„Dunărea de Jos”University of Galați

Doctoral School of Fundamental Sciences and Engineering

PH.D. THESIS

SUMMARY

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Series I 1. BIOTEHNOLOGIES No. 16
GALAȚI
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Keywords: red grape skins, biologically active compounds, anthocyanins, microencapsulation,natural ingredients, value-added products

INTRODUCTION

Nutrition and food selection for a healthy lifestyle have been the main concerns of human society since ancient times. A correct and adequate nutrition is crucial for health, especially when the immune system needs support. Limited access to nutritious and functional foods can compromise the opportunities for a healthy and varied diet, thus affecting the nutritional and functional value of food. The socio-economic and lifestyle changes can lead to an increased consumption of highly processed foods, which tend to be rich in fat, sugars, and salt. However, even with limited bioactive ingredients, the consumer can still follow a diet that supports a healthy lifestyle. Fruits and vegetables are recommended food sources for a healthy diet. They are eaten fresh, partially or fully cooked, depending on their nature and processing techniques. With the changing diets and population growth, the production, as well as the processing method of plant products, have improved substantially to meet consumers' demand. From this perspective, a high quantity of by-products is generated by the processing of fruits and vegetables, with an impact on the nutritional and economic lossess, and the environmental pollution. Fruits and vegetables processing generates a high amount of waste, representing 25-30% of the processed raw material's weight. Grapes are amongst the world's most widely grown fruit, with an estimated production of more than 79 millions of tonnes in 2018, according to the Food and Agriculture Organization (FAOSTAT).

Grape consumption is beneficial for the human health due to the high content of bioactive substances with functional role (Sousa et al., 2014). Approximately 75% of the grapes' production is used for the wine production, resulting in 20-30% residual by-products (García-Lomillo and González-SanJosé, 2017). The main by-products from winemaking are known as pomace, which consists of the grape's epicarp (skin or peel), pulp, seeds, and bunches (Balbinoti et al., 2020). The grapes' skins are considered a valuable by-product, approximately 65% of it being found in the composition of grape pomace. The skin of red grapes is rich in bioactive compounds, mainly anthocyanins, which act as pigments and have antioxidant capacity (Yilmaz and Toledo, 2004). Anthocyanins from red grapes have a highly technological and functional value (Leong et al., 2016; Zhang et al., 2018). In this sense, the extraction of these compounds represents an alternative for obtaining products with added value, which can be used as ingredients in the food, cosmetic, and pharmaceutical industries based on the biorefining principles.

The PhD thesis, entitled ***The valorization of various biologically active compounds from red grapes´ processing by-products and the obtainment of certain value-added ingredients***, had as a main objective the study of the main biologically active compounds present in the epicarp (skin) of red grapes (*Băbească neagră variety*), namely anthocyanins, flavonoids, and total polyphenols, in order to obtain functional composites for the food industry. The thesis aimed to obtain products

that are not available on the national market (gluten-free biscuits, and beer enriched with red grapes' skin extract). Different extraction techniques (conventional solvent extraction, ultrasound-assisted ethanolic extraction, microwave-assisted ethanolic extraction, enzyme-assisted ethanolic extraction) have been used to extract the bioactive compounds from red grapes. Structural and conformational changes induced on the bioactive compounds after their extraction from the natural matrix were studied using high performance liquid chromatography, and molecular modeling techniques.

The main ***scientific objectives*** of the studies were:

- The testing of different extraction methods of the bioactive compounds (total polyphenols, monomeric anthocyanins, flavonoids) from the epicarp of red grapes belonging to *Băbească neagră* variety to obtain extracts with functional potential, mainly with superior antioxidant activity.
- The evaluation of the behavioural pattern of the biologically active compounds extracted from the epicarp of red grapes of *Băbească neagră* variety by heat treatment, aiming to elucidate the degradation mechanisms and to optimize the conditions to obtain and store the extracts rich in biologically active compounds.
- The obtainment of functional composites through microencapsulation techniques of the extract rich in biologically active compounds within natural polymeric matrices for the formulation of bioingredients with an added value and a high stability of the bioactive potential.
- The development of various technologies to obtain several products (gluten-free biscuits, beer assortments) with an added value using the powders and extracts from grape skins (*Băbească neagră*), samples rich in bioactive compounds and with superior antioxidant activity.

I. The PhD thesis is structured in two parts and seven chapters as follows: **DOCUMENTARY STUDY (Chapter 1)**, entitled ***Theoretical considerations on the separation, bioactive properties and stability of bioactive compounds from red grape epicarp***, is structured into three sub-chapters that present the most recent data from the literature on the characterisation of bioactive compounds from the epicarp (skin) of red grapes, *Băbească neagră* variety, and their technological and functional impact. Moreover, the techniques used for the extraction and microencapsulation of the bioactive compounds from grapes' epicarp are also described.

II. EXPERIMENTAL STUDY, which includes the investigations carried out during the doctoral studies and the obtained results that are divided in four chapters as follows:

Chapter 2, **Comparative evaluation of the extraction methods for the bioactive compounds from the epicarp of grapes (*Băbească neagră* variety),** displays the experimental setup in order to extract and separate the biologically active

compounds (monomeric anthocyanins, flavonoids, and total polyphenols) from the red grapes skins (*Băbească neagră*), from the perspective of improving the extraction yield and obtaining extracts with superior functional value (antioxidant activity). Four extraction methods were studied: conventional solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction, and extraction with the addition of enzymatic preparations, and a series of technological parameters with impact on the bioactive compounds' release were varied, namely: concentration of solvents and their combination, temperature, and extraction time. The use of ethanolic solutions (50% , 70%, and 96%), mixed with acids (glacial acetic acid, 99.5% citric acid, 0.1N hydrochloric acid), was very effective for the extraction of the targeted bioactive compounds, considering the conventional method, as well as the combined extraction with microwaves, ultrasounds or enzymatic treatment. The extraction yield of the targeted compounds and the antioxidant activity of the extract varied, in correlation to the specific extraction parameters, which were optimized according to the intended purpose.

Chapter 3, entitled **The chemical characterization and evaluation of the thermal stability and bioactive potential of the extract from the epicarp of red grapes (*Băbească neagră variety*),** shows the results of the phytochemical composition of the extract obtained by a conventional solvent extraction technique (96% ethanol mixed with 0.1 N HCl at 50°C for 55 minutes). The thermal stability of the bioactive compounds from the extract has been evaluated by kinetic modelling and thermodynamic studies. A superior thermal stability of the anthocyanins was demonstrated between 80-120°C, correlated also to the maintenance of the antioxidant activity's stability. The extract's capacity to inhibit a number of enzymes with metabolic impact (α-amylase, α-glucosidase, lipase, and lipoxygenase), in which the anthocyanin malvidin-3-*O*-glucoside plays an important role was assessed, this effect being demonstrated by *in silico* molecular docking studies. The heat treatment at 140°C significantly reduced the inhibitory effect of the enzymes involved in the metabolic syndrome.

Chapter 4, etitled **The valorisation of the red grapes (Băbească neagră variety) extract to obtain bioactive ingredients by microencapsulation,** presents the approach to obtain functional composites, by microencapsulating the rich in bioactive compounds (total polyphenols, anthocyanins, and flavonoids) extract obtained through conventional ultrasound-assisted extraction within polymeric matrices (whey protein isolate, whey protein, carboxymethyl cellulose, pectin, gum arabic, and maltodextrin), in different combinations. The obtained results confirmed that anthocyanins were the compounds that bound efficiently to the tested substrates. The encapsulation efficiency depended, nonetheless, on the tested type of materials and on their ratio for the matrix formulation. Superior encapsulation efficiency results were obtained for the combination of whey protein isolate with carboxymethyl cellulose. The powders obtained under these conditions showed a higher stability regarding the phytochemical composition and antioxidant activity, after 28 days of

storage at room temperature, by limitating the access of oxygen and light.

Chapter 5, entitled **The obtainment of various value-added food products through the valorisation of the biologically active compounds extracted from the epicarp of red grapes (*Băbească neagră variety*)**, describes the experiments performed and the results obtained in the elaboration of two technologies for food products with an improved functional value through the contribution of the bioactive compounds extracted from the epicarp of red grapes, namely gluten-free biscuits and beer with a superior functional potential. The stability of the bioactive characteristics was demonstrated during the storage of the products (biscuits - 28 days at 24°C; beer - 21 days at 4°C). The obtained results certify that the extract and the freeze-dried powder can be used as natural ingredients with bioactive potential, obtained from the skins of red grapes of the *Băbească neagră* variety, for multiple uses in the food industry, thus promoting the principles of a circular economy.

Each chapter of the experimental study is structured in sub-chapters as follows: *General aspects, Objectives of the study, Materials and methods, Results and discussion, Partial conclusions and References*.

Chapter 6 presents the **Final conclusions** of the studies carried out from the perspective of the obtained results with an innovative value, with a fundamental, and applied impact.

Chapter 7, entitled **Personal contributions and future perspectives**, presents the main contributions of the PhD thesis to the development of the fundamental and applied knowledge through the addressed topics and the new perspectives outlined for further studies.

Chapter 8 presents the **Dissemination of research results** by highlighting the main papers published in relevant journals to the scientific field, and presented at prestigious national or international scientific events. Therefore, the research results have been disseminated through **4 scientific articles** published in WOS indexed journals (*Antioxidants* - IF=7.675, *Sustenability* - IF=3.889, *Inventions*, *The Annals of the University Dunarea de Jos of Galati Fascicle VI-Food Technology*), as well as **15 presentations** at representative scientific events.

During the doctoral studies, the PhD student was part of the research teams of two projects funded by the "Dunărea de Jos" University of Galati, namely:

1. *Innovative and emerging solutions for the design of natural co-microcomposites to improve food functionality*, Funding contract 3637/30.09.2021.

