"Lucian Braga" University of Sibiu
Faculty of Agricultural Sciences, Food Industry and Environmental Protection

DOCTORAL DISSERTATION

SUMMARY

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Sibiu
2017

"Lucian Blaga" University of Sibiu
Faculty of Agricultural Sciences, Food Industry and Environmental Protection

The analysis of biological active compounds from some vegetal products and their usage for dietary supplement production

ACKNOWLEDGEMENT:
Research activities were supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/133675

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KEY WORDS: vegetal products, winemaking by-products, polyphenols, phenolic compounds, dietary supplements

FROM THE AUTHOR

This doctoral dissertation aims to offer information on biological active compounds with antioxidant properties from autochthonous vegetal products, thus turning to account by-products from the winemaking process through the development of a dietary supplement with antioxidant properties.

The research was developed from year 2013 to 2017, the results were synthesized in this dissertation which is 119 pages long, comprising 44 tables and 101 figures.

The dissertation is divided in two parts: the first part is based on the bibliographic study, where previous researches about biological active compounds found in autochthonous plants are highlighted along with their properties in preserving human health, and the second part, based on experimental researches, where the content in these kinds of compounds is determined from autochthonous plants and in by-products of the winemaking process and the development of a dietary supplement that has antioxidant properties.

The experimental research took place in the laboratories of the Faculty of Agricultural Sciences, Food Industry and Environmental Protection from the "Lucian Blaga" University of Sibiu and in the Physical-Chemical laboratory from the company Polisano Pharmaceuticals from Sibiu.

The thesis includes appendixes that contain chromatograms, tables and raw data, also references, the list of notations and symbols used, the list of figures and the list of tables that can be found in the text.

The development of this thesis wouldn't have been possible without the guidance and the support of the scientific coordinator, **Mr. Prof. Eng. Ovidiu Tița, Ph.D.,** to whom I would like to show my appreciation.

Also, I would like to show my appreciation for their help, to the guidance committee, comprised from: **Mrs. Prof. Pharm. Felicia Gligor, Ph.D., Mr. Lect. Eng. Ioan Mironescu, Ph.D.** and **Mr. Prof. Eng. Dan Chicea, Ph.D.,** which during the development of the thesis guided me to accomplishment.

I would like to show my appreciation for their support and advices to **Mrs. Prof. Pharm. Felicia Gligor, Ph.D** and to **Mrs. Lect. Eng. Cecilia Georgescu, Ph.D.** They advised me during the whole process, up to the finalization of the thesis.

I received a lot of help from **Mrs. Lect. Eng. Ecaterina Lengyel, Ph.D.** and from **Mrs. Eng. Diana Stegăruș, Ph.D.** to whom I wish to show my gratitude.

I would also like to thank **Mrs. Eng. Laurența Tiniș,** for helping me during my experimental research.

I would also like to show my appreciation to the staff from Polisano Pharmaceuticals, for allowing me to develop my research in their laboratory and for their advices.

AIMS AND SCIENTIFIC OBJECTIVES OF THE DOCTORAL DISSERTATION

This thesis aims to identify and quantify the biological active compounds that are found in vegetal products, to extract them and then to use them for obtaining dietary supplements. In order to achieve this, there are some objectives to fulfill:

1. The identification of the methods of analysis and control
2. Adjusting an efficient system for the extractions
3. The identification and quantification of the extracted compounds
4. The identification of the flow sheet for using the isolated biological active compounds in dietary supplements
5. The analysis of the dietary supplements obtained

Chapter I - The analysis of the international state of the biological active compounds extracted from vegetal products

The chemicals extracted from medicinal plants can be classified in primary and secondary metabolites (Yanez, et al. 2013).

The primary metabolites are widely spread in nature and they are used by the plants for their physiological development. The secondary metabolites are derived from the primary metabolites and their distribution within the plants is limited. Usually, they have an ecological purpose. For example, they have a role in the defense mechanism of the plant, being adaptative compounds for the plant against stress factors, they are synthesized by specialized cells, in different stages of the plants growth, in case of diseases or they can be induced by light (Yanez, et al. 2013).

The extracted polyphenols have a great structural diversity. Their structure varies between simple molecules, like phenolic acids, to more complex molecules, with a high degree of polymerization, like tanins (Baruah 2011).

They are divided in compound subclasses, based on their chemical structure. We can nominate: phenolic acids, flavonoids, lignans and stilbens (Xu 2012).

In order to increase the quantity of the chemical compounds able to defend the plant against infection, the plant can be exposed to the fungus *Botrytis cinerea* (Pour Nikfardjam, Laszlo and Dietrich 2006).

Antioxidants, as dietary constituents, can be considered factors in health preservation, by lowering the risks of some chronic diseases, like cardiovascular diseases or cancer (Mehta 2015).

Chapter II - Presentation of the vegetal raw materials that make the study's objective

The antioxidant compounds can be found in various plant species. Some of them were studied thoroughly and their applicability was determined.

Raspberries (*Rubus idaeus* L.) can be consumed raw or in the form of juice, sauce, marmalade, jelly, liquor, or pigments (Pârvu 2006).

Bilberries (*Vaccinium myrtillus* L.), can be consumed raw or in the form of syrup, marmalade, juice, alcoholic beverages (Pârvu 2006).

Red currant (*Ribes rubrum* L.), can be consumed raw or in the form of syrup, jelly, marmalade, juice or sauce (Pârvu 2006).

Industrially, grapes (*Vitis vinifera* L.) are the raw material in the winemaking process, during which different kinds of wines are obtained, distillates, musts, juices or concentrates. Grapes are used in can factories, for the manufacturing of marmalades, sauces or raisins (Pârvu 2006).

In order for this fruit to be exploited, the methods of harvesting, preservation, and storage have to be known for the retaining of the fruit properties as long as possible.

During the winemaking process, in addition to the main products, there are obtained several by-products that represent 25% from the quantity of the processed grapes. Their exploitation is of interest for researchers because of the manufacturing of products that are useful in different areas of the industry. Using the by-products of the winemaking process there can be extracted biological active compounds (enzymes, vitamins, polyphenols) and the pollution of the environment can be avoided (Tița 2002).

Chapter III - The importance of dietary supplements in preserving human health

"Food means any substance or product, no matter if it is processed or not, destined for human consumption, except tobacco, medicinal products, narcotics, psychotropic substances which are controlled by the Member States and are subjected to international conditions" (Banu 2010).

Dietary supplements are defined as dietary products designed to supply the diet, and they are composed of highly concentrated nutrient sources or other substances with nutritional and physiological effects, singularly or combined, commercialized as doses, in different forms, such as capsules, troches, pills or other similar forms, powder cachets, vials containing liquids, dripping flasks and other similar forms of liquids and powders intended to be used in small quantities, as individual doses (Directive 2002/46/CE 2002).

Studies show that a regular consumption of fruit and vegetables can reassure the protection against the development of several chronic diseases, such as: cancer, diabetes, neurodegenerative diseases or cardiovascular diseases (Pandey and Rizvi 2011).

Polyphenols are plant active metabolites that have an important role in maintaining a balanced diet. The consumption of polyphenols is in inverse proportion to the development of several chronic diseases (Pandey and Rizvi 2011).

Chapter IV - Vegetal products processing for analysis

The samples used were: bilberries (*Vaccinium myrtillus* L.), raspberries (*Rubus idaeus* L.), red currant (*Ribes rubrum* L.), harvested from Sibiu County and fermented pomace, Cabernet Sauvignon variety and Feteasca Neagra variety (*Vitis vinifera* L.), grapes harvested from Alba County.

The grapes were harvested in July, when the fruit reached maturity (Fig. 25), and they were preserved by freezing, followed by drying or by drying and keeping at room temperature up to the moment of the analysis.

The conclusion drawn through the comparison of these methods of preservation concerns the stability of the fruit during freezing.

In regard of the fermented pomace, after the processing of the grapes in the winemaking process, the pomace was dried, grinded, frozen and kept like this for 6 months until the analysis.

Chapter V - Extraction methods

The extraction methods detailed in this study are methods used for the extraction of several phenolic compounds from vegetal products. These were adapted in order not to inactivate the heat

sensitive compounds and to streamline the extraction process by acquiring a better yield using as fewer material resources as possible and time as well.

For the sample execution 4 types of extractions were used. Extraction number 1 was an extraction that used a separation funnel and organic solvents and extractions 2, 3 and 4 were executed using ultrasounds and temperature. The solvents used for these extractions were different.

Chapter VI - Analysis of the obtained extractions

Analysis methods

Spectrophotometrical quantification of total polyphenols

The quantification of the polyphenols was accomplished through the Folin-Ciocalteu method adapted after the Europeean Pharmacopeae, edition 8.0.

The results obtained by the spectrophotometric determination of the samples extinctions were extrapolated from a calibration curve obtained by determining the extinctions of increasing concentrations of gallic acid.

Identification and quantification of phenolic compounds using an HPLC personal method

The method used was adapted, after the method of determination and quantification of resveratrol, rutin and quercetin from grape skins of Iacopini and associates (Iacopini, et al. 2008), for the identification and quantification of 10 compounds of interest.

