitrin amount varies in the apple samples of different cultivars. It varied from 1:5.1:1.6 (cultivar Connel Red) to 1:11.9:1.2 (cultivar Lodel).

Monomeric ((+)-catechin and (–)-epicatechin) and oligomeric (procyanidin B1, procyanidin B2, and procyanidin C1) flavan-3-ols were determined in the apple samples. The highest total amount of identified and quantified flavan-3-ols (2.919 mg g^{-1}) was determined in apple samples of the cultivar Lodel. It was 2.55 times higher than the lowest amount (1.143 mg g^{-1}), determined in the apple samples of cultivar Connel Red.

In the scientific literature, it was noted that in apples, the amount of (–)-epicatechin is higher than (+)-catechin (WU *et al*., 2007; DUDA-CHODAK *et al*., 2010; KAHLE *et al*., 2005; PANZELLA *et al*., 2013). The results of our analysis also confirm this. The ratio of (+)-catechin and (–)-epicatechin in the samples of different cultivars varied from 1:4.3 (cultivar Rajka) to 1:21.9 (cultivar Lodel). In the paper by WOJDYŁO *et al*., it is specified that in samples of apples grown in Poland, the amount of (+)-catechin varies from 0.010 to 0.720 mg g^{-1}, and (–)-epicatechin – from 0.066 to 2.760 mg g^{-1} (WOJDYŁO *et al*., 2008)

The amounts of procyanidin B2 and C1 were higher than the amount of procyanidin B1 was in the samples of all analysed cultivars. This predisposition of the quantitative composition variation of these compounds in apples was also presented by other authors (DUDA-CHODAK *et al*., 2011). The ratio of procyanidin B1, B2 and C1 amounts in samples of different cultivars varied from 1:5.4:3.4 (cultivar Aldas) to 1:13.8:7.9 (cultivar Auksis). The quantitative composition of flavan-3-ol group compounds identified in apple samples is presented in Table 5.

Qualitative and quantitative analyses of dihydrochalcone group compounds are extremely important because the compounds of this group may be selected as chemotaxonomic indicators in apple cultivar taxonomy, for apple product identification and for the determination of apple juice and cider quality (SCHIEBER *et al*., 2001; ALONSO-SALCES *et al*., 2004; GOSCH *et al*., 2010). The amount of dihydrochalcone phloridzin in the apple samples of the analysed cultivars varied from 0.101 mg g^{-1} (cultivar Rajka) to 0.268 mg g^{-1} (cultivar Lodel) (Table 5). Similar results were presented by other authors as well-the amount of phloridzin in apple fruit comprises 2-6% of the total amount of quantified phenolic compounds (SANONER *et al*., 1999)

The highest amount of chlorogenic acid (3.074 mg g^{-1}) was determined in the apple samples of the cultivar Aldas. It was 4.99 times higher than the lowest determined amount of this acid (0.616 mg g^{-1}), determined in the apple samples of the cultivar Rajka (Table 6). WOJDYŁO *et al*. analysed the apple samples of apples grown in Poland and indicated similar amounts (0.015-2.960 mg g^{-1}) of this acid (WOJDYŁO *et al*., 2008).

Table 4. Quercetin glycoside quantitative variation in apple samples.

Substance	Quercetin glycoside amount (mg g^{-1}, dry raw material)						Coefficient of variation, %
	Aldas	Auksis	Connel Red	Ligol	Lodel	Rajka	
Hyperoside	0.274±0.005^{a}	0.138±0.002^{c,d}	0.152±0.003^{c}	0.129±0.002^{d}	0.154±0.003^{c}	0.184±0.004^{b}	31.10
Isoquercitrin	0.053±0.002^{a}	0.020±0.001^{c,d}	0.047±0.002^{a,b}	0.025±0.001^{c}	0.016±0.001^{d}	0.044±0.002^{b}	45.92
Rutin	0.036±0.001^{a}	0.015±0.001^{b,c}	0.030±0.002^{a}	0.022±0.001^{b}	0.013±0.001^{c}	0.035±0.002^{a}	39.69
Avicularin	0.226±0.005^{a}	0.120±0.002^{c}	0.099±0.001^{d,e}	0.087±0.001^{e}	0.114±0.002^{c,d}	0.139±0.003^{b}	38.15
Reynoutrin	0.080±0.003^{a}	0.053±0.002^{b}	0.039±0.001^{c}	0.037±0.001^{c}	0.043±0.001^{c}	0.025±0.001^{d}	40.92
Quercitrin	0.142±0.003^{b}	0.082±0.001^{e}	0.103±0.002^{c,d}	0.120±0.003^{c}	0.098±0.001^{d,e}	0.305±0.007^{a}	58.30

Different letters in the same row indicate statistically significant differences of individual substance amounts in apple samples of analysed cultivars (p<0.05).