2. *Innovative strategies for valorisation of agro-food by-products into economically valuable products promoting circular economy principles*, Funding Contract 14888/11.05.2022.

The research activities of the PhD thesis were carried out within the *Integrated Center for Research, Expertise and Technology Transfer (BioAliment-*

TehnIA) (www.bioaliment.ugal.ro), within the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galati.

The thesis was carried out under the scientific co-supervision of Prof.dr.eng. Gabriela-Elena BAHRIM and Prof.dr.eng. Gabriela RÂPEANU, and the scientific committee composed of: Prof. dr.eng. Iuliana APRODU – coordinator of the molecular modelling and docking studies, Prof.dr.eng. Nicoleta STĂNCIUC – coordinator of the spectrophotometric and co-microencapsulation studies, and Associate prof.dr.eng. Oana-Emilia CONSTANTIN – coordinator of value-added product development studies.

CHAPTER 2

COMPARATIVE EVALUATION OF THE EXTRACTION METHODS FOR THE BIOACTIVE COMPOUNDS FROM THE EPICARP OF GRAPES (BĂBEASCĂ NEAGRĂ VARIETY)

2.1 General aspects

World grape production is around 60 million tonnes per year. In terms of bioactive compounds, polyphenols are the majoritary and 75% are found in the berry epicarp (skin) and seeds (Ghafoor et al., 2010). Grape winemaking generates a significant amount of by-products with a high potential for valorization (Teixeira et al., 2014; Lima et al., 2017). Pomace is the main by-product from grape pressing, representing around 20-25% of the weight of the fruit, containing skin, seeds, clusters and other solid components. Physicochemically, grape pomace is a complex substrate containing simple and complex carbohydrates, proteins, pectins, phenolic compounds and insoluble proanthocyanidins (Dávila et al., 2017). In addition, grape tescovin is a complex matrix that brings together different categories of polyphenolic compounds, including both simple molecules such as phenolic acids and flavonoids (Cheynier et al., 2012; Rockenbach et al., 2011).

There is a great potential for the valorization of by-products from the winemaking process through bio-refining and bio-valorization processes, with particular attention on grape pomace and residual wine yeast (Ali et al., 2010). Red grape epicarp or skin (GS) is valuable due to its significant content in monomeric and polymeric molecules such as anthocyanins, flavan-3-ols, flavonols, dihydroflavonols, hydroxybenzoic acids and hydroxystilbene (Wang et al., 2003). Thus, GS is considered a potential source for the separation of natural bioactive compounds by biorefining (Wang et al., 2003). The bioactive composition of GS is correlated to the wine variety and the horticultural conditions of the grape production. From a nutraceutical point of view, an interesting category of compounds found in grapes are phenolic compounds with remarkable antioxidant activity, their concentrations varying according to the analyzed by-product, higher concentrations being determined in the skins and seeds compared to the grape pulp (Xia et al., 2010). Since white grapes do not contain anthocyanins, their polyphenol content is often lower compared to red grapes. According to the study published by Vidal et al. (2004), anthocyanins are polyphenolic compounds belonging to the flavonoid class and are responsible for the specific colour of red grapes, while the oligomers of procyanidins and low molecular weight flavan-3-ols are responsible for the bitter taste (Wang et al., 2003).

2.2. Objectives

The main specific objectives of this study were to test four methods of extraction of the biologically active compounds from red GS (Băbească neagră variety), namely: a) conventional extraction with organic solvents; b) ultrasound-assisted ethanolic extraction; c) microwave-assisted ethanolic extraction; d) enzyme-assisted extraction.

The extraction efficiency was determined through a comparative evaluation of the phytochemical profile, by determining the total monomeric anthocyanin (TMA) content, total flavonoid content (TFC), total polyphenol content (TPC) and the antioxidant activity of the obtained extracts.

Using the Response Surface Analysis Method, the ultrasound-assisted extraction conditions were optimized by testing the influence of four factors: ethanol and citric acid concentration, extraction time and temperature.

2.3. Materials and methods

The investigation methods were:

- Extraction of the red GS (Băbească neagră variety) biologically active compounds was achieved by conventional ultrasound-assisted ethanolic extraction, microwave-assisted ethanolic extraction and the extraction with the addition of enzymatic preparations. For the phytochemical characterization of the extracts, the TAC content was determined by the differential pH spectrophotometric method, the results being as mg cyanidin-3-*O*-glucoside equivalent (C_3G)/g d.w.. The colorimetric method, based on $AlCl_3$, was used to determine the TF content and the results were reported as mg catechin equivalent (CE)/g d.w.. The TP content was determined by the Folin-Ciocalteu colorimetric method and the results were expressed as mg gallic acid equivalent (GAE)/g d.w. while for the antioxidant activity determination of the extracts, DPPH was used and the results were expressed as mM Trolox/g d.w..

- Optimization of the ultrasound-assisted extraction conditions through mathematical modeling and statistical analysis using the Response Surface Analysis Method, Design Expert software, version 13 (Stat-Ease, Inc., Minneapolis, MN, USA).

2.4. Results and discussion

2.4.1. Phytochemical profile of the extracts obtained by solvents' extraction

The bioactive compounds and the antioxidant potential of the extracts obtained using ethanol solutions, in three different concentrations: 50%, 70%, 96%, in the presence of acetic acid, citric acid and hydrochloric acid were analyzed. The two parameters that have been varied were the temperature and the extraction time.

The extraction yield of TAC was variable depending on the extraction parameters. The combination of conventional solvent extraction with the ultrasound treatment (40 kHz) allowed the obtainment of an anthocyanin content of 4.29 ± 0.04 mg C_3G/g d.w., using a 96% ethanol acidified with 0.1 N HCl as solvent, after 55 minutes of extraction, at 50°C. In the case of the TF content and antioxidant activity, the extraction with the addition of enzymatic preparations highlighted remarkable results. In regards to the total flavonoids' extraction, a significant amount of 17.31 ± 8.42 mg CE/g d.w., was quantified following extraction with the addition of the Zymorouge preparation, after three hours of extraction. Regarding the antioxidant activity, the enzymatic hydrolysis with cellulase led to higher values. Thus, after only one hour of extraction, an extract with an antioxidant activity of 61.48 ± 1.19 mM Trolox/g d.w. has been obtained.

The highest recovery of TPC (49.01 ± 5.08 mg GAE/g d.w.) from the red GS was obtained by ultrasonic extraction performed at 50°C, after 55 minutes of treatment using 50% ethanol acidified with 1% citric acid solution as solvent. For both of the conventional solvent extraction and the ultrasound-assisted extraction, it has been found that, in most of the cases, the temperature of 50°C resulted in the extraction of a higher content of compounds by increasing the permeability and solubility of the cellular walls and decreasing the viscosity of the solvent, compared to the temperature of 25°C.

2.4.5. Optimization of the bioactive compounds' extraction by ultrasound-assisted technique using the Response Surface Methodology

The experiments were designed using the Central Composite Design technique, and the responses evaluated were: the total monomeric anthocyanin (TMA) content, total polyphenols content (TPC) and antioxidant activity (AA) of the extracts obtained by varying the analyzed parameters (independent variables).

Table 2.14. Extraction parameters and responses variation obtained by experimental design

Sample	Independent variabile				Answers		
	A	B	C	D	TAC mg C_3G/g d.w.	TPC mg GAE/g d.w.	AA Mm Trolox/ g d.w
1	0.10	85	25	65	2.09 ± 0.08	29.01 ± 1.70	19.76 ± 0.25
2	1	67	13.06	45	2.15 ± 0.11	29.97 ± 1.55	18.24 ± 0.13
3	1	67	71.90	45	2.29 ± 0.02	40.97 ± 1.70	15.95 ± 0.39
4	1	67	42	45	2.24 ± 0.02	36.94 ± 1.67	17.26 ± 0.14
5	1	67	42	45	2.25 ± 0.18	37.04 ± 1.49	17.25 ± 0.16
6	0.10	50	25	25	2.45 ± 0.03	29.93 ± 0.38	19.65 ± 0.66
7	2	50	25	65	2.64 ± 0.18	30.12 ± 1.71	19.05 ± 0.89
8	0.10	85	60	65	2.40 ± 0.39	31.09 ± 0.56	18.91 ± 0.50
9	2	50	60	65	2.17 ± 0.22	31.92 ± 0.89	19.31 ±0.24
10	0.01	67	42	45	2.10 ± 0.09	30.86 ± 1.23	20.16 ± 0.64
11	0.10	50	60	25	2.53 ± 0.29	43.97 ± 0.94	18.38 ± 0.20
12	1	67	42	11.36	2.05 ± 0.10	31.41 ± 1.39	18.77 ± 0.29
13	2.64	67	42	45	2.49 ± 0.15	35.88 ± 0.98	20.49 ± 0.55
14	2	85	25	25	1.71 ± 0.08	24.67 ± 1.11	20.98 ± 0.40
15	1	67	42	78.63	2.32 ± 0.11	26.45 ± 1.92	18.72 ± 0.18
16	2	85	60	25	2.07 ± 0.07	31.1 ± 0.56	17.47 ± 0.50
17	1	67	42	45	2.24 ± 0.02	37.12 ± 1.94	17.26 ± 0.27
18	1	67	42	45	2.25 ± 0.00	37.01 ± 1.15	17.26 ± 0.13
19	1	96.93	42	45	2.59 ± 0.10	42.07 ± 1.66	16.35 ± 1.25
20	1	38.06	42	45	2.03 ± 0.16	32.25 ± 1.44	17.61 ± 0.80
21	1	67	42	45	2.25 ± 0.11	37.14 ± 1.96	17.35 ± 0.21

The interactions between the independent variables and their effect on the studied responses are shown in Figure 2.8.