Results and discussion

The analysis of the extracts obtained from fruit preserved by freezing

The maximum concentration of total polyphenols expressed as mg gallic acid/g dry weight (d.w.) that was determined for all types of samples was extraction number 4, followed by extraction number 3, then extraction number 2 and the lowest concentration of total polyphenols was determined using extraction number 1 (Fig. 1).

The concentrations of total polyphenols, even though they are different, they follow a pattern regarding the extraction methods. Thus it is noted that the total polyphenols extraction is proportional no matter the analyzed vegetal product.

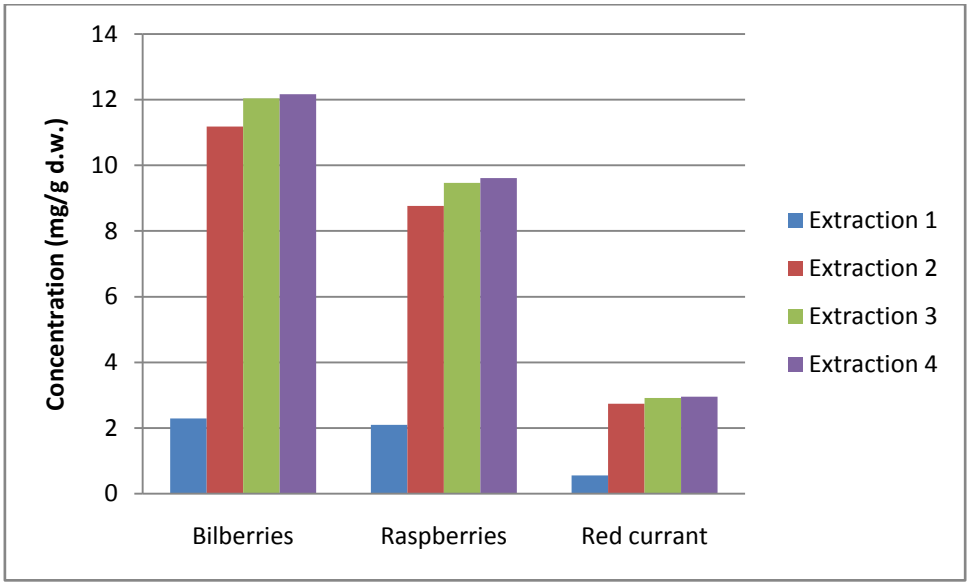

Figure 1. The concentration of total polyphenols of fruit preserved by freezing

Bilberries have the greatest quantity of total polyphenols, followed by raspberries and by red currant regardless of the extraction performed. Thus 2.29 mg total polyphenols (expressed in gallic acid)/g d.w. were determined for bilberries using extraction 1 and 12.16 mg total polyphenols (expressed in gallic acid)/g d.w. using extraction 4. These quantities were the minimum and the maximum quantity of mg total polyphenols (expressed in gallic acid)/g d.w. determined for bilberries. For raspberries, the minimum quantity determined was 2.09 mg total polyphenols (expressed in gallic acid)/g d.w. and the maximum quantity was 9.61 mg total polyphenols (expressed in gallic acid)/g d.w. These values were determined for extraction 1 and 4. For red currant, the minimum and maximum quantities of total polyphenols were determined for the same extraction types like the other two berries. The minimum quantity was 0.56 mg total polyphenols (expressed in gallic acid)/g d.w. and the maximum 2.95 mg total polyphenols (expressed in gallic acid)/g d.w. (Fig. 1).

Identification and quantification of phenolic compounds using an HPLC personal method

For most of the phenolic compounds analyzed the maximum quantity was determined using extraction 4. The rest of them presented the maximum quantity by using extraction 1. For the compounds extracted best using the extraction 4 a tendency of increasing the quantity of phenolic compound was observed from the extraction 1 to extraction 4, and for the ones best extracted by using extraction 1 a tendency of decreasing the quantity in the same order of extractions (Fig. 2).

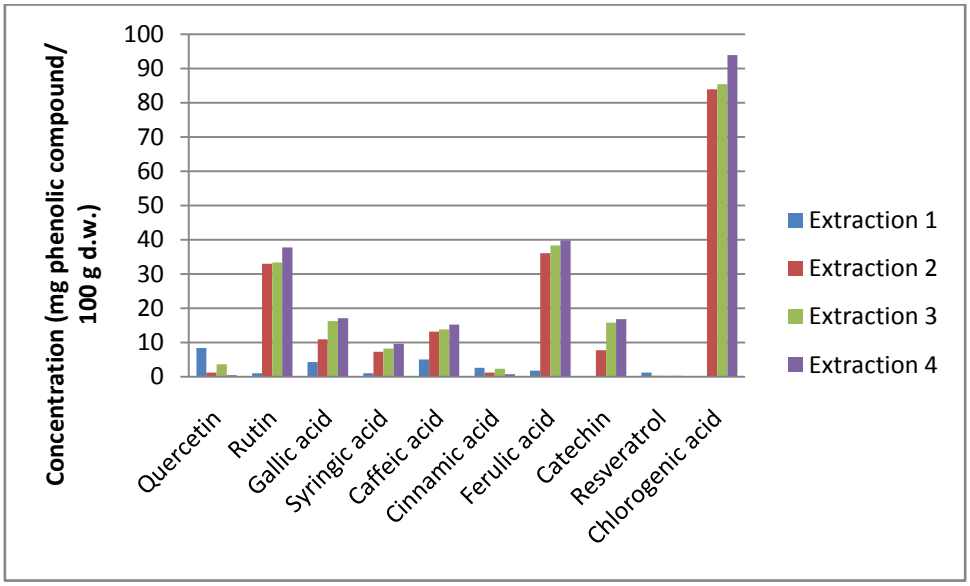

Figure 2. The concentration of the phenolic compounds from bilberry samples preserved by freezing

Between the phenolic compounds analyzed, the greatest quantity was determined for chlorogenic acid: 93.96 mg/100g d.w., followed by ferulic acid and rutin that presented quantities of 39.79 mg/100g d.w. and 37.74 mg/100g d.w. quantities between 10 and 20 mg/100g d.w. were determined for gallic acid, (+)-catechin and caffeic acid, the syringic acid presented a quantity of 9.66 mg/100g d.w. These amounts were obtained using extraction 4. Using this type of extraction, the highest quantity of these phenolic compounds was determined. The quercetin, the cinnamic acid

and the resveratrol presented sub unitary quantities by using extraction 4 and quantities of 8.45 mg/100g d.w., 2.62 mg/100g d.w. and 1.21 mg/100g d.w. using extraction 1. This way, the best extractions for every phenolic compound analyzed was determined for bilberries (Fig. 2).

The behavior of the phenolic compounds found in raspberries, according to the extraction methods is similar to the phenolic compounds found in bilberries, thus the amounts specified will be the maximum ones according to the identified extraction method.

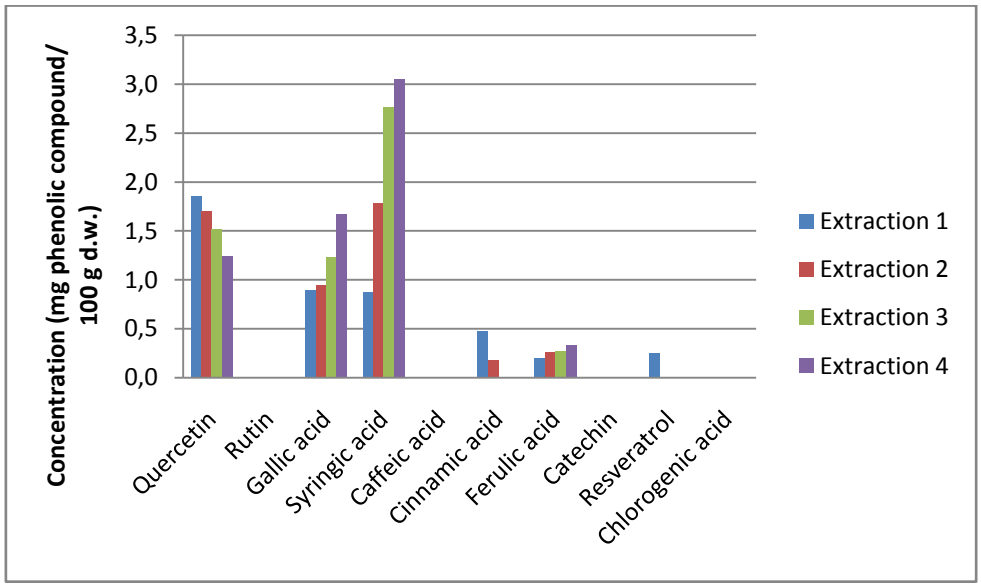

Figure 3. The concentration of the phenolic compounds from raspberry samples preserved by freezing

This way, from the analyzed phenolic compounds, the quantity of syringic acid was proven to be the greatest, followed by gallic acid and quercetin. Sub unitary quantities were determined for cinnamic acid, resveratrol and ferulic acid. Rutin, caffeic acid, (+)- catechin and chlorogenic acid were not found in none of the raspberry samples, no matter the extraction method. This data was obtained by using extraction 1 for quercetin, cinnamic acid and resveratrol and extraction 4 for the rest of the phenolic compounds.