Table 5. Flavan-3-ols, chlorogenic acid and phloridzin quantitative variation in apple samples.

Substance	Flavan-3-ols, chlorogenic acid and phloridzin amount (mg g^{-1}, dry raw material)						Coefficient of variation, %
	Aldas	Auksis	Connel Red	Ligol	Lodel	Rajka	
(+)-Catechin	0.092±0.002^{b}	0.077±0.002^{c}	0.034±0.001^{d}	0.033±0.001^{d}	0.042±0.001^{d}	0.121±0.003^{a}	54.26
(–)-Epicatechin	0.720±0.015^{b}	0.448±0.009^{d}	0.315±0.006^{e}	0.311±0.004^{e}	0.928±0.019^{a}	0.525±0.010^{c}	44.86
Procyanidin B1	0.157±0.003^{a}	0.170±0.004^{a}	0.035±0.001^{d}	0.064±0.001^{c}	0.094±0.002^{b}	0.097±0.002^{b}	50.84
Procyanidin B2	0.854±0.017^{c}	0.990±0.021^{b}	0.484±0.007^{e}	0.676±0.014^{d}	1.279±0.023^{a}	0.834±0.015^{c}	31.81
Procyanidin C1	0.534±0.020^{a}	0.366±0.012^{b}	0.275±0.008^{c}	0.258±0.011^{c}	0.576±0.021^{a}	0.249±0.007^{c}	38.56
Phloridzin	0.188±0.004^{b}	0.124±0.002^{d}	0.135±0.002^{d}	0.157±0.003^{c}	0.268±0.005^{a}	0.101±0.002^{e}	36.82
Chlorogenic acid	3.074±0.068^{a}	2.498±0.052^{b}	0.773±0.013^{e}	1.249±0.020^{d}	1.629±0.035^{c}	0.616±0.011^{e}	59.41

Different letters in the same row indicate statistically significant differences of individual substance amounts in apple samples of analysed cultivars (p<0.05).

The study results confirmed the hypothesis of CEYMANN *et al*., that apple cultivars can be classified based on what types of compounds phenolic acids or flavan-3-ols are predominant in the apple samples (CEYMANN *et al*., 2012). The predominant compound in the apple samples of the cultivars Aldas and Auksis was chlorogenic acid, and in the apple samples of other cultivars, it was flavan-3-ol group compounds. The coefficient of variation, which reflects the variation amplitude of every compound, was calculated to evaluate the variation of the quantitative composition of the phenolic compounds in the apple samples of different cultivars. It varied from 31.10% to 59.41% (Tables 5 and 6). The highest calculated coefficient of variation was for chlorogenic acid, and the lowest was for hyperoside.

4. CONCLUSIONS

In this study, we applied RSM for the extraction of flavonoids from lyophilised apples. The results showed that the independent variables (temperature, extraction time and ultrasonic power), and the quadratic terms of temperature and extraction time had a statistically significant effect on the efficacy of apple flavonoid extraction. A second-order (quadratic) polynomial model was employed to optimize flavonoid extraction from lyophilised apple samples. The projected optimal extraction conditions following statistical modelling were as follows: temperature 44.61ºC, extraction time 26.90 min and ultrasonic power 480 W. The experimental yield of flavonoids was 6.58 mg RE g^{-1}, which was close to the predicted yield value of flavonoids 6.69 mg g^{-1}. By applying RSM selected via statistical modelling, it was determined that the apple flavonoid extraction yield was 37.12% higher compared with sample extraction when extraction conditions were selected empirically. Optimised extraction conditions were applied for the HPLC analysis of the apple samples of six cultivars grown in Lithuania. The highest total amount of identified phenolic compounds (6.430 mg g^{-1}) was determined in the apple sample extracts of the cultivar 'Aldas'.

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