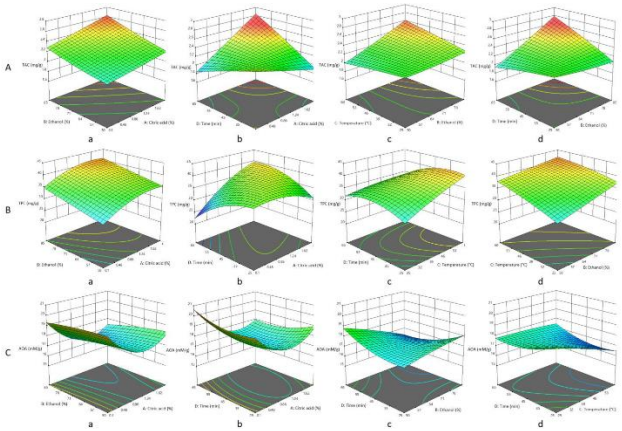


Figure 2.8. Three-dimensional surface plots screening the effect of the variables on the TAC ((**A**)—(**a**): citric acid–ethanol; (**b**): citric acid–time; (**c**): ethanol–temperature; (**d**): ethanol–time), TPC ((**B**)—(**a**): citric acid–ethanol; (**b**): citric acid–time; (**c**): temperature–time; (**d**): ethanol–temperature) and AOA ((**C**)—(**a**): citric acid–ethanol; (**b**): citric acid–time; (**c**): ethanol–time; (**d**): temperature–time).

Optimization of the monomeric anthocyanins extraction

Through mathematical modeling and statistical analysis, the mathematical model described by equation 2.4 was obtained based on the data presented in **Table** 2.14.

$$\text{TAC, mg } C_3G/\text{g d.w.} = 2.26 + 0.1375A + 0.1722B + 0.0380C + 0.1043D + 0.0368AB - 0.06AC + 0.3622AD + 0.1324BC + 0.2475BD - 0.0750CD + 0.0038A2 + 0.0225B2 - 0.008C2 - 0.0200D2 \quad (\mathbf{2.4})$$

After the model's analysis for the TAC extraction, it was concluded that the ethanol concentration is the factor with the greatest influence on the anthocyanins content. Also, the citric acid's concentration and extraction time positively influenced TAC extraction. The interaction between the citric acid and ethanol concentrations (AB) as well as between the citric acid concentration and the extraction time (AD), respectively the interaction between the ethanol concentration and the extraction time (BD) have been shown to be beneficial to achieve the intended objective. This response is negatively influenced, as shown by equation 2.4, by the interaction between the citric acid concentration and the temperature (AD) and of that between the temperature and extraction time (CD). The analysis of the response surfaces in Figure 2.8 (Aa-Ad) showed that the enhanced TAC extraction was achieved by the interaction between the citric acid concentration and the extraction time. The maximum concentration of TAC in the extract was obtained when using a citric acid concentration of 2% w/v and an extraction time of about 65 min, keeping the other extraction parameters constant (ethanol 50%, 25°C).

The negative effect of the interaction between the extraction time and temperature has also been identified by Li et al. (2019), who observed that in the temperature 40-51°C range, there was a significant increase in the extraction yield of TAC, whereas at a temperature higher than 51°C, the yield started to decrease. Moreover, as shown in Figure 2.8 (Ad), the lower extraction times (25 min) and higher ethanol concentrations (85%) led to a lower value of TAC in the extract, a result that was mainly influenced by the ethanol concentration compared to the effect caused by the temperature variation **(Figure** 2.5 Aa, Ac). Curve B in the perturbation plot also demonstrated the influence of ethanol concentration on the TAC extraction yield. Curves A and D, revealed the impact of the extraction time and citric acid addition respectively, which had an insignificant influence compared to the ethanol concentration in the solvent **(Figure** 2.9 A).

Optimization of total polyphenols extraction

In the studied experimental variants, TPC ranged from 24.67 to 43.97 mg GAE/g d.w. (**Table** 2.14). The mathematical model obtained corresponds to equation 2.5.

$$TPC, mg\ GAE/g\ d.w. = +37.41 + 3.19A + 2.88B + 3.10C - 1.10D - 0.1614AB - 0.9895AC + 5.39AD - 0.9180BC + 5.21\ BD - 2.07CD - 2.42A^2 - 0.6054C^2 - 2.89D^2 \quad (\mathbf{2.5})$$

The data presented in **Table** 2.14 demonstrated that for TPC, the analysed factors and the mathematical model were characterised by significant probability values ($p<0.05$), which confirmed the appropriate selection of the independent variables. The coefficients of the regression equation demonstrated that, the citric acid addition, ethanol concentration and extraction temperature positively influenced the polyphenols extraction. The interactions between the citric acid concentration and the extraction time (AD) and between the ethanol concentration and the extraction time (BD) had a positive effect on the TPC extraction yield. On the contrary, the linear effect of the extraction time had a negative impact on the TPC. Also, the interactions between the citric acid concentration and the ethanol concentration (AB), the citric acid concentration and the extraction temperature (AC), and the ethanol concentration and the extraction temperature (BC) presented a negative effect on the yield of total polyphenols. Figure 2.8.Ba-Bd showed the three-dimensional plots of the response surfaces regarding the interactions between the analysed factors, the correlated effects between the citric acid and ethanol concentrations, respectively between the temperature and the extraction time, which had the greatest influence on the TPC extraction. The content of phenolic compounds increased when the ethanol concentration approached the value of 85% and the citric acid concentration was higher than 2% w/v (Figure 2.8.Ba). The citric acid concentration and the ethanol concentration had a greater impact on the polyphenols concentration compared to the extraction duration. A decrease of the content of phenolic compounds was observed at a high citric acid concentration and after an extended extraction time (Figure 2.8. Bb). In addition, the increase in TPC with the increasing temperature was observed at low (<25 min) and moderate (35 min) extraction times. However, the effect of temperature became statistically insignificant at a higher extraction time (>65 minutes) (Figure 2.8. Bc). The data in Figure 2.8 Bd demonstrated a considerable increase in the TPC with the decreasing temperature and the increasing ethanol concentration in the solvent composition. Furthermore, the perturbation plot (Figure 2.9), displaying the effect of each independent variable on the TPC, showed that both citric acid and the concentration of ethanol had a significant influence on the increase of the TPC extraction yield.

Optimization of the extraction to increase the antioxidant activity

The recorded values of the antioxidant activity varied between 15.95 and 20.98 mM Trolox/g d.w.., depending on the influence of the different studied variables (**Table** 2.14). The model obtained for the variation of the antioxidant activity was characterized by equation 2.6.

$$21 - 0.8438A - 0.3931B - 0.6835C - 0.0497D - 11.84AB - 0.4843AD - 0.4184BC - 0.8576BD + 0.5237CD + 1.66A^2 - 0.1089B^2 - 0.0656C^2 + 0.5114D^2 \quad (\mathbf{2.6})$$

The antioxidant activity of the extract was positively influenced by the interaction between the temperature and the extraction time (CD), whereas the interaction between the citric acid concentration and the ethanol concentration (AB) had a significant negative impact on the AA of the extract. The interactions between the citric acid concentration and the extraction time (AD), the ethanol concentration and the extraction temperature (BC), the ethanol concentration and the extraction time (BD) also exerted a negative influence. As shown in Figure 2.8 Ca-Cd, the maximum antioxidant activity was obtained after 25 min of extraction using an 85% ethanol solution as a solvent, correlated to the addition of citric acid (**Figure** 2.8 Ca,Cb), with beneficial effect on reducing the extraction time. The decrease of the AA of the extract was observed at an increased ethanol concentration (**Figure 2.8** Cc). However, at higher extraction times (45 min) and moderate ethanol concentration (64%) an increase of the AA was observed (**Figure 2.8** Cc). Higher temperatures increased the solubility of phenolic compounds, leading to an increase of the AA. However, using higher extraction temperatures and longer extraction time, the extracted phenolic compounds started to degrade, which was reflected in the decreased AA (**Figure** 2.8 Cd). Similar results were obtained by Li et al. (2019), who reported a negative correlation between an increasing ethanol concentration and an increasing temperature on the AA of the extract. Moreover, the D curve in the perturbation plot also demonstrated the significant influence of the extraction time on the AA of the extract (**Figure** 2.9C). After 25 minutes of extraction, at 25°C, using an 85% ethanol and 2% w/v citric acid solution as solvent, the extract with the highest AA of 24.67 mM Trolox/g d.w. (DPPH method) was obtained. For the same grape variety, from the 2012 harvest, Constantin et al., 2015, reported a value of 4.89 ± 0.02 μM Trolox/g d.w. (ABTS method).

Mathematical models' validation

The cumulative debiasing value of 0.926 demonstrated the fidelity of the processes in the rigorous selection of the studied experimental conditions. Thus, the ideal conditions to increase the extraction yield of the anthocyanins and polyphenols, as well as the antioxidant activity of the extract, using the conventional ultrasound-assisted extraction were: solvent - 85% ethanol, citric acid - 0.85%, extraction temperature - 57°C, extraction duration - 52 minutes.

Table 2.16. Validation of the employed mathematical models

Dependent Variable	Predicted Value	95% Confidence Intervals	Experimental Value
TAC (mg C_3G/g DW)	2.25	2.23–2.27	2.26
TPC (mg GAE/gDW)	37.41	37.09–37.73	37.22
AOA (mM TE/g DW)	17.20	17.08–17.32	17.11

The experimental data obtained by performing three independent measurements demonstrated that the resulting mathematical models were validated, under the optimized extraction conditions, higher values of the total monomeric anthocyanins, total polyphenolic compounds and antioxidant activity of 2.26 mg C_3G/g d.w., 37.22 mg GAE/g d.w., 17.11 mM Trolox/g d.w. being obtained.

2.5. Partial conclusions

The experimental results presented in this chapter allowed the following partial conclusions to be drawn:

- This study aimed to analyse the extraction conditions of the bioactive compounds from the skins of red grape berries (*Băbească neagră variety*) using conventional and modern extraction techniques, by increasing the extraction yield of anthocyanins and polyphenolic compounds in correlation to an improved antioxidant activity.