It was determined that resveratrol and cinnamic acid were not detected for extractions 3 and 4, the resveratrol was not detected for extraction 2 either. Also, the tendency of rising of the quantities of gallic acid, syringic acid and ferulic acid from extraction 1 to extraction 4 was determined. The tendency of quercetin and cinnamic acid being the other way around, thus the largest quantity of phenolic compound was found using extraction 1, followed by extraction 2, then extraction 3 and the smallest quantity was determined using extraction 4.

For red currant, the largest quantity of phenolic compound was determined for (+)-catechin, followed by syringic acid. Their amounts were 43.37 mg/100 g d.w. and 32.66 mg/100 g d.w. For the rest of the compounds analyzed, the amounts were under 10 mg/100 g d.w. as followes: 9.61 mg/100 g d.w. for rutin, 3.99 mg/100 g d.w. for chlorogenic acid and 1.20 mg/100 g d.w. for ferulic acid. Quercetin, cinnamic acid and resveratrol had sub unitary amounts and gallic acid and caffeic were not detected (Fig. 3).

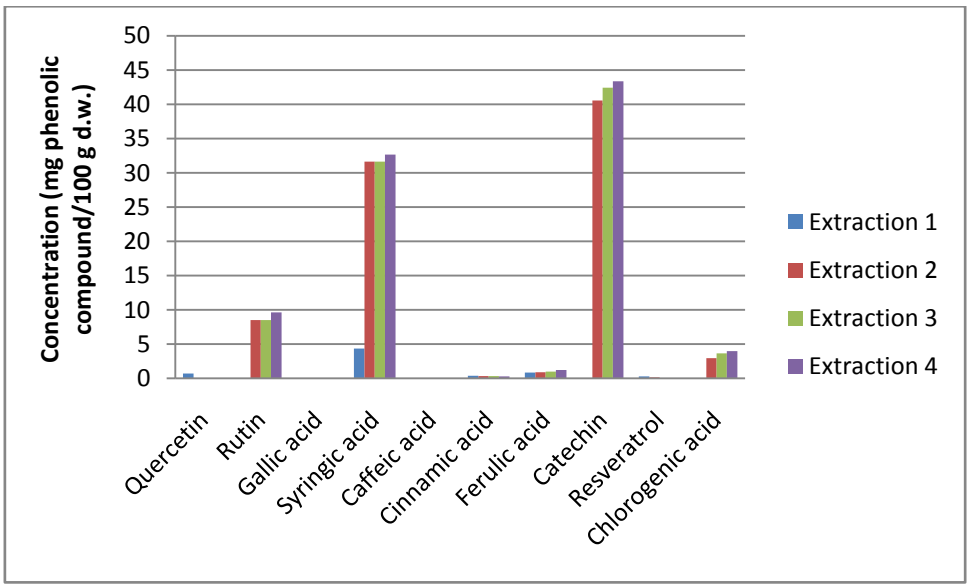

Figure 4. The concentration of the phenolic compounds from red currant samples preserved by freezing

Regarding the best fitted extractions for the analysis of phenolic compounds, there were identified extraction 1 and 4, these being the extractions that provided the largest quantity of different phenolic compounds. Extraction 1 was identified as best for extracting quercetin, cinnamic acid and resveratrol. It can be observed the fact that the amount of these compounds decreases form extraction 1 to extraction 4. The rest of the phenolic compounds analyzed were best extracted using extraction 4. It can be observed the fact that the amount of these compounds decreases from extraction 4 to extraction 1 (Fig. 4).

Bilberries have the greatest quantity of phenolic compounds analyzed, followed by red currant and then, raspberries (Fig. 5).

The results used were the ones obtained from extraction 1 for quercetin, cinnamic acid and resveratrol and from extraction 4 for rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)-catechin and chlorogenic acid, these being the largest amounts of phenolic compound determined for each compound.

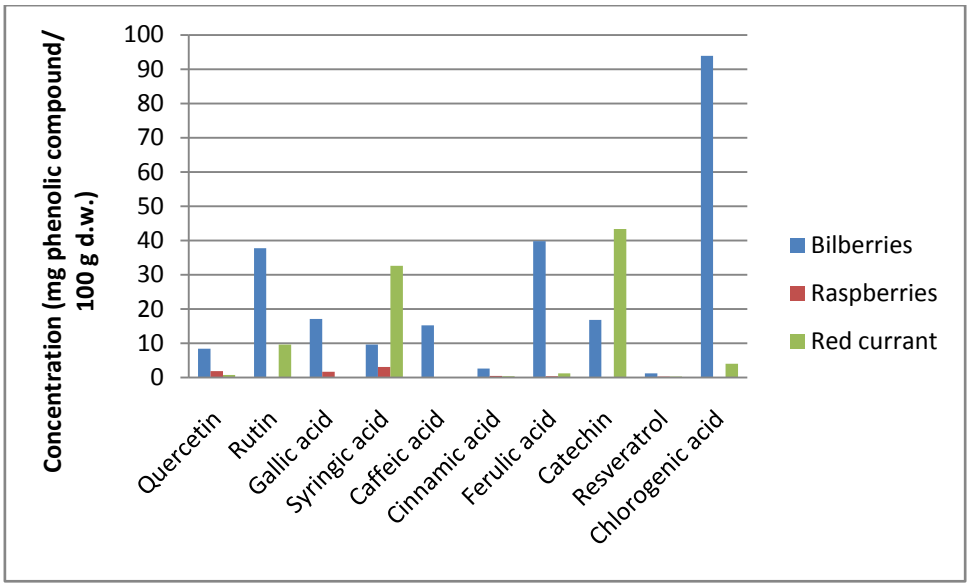

Figure 5. The concentration of the phenolic compounds from fruit samples preserved by freezing

The largest quantity of quercetin, rutin, gallic acid, caffeic acid, cinnamic acid, ferulic acid, resveratrol and chlorogenic acid were determined in bilberry samples and the largest quantity of syringic acid and (+)- catechin were determined in red currant (Fig. 5).

Using raspberry samples there were identified the compound mentioned above, minus rutin, caffeic acid, (+)- catechin and chlorogenic acid. In red currant extracts there were not determined gallic acid and caffeic acid (Fig. 5).

Conclusions

Between the three analyzed berries, the largest quantity of total polyphenols and phenolic compounds analyzed was determined in bilberries. Concerning the quantity of total polyphenols raspberry extracts come next and then red currant, even though the quantity of phenolic compounds is grater in red currant than in raspberries.

The greatest amount of total polyphenols was extracted using extraction 4, followed by extraction 3, then extraction 2 and the lowest quantity was extracted using extraction 1, this one being the most selective.

It has been shown that quercetin, cinnamic acid and resveratrol were best extracted using extraction 1 and rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)- catechin and chlorogenic acid were best extracted using extraction 4.

The analysis of the extracts obtained from fruit preserved by drying

Spectrophotometrical quantification of total polyphenols

Using the data from figure 54, there can be observed that bilberries have the greatest quantity of extracted total polyphenols, followed by raspberries and then red currant. Bilberries have an amount of 2.34 mg total polyphenols (expressed in gallic acid)/g d.w. determined using extraction 1 and 12.44 mg total polyphenols (expressed in gallic acid)/g d.w. using extraction 4. Raspberries have a minimum quantity of 2.03 mg total polyphenols (expressed in gallic acid)/g d.w. and a maximum quantity of 9.60 mg total polyphenols (expressed in gallic acid)/g d.w., and for red currant there was determined a minimum of 0.60 mg total polyphenols (expressed in gallic acid)/g d.w. and a maximum of 2.99 mg total polyphenols (expressed in gallic acid)/g d.w. The minimum quantities were determined using extraction 1 and the maximum ones using extraction 4 (Fig. 6).

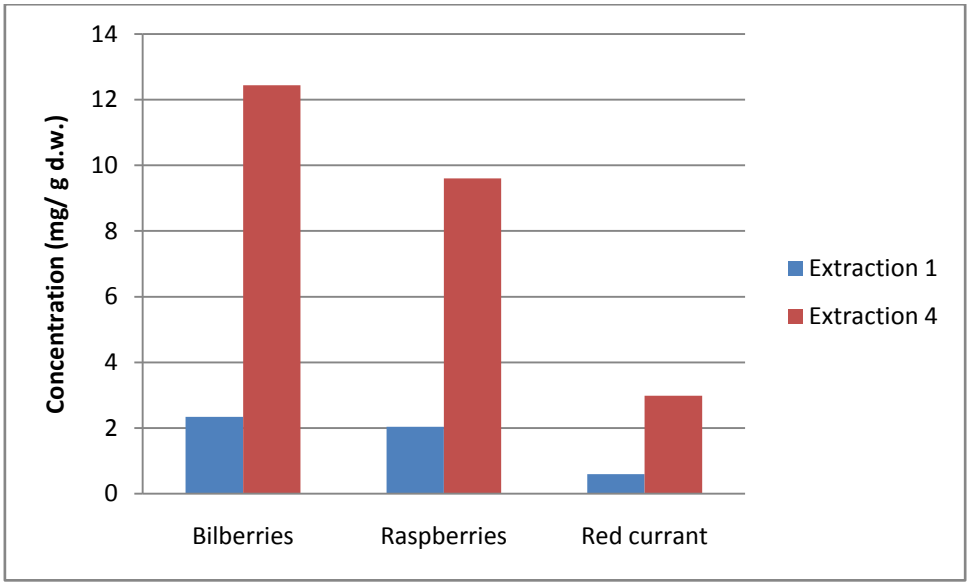

Figure 6. The concentration of total polyphenols of fruit preserved by drying

Identification and quantification of phenolic compounds using an HPLC personal method

The chlorogenic acid is the phenolic compound determined from bilberries that has the largest quantity, 94.00 mg/100 g d.w., followed by ferulic acid with 38.80 mg/100 g d.w. and then rutin with 34.43 mg/100 g d.w. For gallic acid, (+)- catechin and caffeic acid there were determined quantities between 10 and 20 mg/100 g d.w. and quantities lower than 10 mg/100 g d.w. were determined for syringic acid, quercetin, cinnamic acid and resveratrol (Fig. 7).