- Four extraction methods were studied, conventional solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction and extraction with the addition of enzymatic preparations, while a series of technological parameters with an impact on the bioactive compounds release were varied, namely: the concentration of solvents and their combination, temperature and extraction time.

- The results showed that the antioxidant activity of the extracts has been considerably influenced by the concentration of ethanol as solvent and the time period of the extraction process. The increase of the extraction temperature had no positive influence on the antioxidant potential of the extracts obtained under the conditions tested in this study.

- A higher extraction yield of the total monomeric anthocyanins of 4.29 ± 0.04 mg C_3G/g d.w. was obtained by ultrasound-assisted extraction using 96% ethanol mixed with 0.1 N HCl, after 55 min of extraction, at 50°C.

- The highest concentration of the extracted flavonoids, 9.55 ± 2.10 mg CE/g d.w., was obtained through the conventional extraction method, using a 96% ethanol solution mixed with glacial acetic acid, after 120 minutes of treatment at 50°C.

- The extraction with a 50% ethanol solution, mixed with 1% citric acid solution together with the effect of ultrasounds resulted in the extraction with the highest yield of total polyphenols, 49.01 ± 5.08 mg GAE/g d.w., after 55 minutes of extraction, at 50°C.

- The use of the commercial cellulase enzyme preparation resulted in an extract with an antioxidant activity of 61.48 ± 1.19 mM Trolox/g d.w. after only one hour of extraction.

- Ultrasounds have been proven to be the most effective method to extract the bioactive compounds. This extraction process was subjected to mathematical modelling and statistical analysis using the Central Composite Design and Response Surface

Analysis Method, Design Expert software. The mathematical models obtained show that maximum values for the total monomeric anthocyanins (2.26 mg C_3G/g d.w.), total polyphenolic compounds (37.22 mg GAE/g d.w.) and DPPH free radical scavenging activity (17.11 mM Trolox/g d.w.), values that could be obtained by the conventional ultrasound-assisted extraction under the following conditions: 85% ethanol solution, 0.85% citric acid solution, temperature 57°C, and an extraction time of 52 minutes at an ultrasound frequency of 40 kHz.

- Depending on the targeted bioactive compounds for their extraction the most effective method and the optimal process parameters can be selected on the principle of the increased extraction yield and antioxidant activity of the extracts, the productivity and economic efficiency.

CHAPTER 3.

THE CHEMICAL CHARACTERIZATION AND EVALUATION OF THE THERMAL STABILITY AND BIOACTIVE POTENTIAL OF THE EXTRACT FROM THE EPICARP OF RED GRAPES (BĂBEASCĂ NEAGRĂ VARIETY)

3.1. General aspects

World's wine production generates a high quantity of by-products (pomace, seeds, skins, residual yeast, etc.) with a high content of bioactive compounds with functional value. Amongst these bioactive compounds, flavonoids, anthocyanins and stilbene derivatives are the compounds of interest for the food, cosmetic and pharmaceutical industries, due to their antioxidant, antimicrobial, including antiviral or antitumor properties (Teixeira et al., 2014).

The recovery and valorisation of the by-products resulting from the winemaking process into value-added products is considered to be an effective strategy used with the aim of eliminating agro-food waste and preventing the environmental contamination (Devesa-Rey et al., 2011). According to the studies in the literature, the food processors pay a particular attention in obtaining foods with enhanced sensorial and nutritional characteristics enriched with bioactive compounds. This trend is followed by the health relevance of the bioactive compounds, in particular polyphenols, for consumers. Multiple studies have demonstrated various biological activities of anthocyanins, such as anticarcinogenic, antioxidant, anti-inflammatory, antimutagenic activity, etc. (Lao et al., 2017).

3.2. Objectives of the study

The specific objectives of the studies presented in this chapter were:

- The advanced qualitative and quantitative evaluation of the phytochemical profile of the extract obtained from red GS (Băbească neagră variety) by ultrasound-assisted extraction.
- The evaluation of the biologically active compounds (monomeric anthocyanins, total polyphenols, flavonoids and antioxidant capacity) stability of the extract, in the temperature range 80 - 140°C, at a holding time between 0 - 40 minutes.
- The thermal degradation kinetics of the anthocyanins in the extracts, in correlation to their antioxidant activity, from the perspective of evaluating the processing behaviour and he tuse of extracts as ingredients in the food industry.

- The determination of thermodynamic parameters associated to the degradation of the classes of compounds (polyphenols, flavonoids, anthocyanins) analysed, in order to assess whether the kinetic model related to the process is a thermodynamic model.
- The molecular modelling investigations on their behaviour, during the heat treatment of GS anthocyanins.
- The evaluation of some biological properties of the extract by assessing the inhibition potential of some enzymes involved in the metabolic syndrome.

3.4. Results and discussion

3.4.1. Phytochemical composition of the extract

The chromatographic profile of the extract from red GS, *Băbească neagră* variety, is shown in **Figure** 3.1. The presence of nine anthocyanins was highlighted (Table 3. 1): three main compounds (cyanidin, peonidin and malvidin - peaks 6, 8, 9), and six glucoside derivatives (delphinidin, cyanidin, petunidin, pelargonidin, malvidin and peonidin 3-*O*-cumaryl glucoside - peaks 1, 2, 3, 4, 5, 7). Similar profiles were obtained for the extracts from 14 grape varieties from the Eastern Adriatic region; the anthocyanins identified being delphinidin, cyanidin, petunidin, peonunidin and malvidin, together with their derivatives with 3-monoglucosidic, acetyl and p-cumaryl groups (Budic-Leto et al, 2018). The major peak (5) was identified as malvidin 3-*O*-glucoside, with a concentration of 13.83 ± 0.11 mg concentrated extract from GS/g d.w. Similar results were obtained for the extracts from two grape varieties, Gros noir and Muscat noir, in which malvidin 3-*O*-glucoside was, also, the predominant anthocyanin (Benmeziane et al., 2016). Kharadze et al. (2018) obtained a similar profile for five red grape varieties (Alexandrouli, Mujuretuli, Saperavi, Otskhanuri Sapere, Ojaleshi), in which malvidin 3-*O*-glucoside was the major compound.

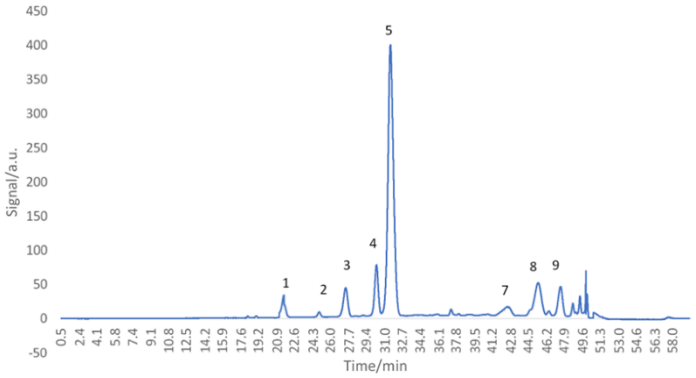


Figure 3.1. HPLC chromatograms of anthocyanin/anthocyanidin profile of *Băbească*

neagră grape skin

Table 3.1. Qualitative and quantitative analysis of the anthocyanins in the GS extract (Băbească neagră) extract by HPLC chromatography

Peak	Compound	RT, min	Anthocyanins, mg/g
1	Delphinidin 3-*O*-Glucoside	21.02 ± 0.02	1.25 ± 0.02
2	Cyanidin 3-*O*-Glucoside	25.01 ± 0.01	0.58 ± 0.00
3	Petunidin 3-*O*-Glucoside	27 ± 0.03	NQ
4	Pelargonidin 3-*O*-Glucoside	29.9 ± 0.01	2.91 ± 0.01
5	Malvidin 3-*O*-Glucoside	30.9 ± 0.02	13.83 ± 0.11
6	Cyanidin	37.8 ± 0.01	0.61 ± 0.00
7	Peonidin 3-*O*-Coumaryl Glucoside	42.2 ± 0.02	NQ
8	Peonidin	45.1 ± 0.04	2.26 ± 0.03
9	Malvidin	47.1 ± 0.04	2.14 ± 0.01

NQ—Not Quantified.

3.4.3. Kinetic behavior of the bioactive compounds under the heat treatment's influence

Influence of the heat treatment on anthocyanins

The main factors affecting the anthocyanins' stability were: chemical structure, concentration, temperature, pH, light, presence of oxygen, nature of solvent, presence of enzymes, flavonoids, proteins or metal ions (Karasu et al., 2016). The effect of the heat treatment on the anthocyanins content of the extract obtained from red GS in the temperature range 80 - 140°C is shown in **Figure 3.2**.

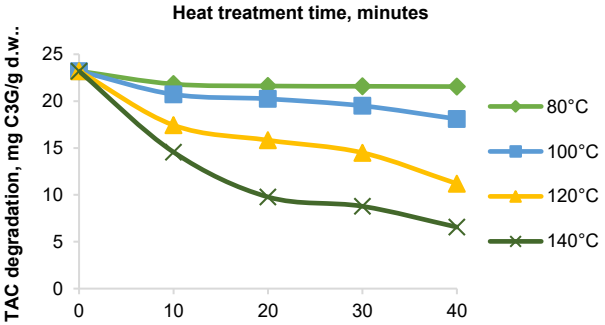


Figure 3.2. Influence of the heat treatment on the stability of TAC from red GS

extract (*Băbească neagră variety*) at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

The obtained results were linearized by logarithmation and the applicability of the angular model of order I was checked, using the linear regression method (**Figure** 3.3).