The amounts expressed above are obtained by using extraction 1 for quercetin, cinnamic acid and resveratrol and extraction 4 for the rest of the phenolic compounds analyzed.

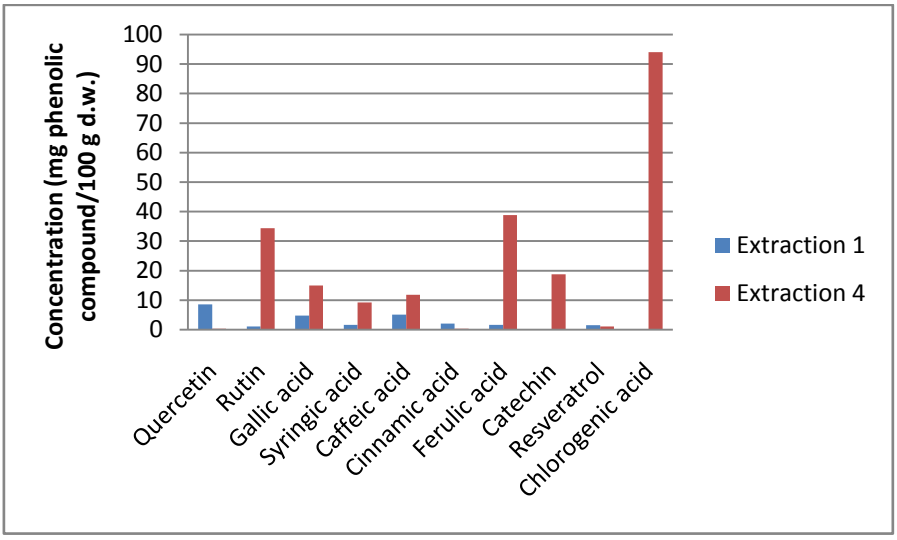

Figure 7. The concentration of the phenolic compounds from bilberry samples preserved by drying

For raspberries, it was observed that syringic acid was the phenolic compound that had the greatest amount, followed by quercetin and gallic acid. For the other compounds there were found sub unitary quantities, rutin, caffeic acid, chlorogenic acid and (+)- catechin being excepted because they were not detected during these analysis (Fig. 8).

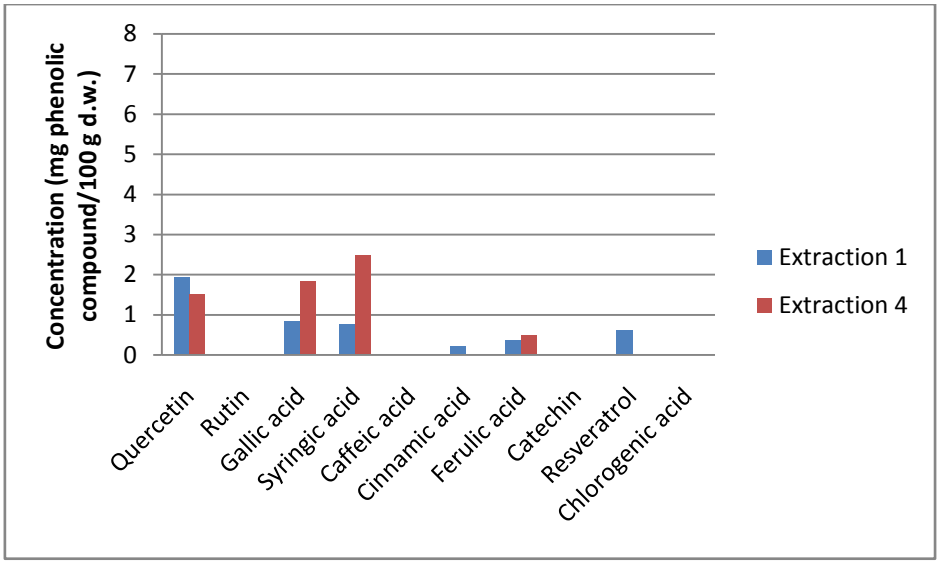

Figure 8. The concentration of the phenolic compounds from raspberry samples preserved by drying

The phenolic compound that was found having the greatest amount in raspberry samples was (+)-catechin, which presented an amount equal to 40.38 mg/100 g d.w., followed by syringic acid, with a quantity of 31.57 mg/100 g d.w., then, by rutin, with a quantity of 10.08 mg/100 g d.w. and quercetin, cinnamic acid and resveratrol with sub unitary values. Gallic acid and caffeic acid were not detected (Fig. 8).

By comparing the quantities of phenolic compounds determined from the analyzed berries (Fig. 9), dried and grinded soon after harvesting, the largest quantity was attributed to bilberries, followed by red currant and then raspberries.

The used data is attributed to the types of extractions that provide the largest amount of each phenolic compound analyzed.

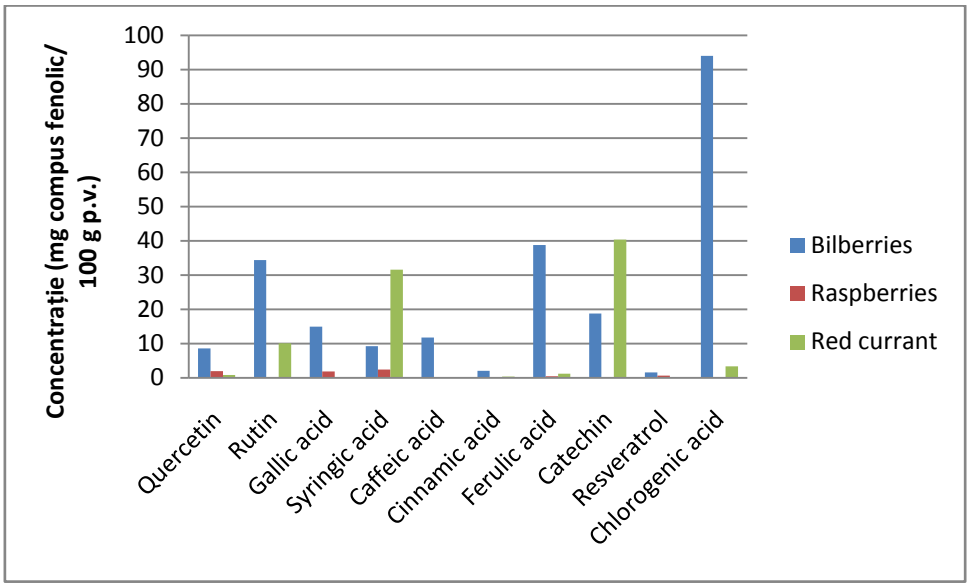

Figure 9. The concentration of the phenolic compounds from fruit samples preserved by drying

Quercetin, rutin, gallic acid, caffeic acid, cinnamic acid, ferulic acid, resveratrol and chlorogenic acid were the phenolic compounds found to have the greatest amount in bilberry samples. Rutin, caffeic acid and chlorogenic acid were not identified in raspberry samples and gallic acid and caffeic acid in red currant.

Syringic acid and (+)-catechin were determined to have the greatest amount in red currant samples and (+)-catechin could not be found in raspberry samples.

Conclusions

Among the three analyzed berries, the quantity of total polyphenols was the greatest in bilberries, followed by raspberries and the by red currant. The total of phenolic compounds analyzed determined was the greatest in bilberries, followed by raspberries and then red currant. The total of phenolic compounds analyzed was determined as being the greatest in bilberries, followed by red currant and then raspberries. So, the phenolic compounds analyzed, determined from red currant have a greater part of the total polyphenols than in raspberries or bilberries, and raspberries posses the lowest part from the three berries analyzed.

The extractions used were extraction 1 and 4. The larger quantity of total polyphenols was extracted using extraction 4, extraction 1 being considered to have a higher degree of selectivity. Regarding the phenolic compounds analyzed, quercetin, cinnamic acid and resveratrol had the greatest quantity by using extraction 1 and rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)-catechin and chlorogenic acid by using extraction 4. These extractions are the best ones for the quantification of the mentioned phenolic compounds.