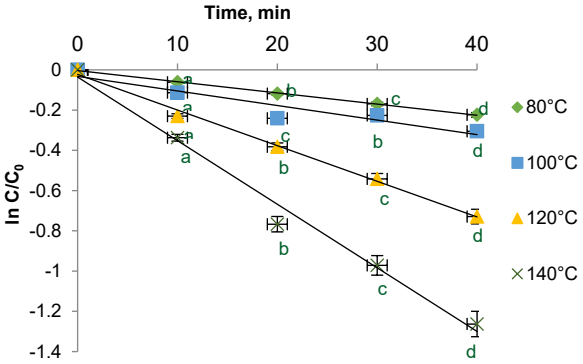


Figure 3.3. Isothermal degradation of the TAC from red GS (*Băbească neagră variety*) extract at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

For all the studied temperatures, the heating for 10 minutes resulted in a significant anthocyanins loss (**Figure** 3.2). At 80°C, after 10 minutes of treatment, a 6% reduction was observed, while at 140°C, a reduction up to 28.60% was assessed. By increasing the heating time to 20 minutes, there was a substantial reduction of the TAC from 11.17% to 53.57% within the whole studied temperature range, from 80°C to 140°C. Similar results have been obtained in other studies carried out on different plant matrices.

Table 3.2. Variation of the thermal degradation kinetic parameters on the total monomeric anthocyanins content(TAC)

Temperature °C	k ·10^{-2} (min^{-1})	$t_{1/2}$ (min)	D (min)	Ea (kJ·mol^{-1})
80	0.56 ± 0.01 ^{d}	123.77 ± 0.21 ^{a}	411.17 ± 4.54 ^{a}	36.63 ± 0.07 ^{a}
100	0.72 ± 0.01 ^{c}	96.27 ± 0.11 ^{b}	319.80 ± 0.51 ^{b}	
120	1.77 ± 0.48 ^{b}	39.16 ± 0.01 ^{c}	130.08 ±1.01 ^{c}	
140	3.16 ± 0.01 ^{a}	21.93 ± 0.30 ^{d}	72.86 ± 0.81 ^{d}	

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05)*

The data presented in **Table 3.2** showed that the anthocyanins extracted from the epicarp of red grape berries (*Băbească neagră variety*) degraded at a slower rate, with k parameter values ranging from 0.56 ± 0.01-10-2 min^{-1} at 80°C to 3.16 ± 0.01-10-2 min^{-1} at 140°C.

Thermal stability of flavonoids

The flavonoids concentration changes (TFC) of the red GS extract after the heat treatment, in the temperature range 80-140°C, as a function of the processing time are shown in **Figure 3.4**.

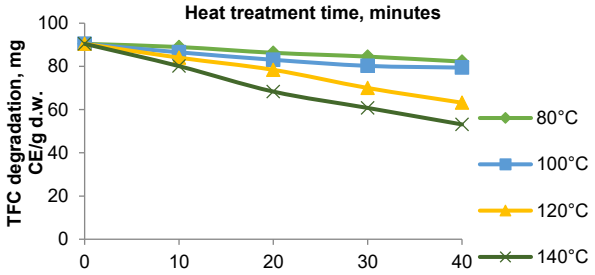


Figure 3.4. Influence of the heat treatment on the TFC stability of the red GS extract (Băbească neagră variety) at different temperatures (◆ 80°C, ■ 100°C, ▲ 120°C, × 140°C)

The flavonoids content of the extract followed a decreasing trend over the studied temperature range. Thus, by increasing the temperature from 100°C to 140°C for 40 min, a reduction from 12.18% to 41.25% of the TFC was observed. The obtained results were linearized by a logarithmic function and the applicability of the first-order kinetic model was checked using the linear regression method (**Figure 3.5**).

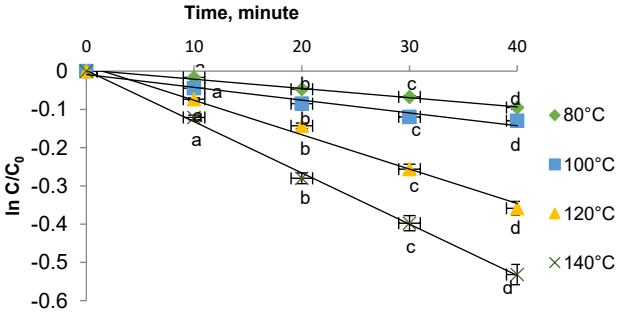


Figure 3.5. Isothermal degradation of TFC in the red GS extract (*Băbească neagră variety*) treated at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140)

The kinetic parameters of the TFC thermal degradation process are presented in **Table 3.3.**

Table 3.3. Variation of the kinetic parameters of the thermal degradation process of TFC in the GS (*Băbească neagră variety*) extract

Temperature °C	$k \cdot 10^{-2}$ (min^{-1})	$t_{1/2}$ (min)	D (min)	Ea ($kJ \cdot mol^{-1}$)
80	0.24 ± 0.002 [d]	288.81 ± 0.40 [a]	959.41 ± 3.71 [a]	37.22 ± 0.02 [a]
100	0.33 ± 0.01 [c]	210.04 ± 0.93 [b]	697.75 ± 1.71 [b]	
120	0.90 ± 0.003 [b]	77.01 ± 0.91 [c]	255.84 ± 1.71 [c]	
140	1.34 ± 0.05 [a]	51.72 ± 0.91 [d]	171.83 ± 0.30 [d]	

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences ($p<0.05$)*

The TFC degradation followed the first-order kinetic model. The values of the degradation rate constant (k) increased from 0.24 ± 0.002·10-2 min^{-1} to 1.34 ± 0.01·10-2 min^{-1}, correlating to increasing temperature. The activation energy for the thermal degradation of the total flavonoids was 37.22 ± 0.002 $kJ\text{-}mol^{-1}$. A reduction in the half-life in regards to flavonoids was correlated to the increasing temperature, namely values of 288.81 ± 0.40 min at 80°C and 51.72 ± 0.91 min at 140°C.

Heat treatment influence on the polyphenols

Following the heat treatment in the studied temperature range (80-140°C, for 40 min), the TPC of the extract obtained from the red GS of Băbească neagră variety decreased from 164.61 ± 4.15 mg GAE /g d.w. to 74.83 ± 4.15 mg GAE/g d.w..

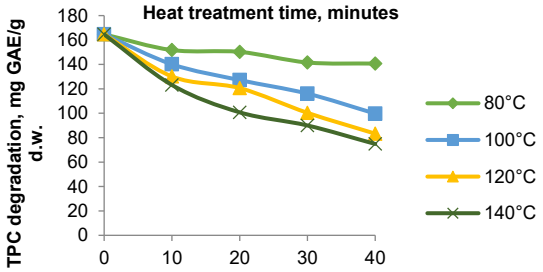


Figure 3.6. The influence of the heat treatment on the TPC of red GS extract (*Băbească neagră variety*) at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

As it can be observed from **Figure 3.6,** by increasing the temperature from 80°C to 140°C and the treatment time from 0 to 40 minutes, a reduction percentage in the total polyphenols content from 14.96% to 45.45% was observed. Also in the case of TPC, the obtained results were linearized by a logarithm and the applicability of the first-order kinetic model was checked using the linear regression method (**Figure** 3.7).

After 10 minutes of heating, the TPC degradation started and progressed rapidly, with a significant reduction from 7.80% at 80°C to 23.50% at 140°C (**Figure 3.7**). A reduction of 22.70% and 38.80% of the concentration of the polyphenols in the extracts was observed by the heat treatment of the samples at 100°C and 120°C for 30 minutes, respectively. Similarly, TPC of a plum (*Prunus domestica*) extract reported by Turturică et al., 2016, was reduced by 4% to 23% by the heat treatment in the 70-90°C temperature range, and the degradation rate reached up to 43-72% in the 100-110°C temperature range.

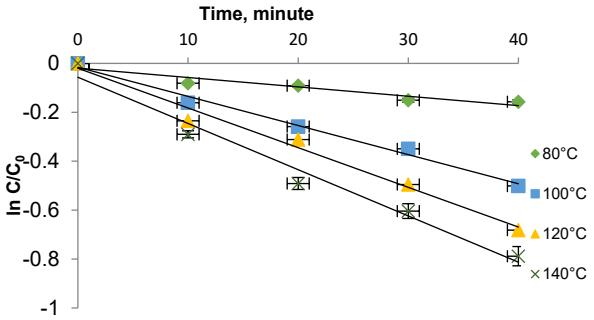


Figure 3.7. Isothermal degradation of the TPC in the red GS extract (*Băbească neagră variety*) treated at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

Table 3.4. The calculated kinetic parameters for the TPC thermal degradation of the red GS extract (*Băbească neagră variety*)

Temperature °C	k ·10^{-2} (min^{-1})	$t_{1/2}$ (min)	D (min)	Ea (kJ·mol^{-1})
80	0.38 ± 0.002 ^{d}	182.40 ± 6.04 ^{a}	605.94 ± 0.20 ^{a}	31.61 ± 0.07 ^{a}
100	1.19 ± 0.01 ^{c}	58.24 ± 3.01 ^{b}	193.49 ± 0.51 ^{b}	
120	1.62 ± 0.003 ^{b}	42.78 ± 1.01 ^{c}	142.13 ± 0.91 ^{c}	
140	1.89 ± 0.001 ^{a}	36.67 ± 3.03 ^{d}	121.82 ± 0.41 ^{d}	

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05)*

For thermal degradation of TPC, the value of the degradation rate constant (k) increased from 0.38 ± 0.002·10-2 min^{-1} to 1.89 ± 0.001·10-2 min^{-1}.

By increasing the temperature from 80°C to 140°C, a substantial reduction of the half-life (t1/2) resulted, which ranged from 79.20 ± 0.004 min at 80°C to 15.92 ± 0.003 min at 140°C.

Influence of the heat treatment on the antioxidant activity

Following the heat treatment, the antioxidant capacity, expressed as anti-radical activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH method) decreased. In the studied temperature range, 80-140°C, there was a decrease of the antioxidant capacity of 6.52-46.89% for the treatment time of 40 minutes (**Figure 3.8**). By increasing the thermal processing time, a gradual decrease of the antioxidant capacity was observed. Thus, after 40 minutes of treatment at 120°C, a decrease in the antioxidant capacity of 38.41% was observed and after 40 minutes of treatment at 140°C, a decrease in the antioxidant capacity of 46.89% was also assessed.

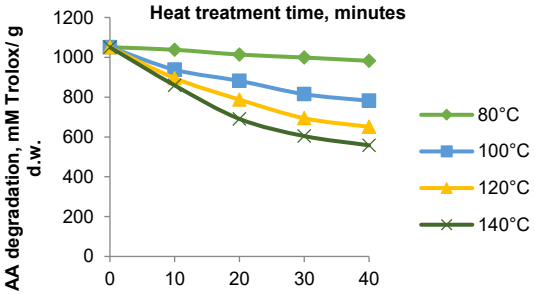


Figure 3.8. Influence of the heat treatment on the AA stability of red GS extract (*Băbească neagră variety)* at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

As it can be seen from **Figure 3.8**, the AA of the extract was influenced by the temperature. Thus, in the temperature range 80-140°C, there was a reduction of AA values, which ranged within 1.21-18.25%, after 10 minutes. The degree of the degradation was influenced by the temperature but also by the heat treatment time (**Figure 3.9**). Thus, after 40 minutes of treatment at 120°C, a 38.05% decrease of AA values was observed and a 44.02% decrease by treatment at 140°C, considering the same processing time.

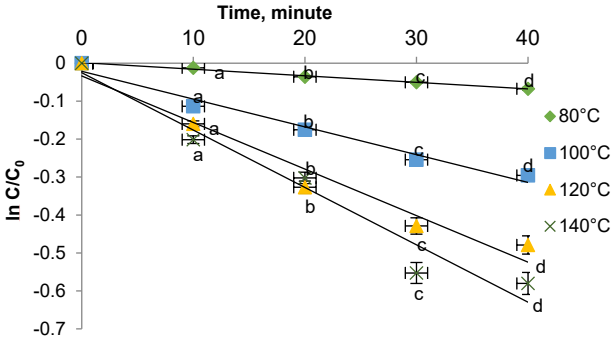


Figure 3.9. Isothermal degradation of the AA in the red GS extract (*Băbească neagră variety*) at different temperatures (◆ 80°C, ■100°C, ▲120°C,× 140°C)

These variations were correlated to the degradation of bioactive compounds with antioxidant capacity. Table 3.5 shows the variation of the kinetic parameters of AA of the extract by thermal degradation. The values of the degradation rate constant (k) increased with the increasing temperature, suggesting a high thermal sensitivity of the bioactive compounds in the red GS (*Băbească neagr*ă variety). For the AA, an increase of the k parameter value was observed, from 0.17 ± 0.03 -10-2 min^{-1} to 1.51 ± 0.01-10-2 min^{-1}.

Table 3.5. Kinetic parameters of the antioxidant activity (DPPH method) variation by the heat treatment of the red GS extract (*Băbească neagră variety*)

Temperature °C	k ·10^{-2} (min^{-1})	$t_{1/2}$ (min)	D (min)	Ea (kJ·mol^{-1})
80	0.17 ± 0.03 ^{d}	407.73 ± 1.20 ^{a}	1354.46 ± 3.01 ^{a}	43.59 ± 0.02 ^{a}
100	0.73 ± 0.01 ^{c}	94.95 ± 0.26 ^{b}	315.42 ± 0.71 ^{b}	
120	1.23 ± 0.01 ^{b}	56.35 ± 0.20 ^{c}	187.20 ± 0.91 ^{c}	
140	1.51 ± 0.01 ^{a}	45.90 ± 1.12 ^{d}	152.48 ± 0.02 ^{d}	

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05)*

3.4.4. Study of the thermodynamic behavior of the bioactive compounds in the extract

For the total monomeric anthocyanins (TAC), the data presented in Table 3.6, showed that ΔH varied with the temperature from 33.69 ± 0.41 kJ/mol to 33.20 ± 0.25 kJ/mol. The positive values of ΔH certified the degradation of the anthocyanins

by an endothermic reaction (Mercali et al., 2015). The spontaneity of the chemical reaction was described by the constant ΔG, which in this study ranged from 120.01 ± 1.04 kJ/mol to 113.94 ± 1.04 kJ/mol. This indicated that the TAC degradation was an irreversible and non-spontaneous reaction. The activation entropy (ΔS) represents the disorder degree of the molecules in a system and is related to the number of molecules with an adequate energy that can react (Peron et al., 2017).

Table 3.6. Thermodynamic parameters describing the TAC degradation in the red GS extract (*Băbească neagră variety*)

Temperature (˚C)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
80	33.69 ± 0.41 [a]	-193.50 ± 2.11 [a]	120.01 ± 1.04 [a]
100	33.53 ± 0.25 [a]	-197.44 ± 2.12 [c]	107.17 ± 1.06 [d]
120	33.36 ± 0.17 [a]	-195.39 ± 3.06 [b]	110.15 ± 1.08 [c]
140	33,20 ± 0,25[a]	-195.50 ± 2.09[b]	113,94 ± 1,04[b]

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05)*

Table 3.7 shows the values of enthalpy of activation (ΔH), free decay energy (ΔG) and entropy of activation (ΔS) for the thermal behaviour of flavonoids at different temperatures. From the analysis of the obtained data, it can be seen that ΔG varied between 104.49 and 116.88 kJ/mol with the increasing temperature in the range of 80-140°C.

The ΔS values were correlated to the number of molecules with an adequate energy that could react efficiently. The negative ΔS values indicated a smaller structural freedom than the reactants and also confirmed that the process was irreversible. The ΔS values ranged from -198.88 J/mol-K at 80°C to -201.20 J/mol-K at 140°C.

Table 3.7. The thermodynamic parameters describing the TFC degradation in the red GS extract (Băbească neagră variety)

Temperature (˚C)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
80	34.28 ± 0.21 [a]	−198.88 ± 0.79 [a]	104.49 ± 1.04 [d]
100	34.12 ± 0.14 [a]	−202.34 ± 0.80 [c]	109.59 ± 1.06 [c]
120	33.95 ± 0.20 [a]	−199.51 ± 0.95 [ab]	112.36 ± 1.12 [b]
140	33.79 ± 0.14 [a]	−201.20 ± 0.79 [bc]	116.88 ± 1.14 [a]

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05)*

Table 3.8. The thermodynamic parameters describing the TPC degradation in the red GS extract (*Băbească neagră variety*)

Temperature (˚C)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
80	28.68 ± 0.14 [a]	−210.94 ± 0.54 [c]	103.14 ± 1.00 [d]
100	28.51 ± 0.17 [a]	−206.71 ± 0.21 [a]	105.62 ± 1.12 [c]
120	28.34 ± 0.09 [a]	−208.90 ± 0.14 [b]	110.44 ± 1.16 [b]

140	28.18 0.14 [a]	−211.920.14 [c]	115.70 ± 1.18 [a]

**The results are the mean of 3 independent determinations ± standard deviation. Different letters per column indicate significant differences (p<0.05).*

The low values of the ΔH activation in terms of the TPC degradation (**Table 3.8**) indicated that the formation of the activated complex was favoured because the energy barrier to reach the transition state was low. The activation enthalpy values were similar for all the experimental conditions, ranging from 26.68 kJ/mol to 28.18 kJ/mol. The positive sign of ΔH indicated an endothermic degradation process of the TPC.

The low values of the ΔH obtained for the degradation of the antioxidant activity (**Table 3.9**) indicated that the formation of the activated complex was favoured because the energy barrier required to reach the transition state was low. The activation enthalpy values were low for all the experimental conditions, ranging from 40.66 ± 0.15 kJ/mol to 40.16 ± 0.17 kJ/mol. In general, the ΔH is related to the strength of bonds broken and formed in the transition state between the reactants, as well as to the solvation effect, which may differ for a given molecule.

The ΔG values increased with the increasing temperature, indicating an unsteady reaction. The negative ΔS values suggested that the temperature-induced transition state had less structural freedom compared to the reactants, and the changing process of the antioxidant activity in terms of the temperature effect was irreversible.

Table 3.9. The thermodynamic parameters describing the AA variation in the red GS extract (*Băbească neagră variety*)

Temperature (˚C)	ΔH (kJ/mol)	ΔS (J/mol·K)	ΔG (kJ/mol)
80	40.66 ± 0.15 [a]	−183.68 ± 2.08 [c]	105.50 ± 1.04 [d]
100	40.49 ± 0.52 [a]	−178.65 ± 2.14 [a]	107.13 ± 1.07 [c]
120	40.33 ± 1.02 [a]	−180.69 ± 2.02 [b]	111.34 ± 1.09 [b]
140	40.16 ± 0.17 [a]	−184.77 ± 2.07 [c]	116.47 ± 1.04 [a]

**The results are the mean of 3 independent determinations ± standard deviation.vDifferent letters per column indicate significant differences (p<0.05)*

3.4.5. Evaluation of the extract's potential to inhibit some enzymes involved in metabolic disorders

Digestive enzymes are key enzymes that influence the digestion and absorption of nutrients. The decreased activity of the α-amylase and α-glucosidase enzymes lead to a reduced carbohydrate metabolism (Yang et al., 2020).

The metabolic syndrome is a metabolic disorder with multiple etiologies, characterized by chronic hyperglycemia and impaired carbohydrate and fat metabolism (Costamagna et al., 2016).