Comparison of the fruit preservation methods

Spectrophotometrical quantification of total polyphenols

The comparison was made using the quantities of total polyphenols determined for preservation by using freezing and then drying and by drying alone immediately after harvesting depending on the type of extraction.

The data presented in figure 10 reveals that in case of bilberries, the quantity of total polyphenols is larger in case of preservation by drying alone immediately after harvesting than by freezing and then drying. The differences between the two types of preservations did not exceed 0.28 mg/ g d.w. In the case of raspberries it can be observed a slight increasing of the quantity of total polyphenols for both of the extractions of the fruit that were frozen and then dried compared to the ones dried immediately after harvesting. The differences observed in the case of bilberries were lower than in the case of raspberries, the value did not exceed 0.06 mg/ g d.w. For the red currant the differences were lower than presented for raspberries or bilberries, they were 0.04 mg/ g d.w., the preservation by drying immediately after harvesting presented superior values than the one by freezing followed by drying, in the case of both of the extractions.

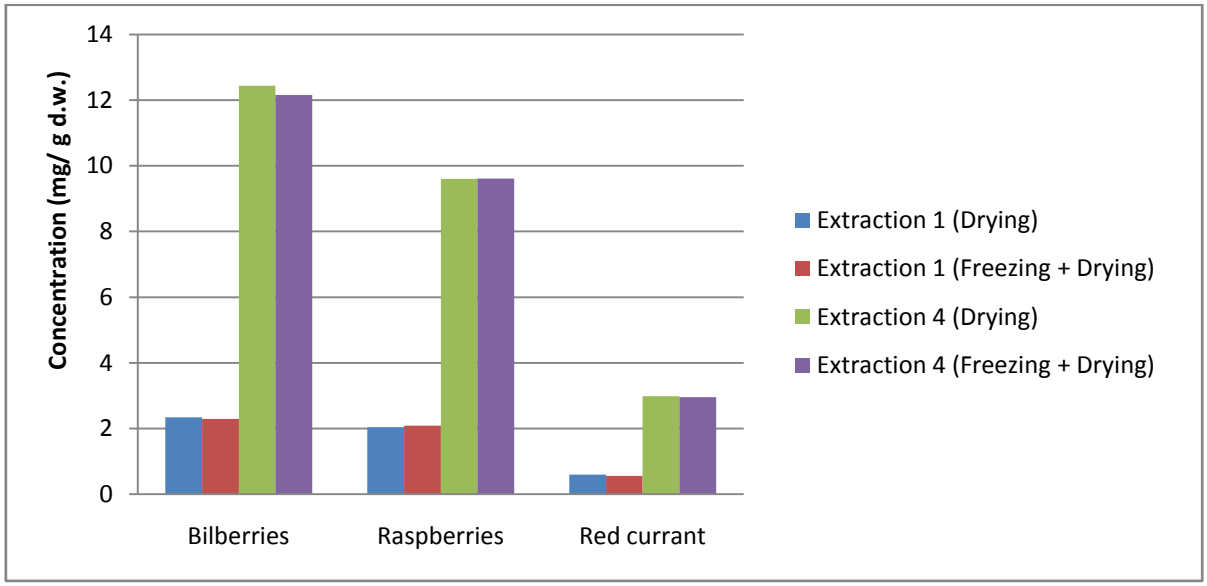

Figure 10. The concentration of total polyphenols of fruit depending of the type of extraction and the method of preservation

Identification and quantification of phenolic compounds using an HPLC personal method

The data used for the comparison were the ones identified as the best concerning specific phenolic compounds, and are detailed above.

It can be observed that the variations of the quantities of the phenolic compounds analyzed, concerning both of the best extractions used were minor for all of the analyzed berries (Fig. 11-13).

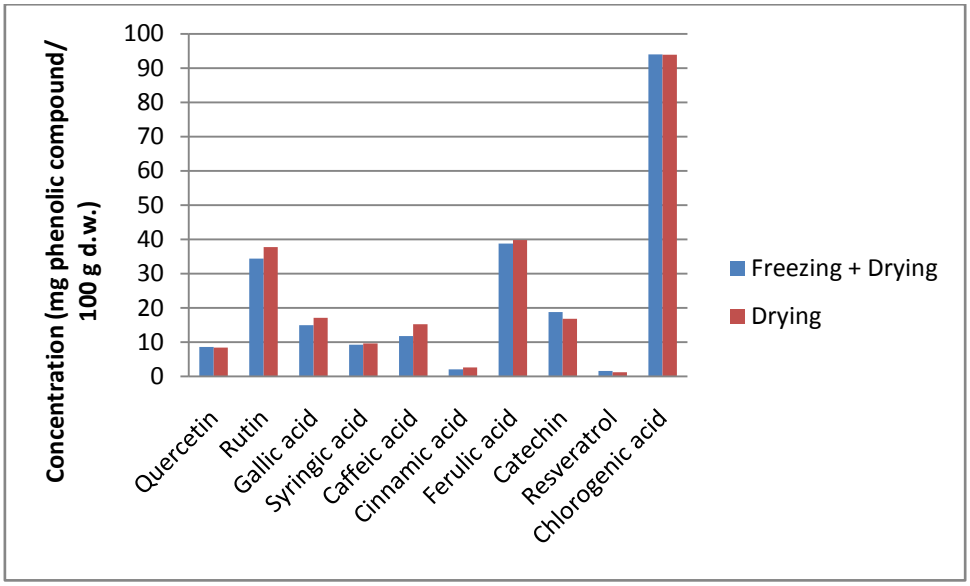

Figure 11. The concentration of the phenolic compounds in bilberries regarding the method of preservation

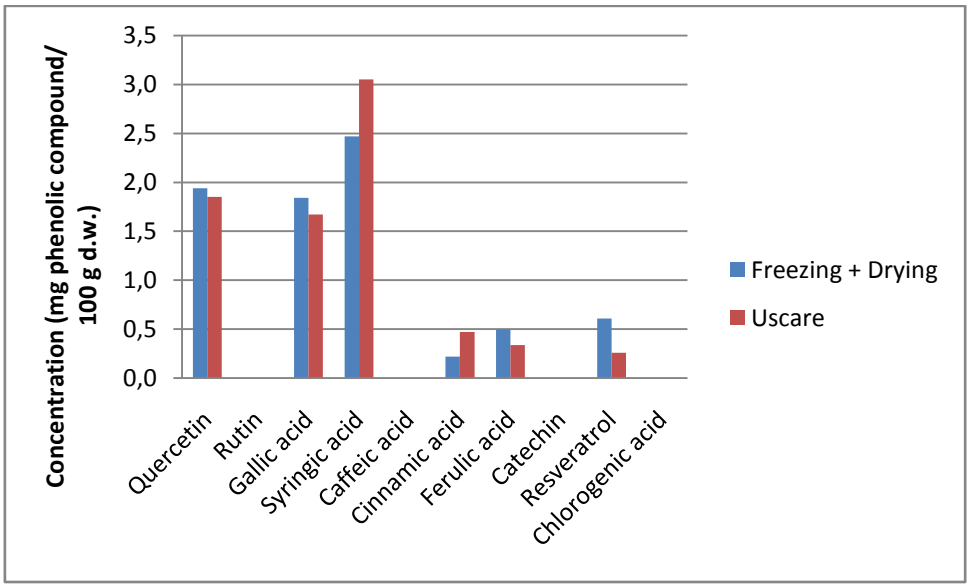

Figure 12. The concentration of the phenolic compounds in raspberries regarding the method of preservation

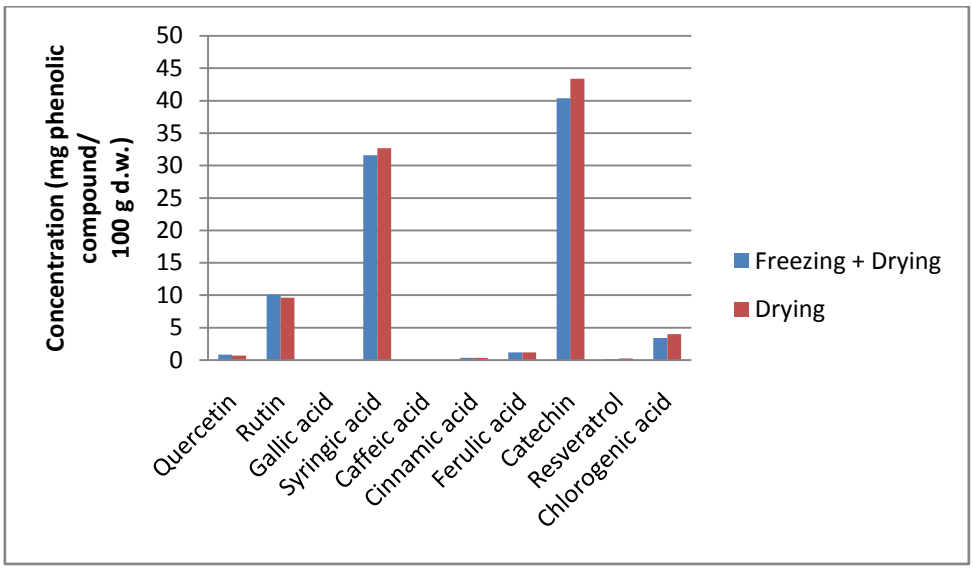

Figure 13. The concentration of the phenolic compounds in red currant regarding the method of preservation

Conclusions

The analysis was performed regarding the total polyphenols using extraction 1 and 4 in order for the similarity to be revealed between the type of extraction and between the methods of preservation. The data used for the comparison of the two methods of preservation regarding the phenolic compounds analyzed were obtained using the extractions determined to be the best ones in the quantification of specific phenolic compounds.