The in vitro inhibitory effect on the enzymes, α-glucosidase, α-amylase,

pancreatic lipase and lipoxygenase, of the red GS extract (*Băbească neagră variety*) was evaluated before and after the heat treatment at 80°C and 140°C, for 20 minutes. The inhibitory capacity was tested using various extract concentrations of 0.50, 1 and 5 µg/mL and the results are shown in **Table 3.10** and **Table 3.11.**

Table 3.10. The ability of the non-heat-treated red GS (*Băbească neagră variety*) extract to inhibit the enzymes involved in the metabolic syndrome

Sample	IC_{50} (µg/mL extract)			
	α-Amylase	α-Glucosidase	Lipase	LOX
Extract	3.06 ± 0.30[a]	1.06 ± 0.16[b]	7.62 ± 0.86[a]	1.64 ± 0.71[a]
Acarbose	3.91 ± 0.44[a]	1.75 ± 0.14[a]	-	-
Orlistat	-	-	3.18 ± 0.33[b]	-
Quercetin	-	-	-	1.18 ± 0.20[a]

**The results are the mean of 3 independent determinations ± standard deviation.Different letters per column indicate significant differences (p<0.05).*

The untreated extract exhibited a high α-amylase enzyme inhibition capacity at low concentrations. The results indicated that the heat-treated extract could successfully substitute acarbose. The untreated red GS extract had a lower IC_{50} value for α-glucosidase (1.06 ± 0.16 µg/mL) compared to α-amylase (3.06 ± 0.30 µg/mL), with values close to the inhibition capacity of the reference substances.

The extract also exerted an inhibitory effect on the pancreatic lipase. The IC_{50} value of the extract was 7.62 ± 0.86 µg/mL, while for the Orlistat control sample it was significantly lower (3.18 ± 0.33 µg/mL). For the lipoxygenase, the IC_{50} value of the extract was 1.64 ± 0.07 µg/mL, higher than that of the control sample with quercetin (1.18 ± 0.02 µg/mL).

The enzyme inhibition capacity was also tested for the heat-treated extract for 20 minutes, at 80°C and 140°C (**Table 3.11**).

Table 3.11. The ability of the heat-treated of the red GS (Băbească neagră variety) extract to inhibit the enzymes involved in metabolic syndrome

Temperature	IC_{50} (µg/mL extract)			
	α-Amylase	α-Glucosidase	Lipase	LOX
25°C	3.06 ± 0.30[a]	1.06 ± 0.16[b]	7.62 ± 0.86[c]	1.64 ± 0.71[c]
80°C	3.14 ± 0.33[a]	1.02 ± 0.17[b]	8.70 ± 0.38[b]	2.29 ± 0.01[b]
140°C	5.06 ± 0.33[a]	1.79 ± 0.02[a]	13.37 ± 1.71[a]	2.75 ± 0.01[a]

**The results are the mean of 3 independent determinations ± standard deviation. Different letters per column indicate significant differences (p<0.05)*

For each of the tested enzyme, IC_{50} values increased with the increasing treatment temperature ($p<0.05$) (**Table 3.11**). This fact was correlated to the thermal denaturation of the bioactive compounds in the extract. Thus, for α-amylase the IC_{50} value increased from 3.06 ± 0.30 µg/mL extract to 5.06 ± 0.31 µg/mL extract

by increasing the treatment temperature of the extract. A less obvious slight increase in the IC_{50} value with a significant effect ($p<0.05$) was observed for α-glucosidase, from 1.06 ± 0.16 µg/mL extract (25°C) to 1.79 ± 0.02 µg/mL extract, treated at 140°C. The lipase inhibition activity showed the highest increase from 7.62 ± 0.86 µg/mL extract (25°C) to 13.37 ± 1.71 µg/mL extract, treated at 140°C. The lipoxygenase inhibition activity showed an increase from 1.64 ± 0.71 µg/mL extract (25°C) to 2.75 ± 0.01 µg/mL extract, treated at 140°C.

The effect of the anthocyanins in the red GS (*Băbească neagră variety*) extract on the metabolic syndrome-associated enzymes was studied by *in silico* molecular docking experiments. The complexes resulted from the binding of the major anthocyanin, malvidin 3-O-glucoside (M_3G), in the studied extract by the enzymes α-amylase, α-glucosidase, lipase and lipoxygenase are shown in **Figure 3.10.** The analysis of the resulting docking patterns involving the α-amylase enzyme as a receptor indicated that the higher scoring complexes, the M_3G attached to the surface of the enzyme in the close proximity to the catalytic site (**Figure 3.10 a**).

The anthocyanins bound to Trp^{58}, Trp^{59}, Tyr^{62}, Leu^{162}, Thr^{163}, Leu^{165}, Asp^{300}, His^{305} and Gly^{306} residues in the enzyme structure. In the case of α-glucosidase, M_3G binding to the amino acid residues in the catalytic centre was not obvious. The results of the molecular docking simulations where the lipase was, the receptor indicated that the M_3G molecules attach to a surface cavity on the surface of the enzyme with a high affinity, in close proximity to the Phe^{77} amino acid residue located in the catalytic centre (**Figure 5.10 c**). The M_3G binding in the vicinity of the catalytic site influenced the recognition of the specific substrate and its transformation by lipase (Garza et al., 2011).

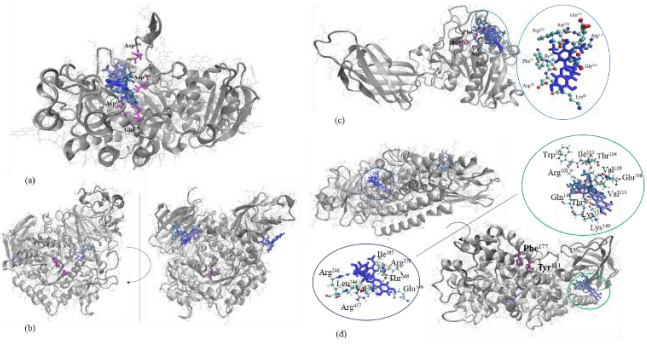


Figure 3.10. The binding of the anthocyanin (M_3G) from the red GS (*Băbească neagră variety*) extract to the α-amylase (a), α-glucosidase (b), lipase (c) and lipoxygenase (d) enzyme molecules demonstrated by molecular docking. Enzyme molecules-grey-silver; M_3G molecule-blue shades, blue-greenish blue; Amino acid residues involved in M_3G binding-purple. The images were obtained

using the VMD software (Humphrey et al., 1996; Serea et al., 2022).

Two different M_3G binding sites of the lipoxygenase were identified (**Figure 3.10 c**). Both of the potential ligand binding sites were located in the narrow areas of the surface of the positively charged protein. The first binding site was polar and involved three residues of the amino acid arginine (Arg) located at positions 246, 370 and 457. The second binding site contained non-polar residues consisting of three positively charged amino acids (Arg^{101}, Lys^{133} and Lys^{140}) and one negatively charged amino acid (Glu^{108}). Both of the ligand binding sites were spaced apart from the Phe^{177} and Tyr^{181} amino acid residues in the catalytic center (Gilbert et al, 2011). Given the bioactive compounds-rich composition of the extract from red GS (*Băbească neară variety*), although their concentration was quite low compared to the M_3G, they might affect the activity of enzymes associated to the metabolic syndrome. Therefore, the experimental results obtained could be explained on the basis of the cumulative effect of the compounds in the extract.

3.5. Partial conclusions

- The thermal stability of the bioactive compounds (anthocyanins, flavonoids, polyphenols) in the red GS extract (*Băbească neagră variety*) obtained by ultrasound-assisted extraction (40 kHz) using 85% ethanol mixed with 0.85% citric acid as a solvent at 57°C for 52 minutes was studied.
- The thermal inactivation kinetics were studied at a frequency of 10 minutes, for 40 minutes, by a treatment at the temperatures of 80°C, 100°C, 120°C and 140°C. The degradation of the anthocyanins, flavonoids and polyphenols, as well as the changes of the antioxidant activity of the extract corresponded to a first-order kinetic model.

➢ The obtained kinetic and thermodynamic parameters demonstrated a high temperature dependence for the bioactive compounds and a moderate dependence for the antioxidant activity of the extract.

➢ The kinetic studies showed a high thermostability of the anthocyanins of the extract at temperatures in the range of 80-120°C, correlated to the maintenance of the stability of the antioxidant activity. The extract has been proven to be effective in inhibiting the activity of some enzymes with a metabolic impact (α-amylase, α-glucosidase, lipase and lipoxygenase), with a higher degree of inhibition compared to the control samples (specific reference compounds). The inhibitory potential was substantially reduced by the heat treatment of the extract at the temperatures between 80°C and 140°C.

➢ By in silico molecular docking, the effect of malvidin 3-*O*-glucoside anthocyanin (M_3G) in inhibiting the studied enzymes was demonstrated, which provided valuable insights into the technological applicability and functional potential of the studied extract, correlated to the superior thermal stability demonstrated for anthocyanins.

CHAPTER 4.

THE VALORISATION OF THE RED GRAPES (BĂBEASCĂ NEAGRĂ VARIETY) EXTRACT TO OBTAIN BIOACTIVE INGREDIENTS BY MICROENCAPSULATION

4.1. General aspects

The development of functional plant-based food ingredients has become a major focus of the modern food industry, in response to the consumers' demands for the need to replace synthetic additives, but also to the awareness of a balanced nutrition's importance for health. Therefore, there is great interest in the identification, isolation, characterization and use of biologically active compounds from plant sources, especially through the valorisation of agro-food by-products (Domínguez et al., 2020).

In recent decades, many research studies have focused on the separation, characterization and use of biologically active compounds in the food industry (Munekata et al., 2020). However, many bioactive compounds from plant sources do not have applications in the food industry due to their low solubility, poor chemical stability or negative impact on the food quality and safety. The compounds from natural sources have antioxidant, antimicrobial, flavouring, colouring and biologically active properties and are widely used in the food industry for technological and functional purposes (Lorenzo et al., 2018). Food processing can result in the decrease or loss of this functionality of these compounds in the food systems.