Regarding the quantity of total polyphenols, although a correspondence between the type of extraction and the method of preservation was not found, it can be concluded that bilberries, raspberries and red currant have a high stability when frozen. This conclusion was based on the low differences between the quantities of total polyphenols found using the two preservation methods.

A correspondence between the phenolic compound analyzed and the preservation method was not found regarding the identification and quantification of the phenolic compounds. Also, the small differences between the preservation methods determined for each compound separately lead us to believe that in terms of the phenolic compounds determined, the analyzed berries are stable if frozen.

Analysis of the winemaking by-products extracts

Spectrophotometrical quantification of total polyphenols

The maximum quantity of total polyphenols (expressed in mg gallic acid)/ g d.w. that was found in the analyzed samples was obtained for extraction 4, followed by extraction 3, then by extraction 2 and the minimum quantity was found using extraction 1 (Fig. 14).

Both varieties of pomace had between 3 and 5 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extraction 1 and between 6 and 8 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extractions 2, 3 and 4.

The variety Cabernet Sauvignon had 4.10 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extraction 1 and 7.99 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extraction 4. The variety Feteasca Neagra had 3.85 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extraction 1 and 6.95 mg total polyphenols (expressed in mg gallic acid)/ g d.w. by using extraction 4 (Fig. 14).

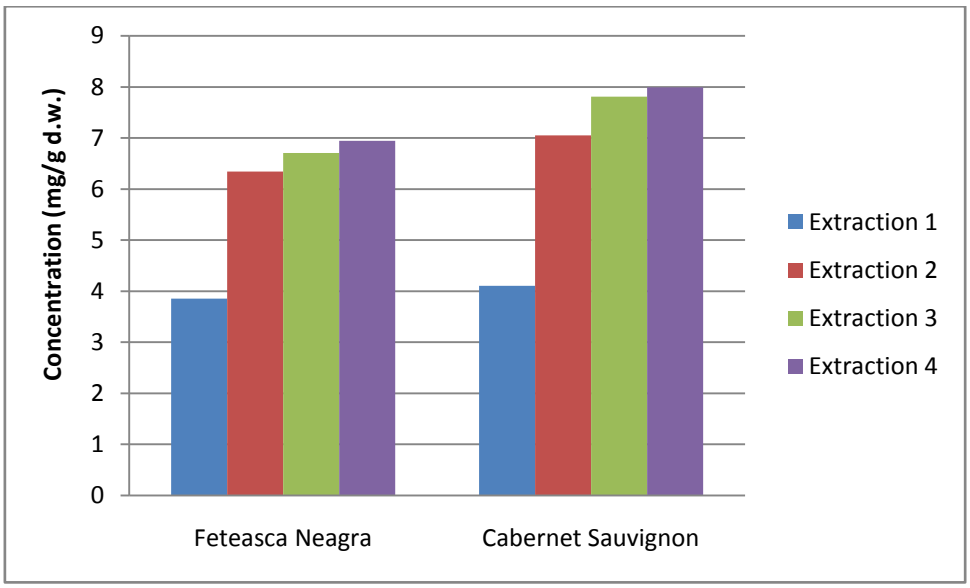

Figure 14. The concentration of total polyphenols of fermented, red pomace

Identification and quantification of phenolic compounds using an HPLC personal method

The highest quantity of rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)-catechin, and chlorogenic acid was extracted using extraction 4. It can be observed that the quantity of these compounds is decreasing from extraction 4 to extraction 1, extraction 4 being the one that delivered the highest quantity and extraction 1, the lowest quantity (Fig. 15).

Quercetin, cinnamic acid and resveratrol are the compounds that had the highest quantity by using extraction 1. For these compounds, the quantity is decreasing from extraction 1 to extraction 4.

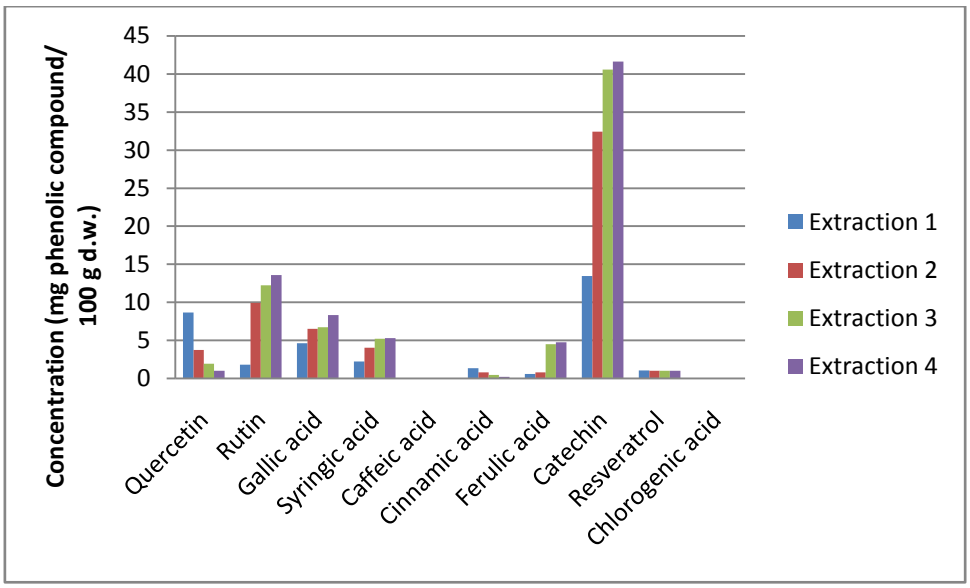

Figure 15. The concentration of phenolic compounds from fermented, red pomace, Cabernet Sauvignon variety

The fermented pomace, Cabernet Sauvignon variety had 41.63 mg/ 100 g d.w. (+)-catechin and 13.57 mg/ 100 g d.w. rutin. Quercetin, gallic acid, syringic acid, ferulic acid, cinnamic acid and

resveratrol had quantities between 1 and 10 mg/ 100 g d.w. Caffeic acid and chlorogenic acid were not identified in this vegetal product. These values were determined by using the suitable extraction for each of the compounds.

The behavior of the phenolic compounds extracted from red, fermented pomace, variety Feteasca Neagra, regarding the extraction type, is similar to the one of the red, fermented pomace, Cabernet Sauvignon variety, this way, the mentioned quantities of phenolic compounds are the ones obtained regarding the type of extraction identified to be the best.

(+)- Catechin is the phenolic compound that had the largest quantity in the red, fermented pomace, Feteasca Neagra variety. It had 49.96 mg/ 100 g d.w. and the gallic acid 11.09 mg/ 100 g d.w. Syringic acid, rutin, quercetin, cinnamic acid, ferulic acid and resveratrol had quantities below 10 mg/ 100 g d.w. Caffeic acid and chlorogenic acid could not be identified (Fig. 16).

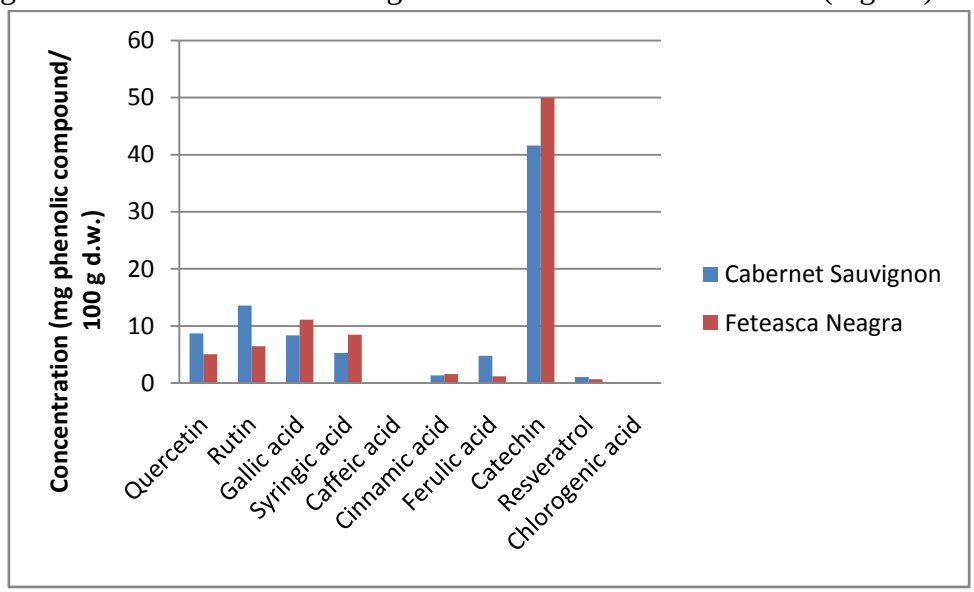

Figure 16. The concentration of phenolic compounds from red, fermented pomace

Using figure 16, it can be observed that both varieties of red, fermented pomace do not have caffeic acid or chlorogenic acid. Quercetin, rutin, ferulic acid and resveratrol had higher values in the pomace of Cabernet Sauvignon and gallic acid, syringic acid, cinnamic acid and (+)-catechin in the pomace of Feteasca Neagra.

Conclusions

Among the two analyzed varieties of pomace, the highest quantity of total polyphenols and total phenolic compounds analyzed was determined in the Cabernet Sauvignon variety even though the quantities were not far apart.

The proposed extraction methods were used to determine the one that is the most appropriate for the extraction of the total polyphenols respectively the phenolic compounds of interest from the red, fermented pomace. This way, extraction 4 was determined to be appropriate for extracting the total polyphenols from the red, fermented pomace as well as rutin, gallic acid, syringic acid, ferulic acid and (+)- catechin. A decrease in quantity of these compounds was observed from extraction 4 to extraction 1, which had the lowest quantity of these compounds.

Regarding quercetin, cinnamic acid and resveratrol, these compounds had the highest quantity when extracted with extraction 1, the decrease in quantity being observed towards extraction 4.

Caffeic acid and chlorogenic acid were not determined during the analysis of red, fermented pomace.

Chapter VII - Description and selection of the best alternative in obtaining a dietary supplement with phenolic compounds

In order to obtain a dietary supplement that could satisfy the needs and demands of consumers a study has been developed. The public opinion regarding the definition of the dietary supplements, the way of administration preferred, the beneficial effects on the human body and the obtained knowledge about the antioxidant effects and the products that may exert this effect were enquired (Frum 2015).

The study was developed using a questionnaire conducted on 144 persons from different social media, and the result interpretation was conducted using the IBM SPSS Statistics v.20 program (Frum 2015).

According to this study, the preferred administration form was determined. This coincides with the form in which the dietary supplement would be. So, it is going to be conditioned in capsules in order to increase the compliance.

The best way to prepare the raw material was determined using the analysis on each product individually, for the 3 types of berries and the 2 types of red, fermented pomace. The chosen vegetal products were bilberries, red currant and both types of pomace in equal proportions, in order to obtain the largest variety of phenolic compounds and a large quantity of total polyphenols. Even though the raspberries had a larger quantity of total polyphenols than the red currant, it does not have a large variety of phenolic compounds.

Chapter VIII - Development of dietary supplement

Raw material acquisition

The raw material was obtained by mixing dried and grinded vegetal products. The preservation of the vegetal products, until obtaining the raw material, was accomplished by drying in air flow at 40º C for bilberries and red currant and for the red, fermented pomace, drying at 20º C followed by grinding.

The mixing was accomplished by vigorous stirring for 10 minutes. The obtained powder can be described as a red-brownish powder, with specific odor.

The raw material obtained was subjected to several analysis in order to determine if it is suitable for encapsulation.

Raw material analysis

Particle size distribution

This analysis was accomplished using different sized sieves. This being one of the most used methods that may classify powders by the particle size distribution.

Table 1. Results of the particle size distribution

Sieve (μm)	Weight (g)	Weight (%)
710	4.25	4.27
224	94.93	95.36
125	0.37	0.37
90	0.00	0.00
63	0.00	0.00

Using the obtained data it can be concluded that the raw material obtained is a coarse powder, due to the fact that 95.36% of the added mass passed through the 710 μm sieve and 0.37% through the 224 μm sieve (Table 1).

The flow properties of the powder

For the determination of the flowing properties of the powders several methods can be used. The most used are: the Carr compressibility index or the Hausner ratio.

Tabel 2. Results for powder flow

Number beats	Volume read (mL)	Carr index	Hausner ratio
0	208	14,71%	1,17
10	184		
500	174		
1000	174		

The flow of the raw material is good according to the results obtained (Table 2). This parameter determines the flowing of the raw material, this way, the encapsulation will not encounter any problems or it will not have to be mixed with excipients that improve this property.

The quantification of the total polyphenols

The samples were prepared using raw material samples, and were extracted by using extraction 1 and 4. The quantification was accomplished by using the spectrophotometrical method.

For the interpretation of the data, the calibration curve presented at the analysis of total polyphenols of fruit was used.

Table 3. The quantification of the total polyphenols from the raw material

Extinction	**Concentration (mg/1000mL)**	**Concentration (mg/g d.w.)**
0,787	2,97	9,07

Results: The raw material obtained from the mixture of equal parts of billberies, red currant and red, fermented pomace (Cabernet Sauvignon and Feteasca Neagra varieties 1:1), had a concentration of total polyphenols (expressed in gallic acid) of 9.07 mg/g raw material (Table 3).

The quantification of the phenolic compounds

Extraction 1 was used for the analysis of quercetin, cinnamic acid and resveratrol. Extraction 4 was used for the analysis of rutin, gallic acid, syringic acid, caffeic acid, (+)-catechin and chlorogenic acid.

The highest quantity of the phenolic compounds analyzed in the raw material was determined for chlorogenic acid, with a quantity of 33.37 mg/ 100 g r.m. (raw material). Values between 10 and 20 mg/ 100 g r.m. were determined for rutin, ferulic acid and (+)- catechin with quantities of 19.89 mg/ 100 g r.m., 17.98 mg/ 100 g r.m. respectively 11.87 mg/ 100 g r.m.. the gallic acid, cinnamic acid, resveratrol, syringic acid, quercetin and caffeic acid had quantities beneath 10 mg/ 100 g r.m. (Fig. 17).

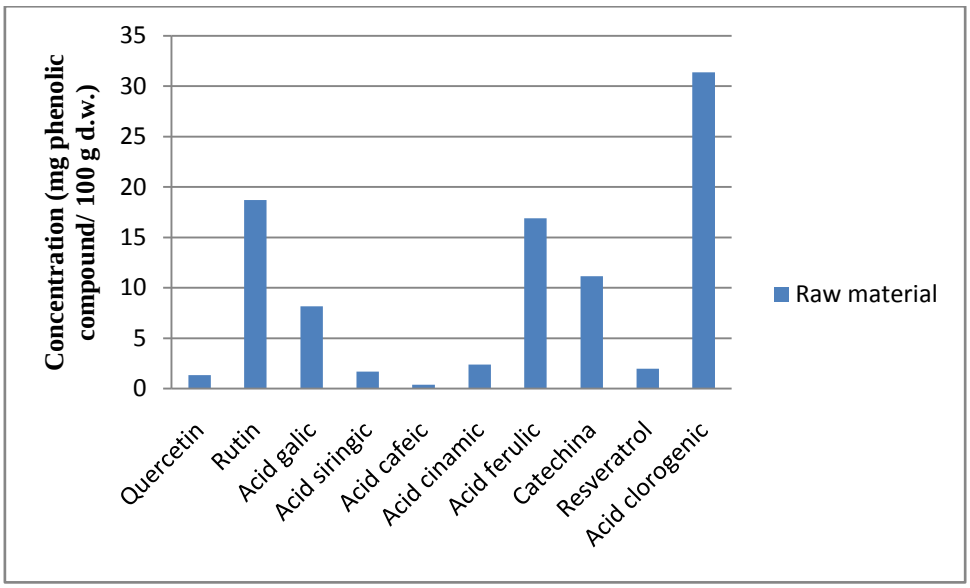

Figure 17. The phenolic compounds analyzed from raw material

Results: Of the total quantity of phenolic compounds analyzed/ capsule: chlorogenic acid was 33.53%, rutin 19.98%, ferulic acid 18.06%, (+)- catechin 11.93%, gallic acid 8.72%, cinnamic acid 2.53%, resveratrol 1.62%, syringic acid 1.80%, quercetin 1.43% and caffeic acid 0.40%.

The obtaining of the end product

Encapsulation

For the filling of the hard capsules it was used a special device designed for this process which is formed of a metallic frame on which there are set several plates: the pressure plate, the capsule guiding plate and the covering plate (Leucuța, et al. 2010).

The analysis of the end product

Average weight and uniformity of mass

Requirements:

The average weight of the intact capsules has to meet the interval: 820.0 mg/capsule ± 7.5% (758.5 – 881.5 mg/cps.) (Table 4).

The average weight of the capsules content has to meet the interval: 700.0 mg/capsule ± 7.5% (647.5 - 752.5 mg/cps.) (Table 4).

At least 18 capsules may have a deviation of maximum ± 7.5% from the determined average mass and at most 2 capsules may have a deviation of maximum ± 15% from the determined average mass.

Table 4. The results for average weight

	Intact capsule (mg)	**Content capsule (mg)**
Average	822,95	704,4

Results: The analyzed capsules meet the requirements regarding the average weight and the uniformity of the mass, the results that were obtained fit in the declared interval.

Disintegration

This test is accomplished for the determination of the required time of disintegration of the capsules in small particles by putting them in a liquid medium. They have to meet the requirements indicated in the monograph of the corresponding capsules (European Pharmacopoeia 2014).

Requirements: the requirements are met if the disintegration time is maximum 30 minutes, for all capsules (6 capsules). If 1 or 2 capsules did not disintegrate completely, the test is repeated for 12 capsules. The test meets the requirements if minimum 16 capsules out of 18 were disintegrated in 30 minutes.

Results: The disintegration of the capsules was achieved in less than 3 minute. The result meets the requirements.

The quantification of the total polyphenols

500 mg end product powder, obtained by triturating the content of 20 capsules are extracted using extraction 1 and 4 and are analyzed using the spectrophotometrical method.

The results are displayed in table 5.

Table 5. The quantification of the total polyphenols from the raw material

Extinction	Concentration (mg/1000mL)	Concentration (mg/capsule)
0.812	3.06	6.60

Requirements: The quantity of total polyphenols (expressed in gallic acid) has to be 6.50 mg/capsule ± 10% (5.85 - 7.15 mg/capsule).

Results: The quantity of total polyphenols (expressed in gallic acid) is 6.60 mg/capsule the result met the requirements.

The quantification of phenolic compounds

500 mg end product powder, obtained by triturating the content of 20 capsules are extracted using extraction 1 and 4 and are analyzed using the HPLC method.

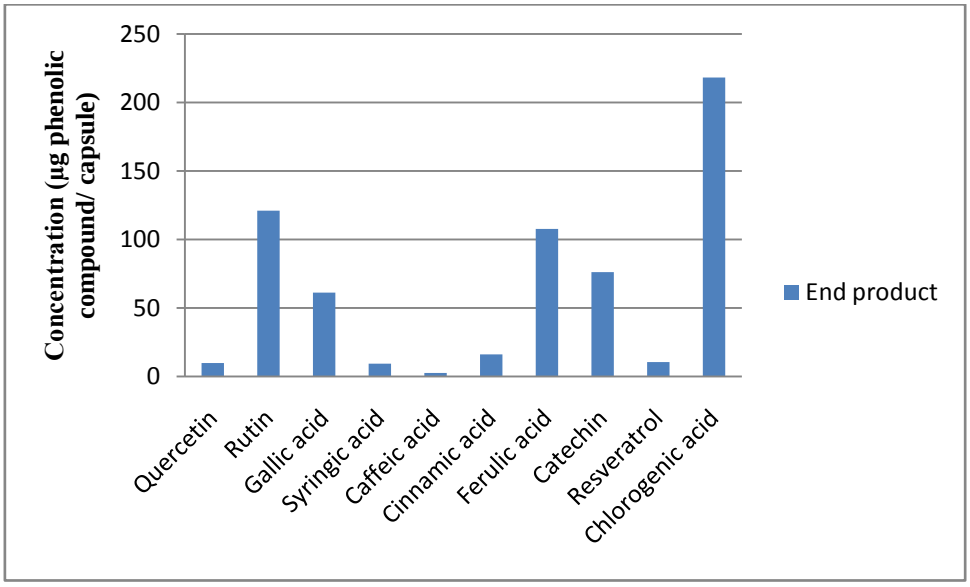

Figure 18. The phenolic compounds analyzed from the end product

Extraction 1 was used for the analysis of quercetin, cinnamic acid and resveratrol and extraction 4, for the analysis of rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)-catechin and chlorogenic acid.

The highest quantity from the phenolic compounds analyzed was determined for chlorogenic acid, having a quantity of 218.33 μg / capsule. Values between 100 and 200 μg / capsule were determined for rutin and ferulic acid: 121.03 μg / capsule respectively 107.75 μg / capsule. (+)-Catechin, gallic acid, cinnamic acid, resveratrol, syringic acid, quercetin and caffeic acid had values beneath 100 μg / capsule (Fig. 18).

Requirements: Of the total quantity of phenolic compounds analyzed/ capsule: chlorogenic acid has to be higher than 30.00%, rutin and ferulic acid higher than 15.00%, (+)-catechin and gallic acid, higher than 7.00%, cinnamic acid, resveratrol, syringic acid and quercetin higher than 1.40% and caffeic acid higher than 0.40%

Results: Of the total quantity of phenolic compounds analyzed/ capsule: chlorogenic acid was 34.51%, rutin 19.13%, ferulic acid 17.03%, (+)- catechin 12.04%, gallic acid 9.66%, cinnamic acid 2.54%, resveratrol 1.65%, syringic acid 1.50%, quercetin 1.55% and caffeic acid 0.40%, the results met the requirements.

Validation report of the phenolic compounds analysis method

This report was accomplished according to the working rules and it certifies the validity of the analysis method for the identification and quantification of the phenolic compounds of interest (Bliesner 2006).

The validation was performed regarding the specificity, precision, linearity, the conformity of the system, the stability of the solutions and robustness.

The developed dietary supplement in relation to other antioxidant products

The product developed in this paper is a natural product 100%, does not have preservative or dying compounds. Also, this product does not contain lactose, this way it can be used by lactose intolerant persons.

The raw material consists of dried and milled fruit, respectively by-products from the winemaking process that were dried and milled too. So this product does not contain even traces of residual solvents that can be toxic and that could have been present in the dietary supplement after the extraction of the active compounds.

The content of total polyphenols and phenolic compounds, like: gallic acid, (+)-catechin, syringic acid, cinnamic acid, resveratrol, chlorogenic acid, caffeic acid, ferulic acid, rutin and quercetin is well known, this way the administered dosage of each compound can be exactly quantified.

Chapter IX - Conclusions and perspectives

Final conclusions

Medicinal plants have been used from the beginning of mankind, being the only sources of preserving human health and to prevent and cure diseases. Nowadays, the usage of natural remedies for preventing and treating certain diseases show the high efficiency of the natural products in increasing the quality of life.

Thus, three types of berries were analyzed: bilberries (*Vaccinium myrtillus* L.), raspberries (*Rubus idaeus* L.) and red currant (*Ribes rubrum* L.), and a by-product from the winemaking process: fermented, red pomace, varieties: Cabernet Sauvignon and Feteasca Neagra (*Vitis vinifera* L.).

The preservation of the vegetal products was achieved by freezing and then drying or by drying immediately after harvesting for the berries, and the pomace was dried, grinded and then frozen. This way, it was determined that regarding the total polyphenols and the analyzed phenolic compounds the methods of preservation applied to the berries do not influence their quantity.

The isolation of the compounds of interest was achieved through four types of extraction. The highest quantity of total polyphenols was extracted using extraction 4, followed by extraction 3, then extraction 2, and the lowest quantity was determined using extraction 1, this one being the most selective.

Regarding the analyzed phenolic compounds, it has been observed that the extraction suitable for quercetin, cinnamic acid and resveratrol was extraction 1 and for rutin, gallic acid, syringic acid, caffeic acid, ferulic acid, (+)- catechin and chlorogenic acid was extraction 4.

All of the analyzed products have compounds that posses antioxidant properties and can be used due to their beneficial effects on the human body.

In order to obtain the dietary supplement, a series of operations and preliminary analysis were performed.

The used raw material was obtained from dried powder of bilberries (*Vaccinium myrtillus* L.), red currant (*Ribes rubrum* L.) and a 1:1 mixture of fermented, red pomace, varieties Cabernet Sauvignon and Feteasca Neagra (*Vitis vinifera* L.). The analysis performed were the particle size distribution, powder flow properties and the quantification of total polyphenols and phenolic compounds.

The encapsulation of the raw material was achieved using hard capsules, and their analysis were based on the average mass uniformity of dosage form, disintegration and quantification of total polyphenols and phenolic compounds.

The HPLC method of quantification of phenolic compounds was validated for the analysis of the end product. The validation was performed regarding the specificity, precision, linearity, the conformity of the system, the stability of the solutions and robustness.

The dietary supplement obtained can be compared to dietary supplements already on the market. The major advantages of this product are that it is 100% natural, it does not have colorants or conservatives and it can be administrated to lactose intolerant people.

Personal contributions

The personal contributions revealed in this paper consist in:

- The identification and quantification of several phenolic compounds from indigenous vegetal products
- The identification of several extraction methods that are suitable for the phenolic compounds analyzed
- The development of an HPLC analysis method suitable for analyzing the ten phenolic compounds
- The validation of the HPLC method
- The development of a dietary supplement with bioactive properties that is 100% natural and it is made by indigenous vegetal products

- The exploitation of by-products from the winemaking industry for the obtaining of dietary supplements
- The application of the developed HPLC method for analyzing the developed dietary supplement

Perspectives for the continuation of the research

For the continuation of the research I propose the following subjects:

- Filling a notification folder regarding the dietary supplement obtained, to the National Institute of Research and Development for Alimentary Bioresources, in order to put it on the market. All the required data are in this doctoral dissertation
- The analysis of other vegetal products with bioactive potential using the methods described in this paper
- The determination of other analysis methods for the bioactive potential from the vegetal products analyzed
- The exploitation of other by-products in order to obtain dietary supplements with bioactive potential

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