4.2. Objectives of the study

In this chapter, different strategies were proposed for the development of multi-functional ingredients that exploit the potential of red grape GS (Băbească neagră variety) as a valuable source of biologically active compounds through microencapsulation. Thus, the polyphenolic compounds from GS were extracted using optimised extraction techniques, presented in Chapter 2, in order to obtain a higher extraction yield of the bioactive compounds and the increase of the antioxidant potential of the extract.

The aim was to stabilize the biological properties of the bioactive compounds by applying freeze-drying as a microencapsulation technique. Several functional composites were obtained in the form of fine, coloured powders with a high functionality. The obtained powders were analyzed in terms of the microencapsulation efficiency of the biologically active compounds, phytochemical composition and antioxidant potential.

4.4. Results and discussion

4.4.1. Comparative analysis of the bioactive compounds' encapsulation efficiency

Microencapsulation was used to obtain high quality functional powdered composites, thus minimizing the oxidative degradation of the targeted biologically active compounds from the red GS extract (*Băbească neagră variety*). The production of the microcapsules was achieved by a combined method using complex coacervation followed by freeze-drying, methods that resulted in fine, purple powders. The resulting powders were characterized in terms of their microencapsulation efficiency, TAC, TFC, TPC and antioxidant activity.

The encapsulation efficiency of the anthocyanins, the major bioactive compounds in the composition of the extract obtained by ultrasound-assisted solvent extraction was determined (Oancea et al., 2018). The data presented in **Table 4.1**, demonstrated that the encapsulation efficiency of the anthocyanins from the red GS extract was influenced by the components of the encapsulation matrix and their ratio, as the mixture with a high concentration of polysaccharides allowed the obtainment of higher values for the microencapsulation efficiency of anthocyanins. Thus, the encapsulation efficiency increased significantly with the carbohydrate concentration, values that ranged from 85.93 ± 0.44% to 93.24 ± 0.65 (V1), 72.25 ± 0.18% to 66.25 ± 1.75% (V2) to 66.13 ± 1.11% to 57.99 ± 1.73% (V3), respectively.

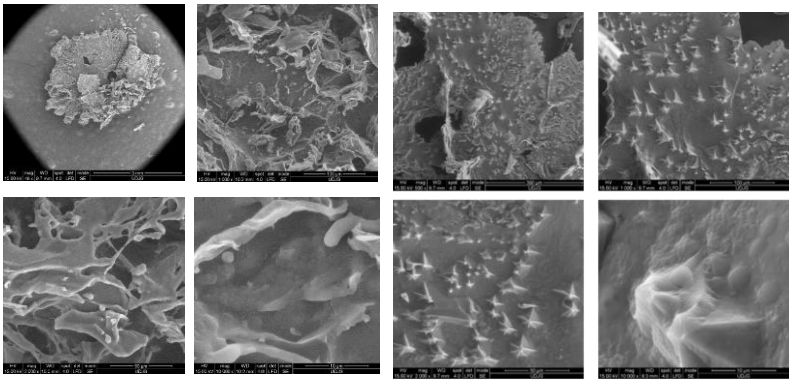
Table 4.1. Encapsulation efficiency of the anthocyanins from the red GS extract (*Băbească neagră varity*)

Experimental variants	Encapsulation matrix formulation variants	Encapsulation efficiency, %
V1	V1-1	85,93 ± 0,44
	V1-2	93,24±0,65
V2	V2-1	72,25 ± 0,18
	V2-2	66,25 ± 1,75
3	V3-1	66,13±1,11
	V3-2	57,99 ± 1,73

4.4.2. Morphological and structural analysis of the composites obtained by the extract's encapsulation

The external morphology of the powders can be visualized in the SEM images shown in **Figure 4.2.** The powders showed significant morphological differences in correlation to the characteristics of the encapsulation matrices. The microstructure of the powders presented different features in terms of their behaviour and the controlled release of the bioactive molecules.

In the case of the obtained powders by experimental variant V1-2 (**Figure 4.2 a**), in which the encapsulating materials were WPI and CMC, both the vesicular and polyhedral formations distributed on the curved surfaces connected by the ridge zone were visible.



(a) (b)

Figure 4.2. SEM images of the composites (powders) obtained by encapsulating the red grape (*Băbească neagră*) skin extract into various matrices: (a) Whey protein isolate and carboxymethyl cellulose (V1-2); (b) Whey protein isolate and gum arabic (V3-2**)**

It can be seen that the outer surface of the microcapsule had a smooth appearance due to the film-forming properties of WPI. **Figure 4.2 b** shows a stellate structure of a non-uniform size, this fact was due to the use of gum arabic and freeze-drying. The indentations on the surface of the microcapsule indicated a higher retention of the microencapsulated extract. Roughness may also occur due to the abrupt temperature change during the drying process, when water evaporated rapidly, causing the powder to shrink in the cooling stage (experimental variants V3-2).

4.4.3. Phytochemical characterisation of the powders and the evaluation of the bioactive potential's stability by preservation

Table 4.2. presents the phytochemical profile of the powders obtained by compositional variation of the bioactive compounds encapsulation matrices.

Table 4.2. Phytochemical profile of the composites obtained with the *Băbească neagră variety* extract

Experimental variants	Encapsulation matrix formulation variants	TAC mg C_3G/g d.w.	TFC, mg CE/g d.w.	TPC, mg GAE/g d.w.	AA, mM Trolox/g d.w.
	V1-1	9.03 ± 1.27	8.67±0.13	61.99±6.66	44.84±0.42
	V1-2	10.35±0.57	10.81±0.17	71.04 ± 4.58	46.19± 0.61
V2	**V2-1**	2.52±0.18	7.60±0.26	26.14±1.93	40.82 ± 0.86
	V2-2	2.94±0.13	6.43 ± 0.66	32.63 ± 2.66	49.56±0.46
V3	**V3-1**	2.94 ± 0.13	6.43±0.66	32.63 ± 2.66	40.81±0.86
	V3-2	3.37 ± 0.09	8.50±1.14	33.08±5.29	44.35±1.58

As it can be seen from Table 4.2, the matrix composition influenced the binding efficiency of the bioactive compounds, with superior results being obtained by combining WPI with CMC in a 1:1 ratio, for all the studied categories of bioactive compounds, except for the antioxidant activity, for which a higher value was obtained in the case of the V2-2 variant (49.56±0.46 mM Trolox/g d.w.), although the encapsulation efficiency was lower around 66.25 ± 1.75%. This fact could be explained by the different distribution of the biologically active compounds within the matrices, with a higher distribution at the microparticles surface in the case of V2-2 compared to V1-1, for which the compounds were concentrated inside the microparticles.

Table 4.3. shows the storage stability (after 28 days, at room temperature) of the phytochemical composition of the composite powders formulated as different encapsulation variants.

Table 4.3. Stability of the bioactive compounds in the composite powders after 28 days storage, at room temperature, in the absence of light

Time of analysis/ Encapsulation variant	TAC mg C_3G/g d.w.	TFC, mg CE/g d.w.	TPC, mg GAE/g d.w.	AA, mM Trolox/g d.w.
		V1-1		
Originally	**9.03 ± 1.27 ^{aA}**	**8.67±0.13^{aA}**	**59.68±6.66$^{a\ A}$**	**44.84 ± 0.42^{aA}**
After 28 days	**9,00 ± 1.24^{aA}**	**8.65 ± 0.13^{aA}**	**61.78±6.63^{aA}**	**44.80 ± 0.42^{aA}**
		V1-2		
Originally	**10.35 ± 0.57^{aA}**	**10.81±0.17^{aA}**	**71.04 ± 4.58^{aA}**	**46.19± 0.61^{bA}**
After 28 days	**10.22 ± 0.55^{aA}**	**10.78 ± 0.17^{aA}**	**71.00 ± 4.48^{aA}**	**46.12 ± 0.58^{bA}**
		V2-1		
Originally	**2.52 ± 0.18^{bA}**	**7.60 ± 0.26 ^{aA}**	**26.14±1.93^{bA}**	**40.82 ± 0.86^{bA}**
After 28 days	**2.48 ± 0.15 ^{bA}**	**7.58 ± 0.24 ^{bA}**	**26.09±1.90^{bA}**	**40.78 ± 0.83^{bA}**
		V2-2		
Originally	**2.94 ± 0.13^{bA}**	**8.36 ± 0.66^{bA}**	**32.63 ± 2.66^{bA}**	**49.56 ± 0.46^{aA}**
After 28 days	**2.90 ± 0.13^{bA}**	**8.28 ± 0.63^{bA}**	**32.60 ± 2.63^{bA}**	**49.36 ± 0.36^{aA}**
		V3-1		
Originally	**2.94 ± 0.13 ^{bA}**	**6.43 ± 0.66^{cA}**	**32.63 ± 2.66^{bA}**	**40.81 ± 0.86^{bA}**
After 28 days	**2.90 ± 0.13 ^{bA}**	**6.40 ± 0.63^{cA}**	**32.58 ± 2.59^{bA}**	**40.71 ± 0.78^{bA}**
		V3-2		
Originally	**3.37 ± 0.09^{bA}**	**8.50 ± 1.14^{bA}**	**33.08 ± 5.29^{bA}**	**44.35 ± 1.58^{bA}**
After 28 days	**3.36 ± 0.08^{bA}**	**8.48 ± 1.12^{bA}**	**33.05 ± 5.24^{bA}**	**44.15 ± 1.38^{bA}**

** For each tested phytochemical compound and each powder variant, the values that are on the same row that do not share the same lowercase letters are statistically different at p<0.05.*

The analysis of the data showed that by storing the powders for 28 days, at room temperature, in the absence of oxygen and light, the bioactive properties were better maintained and the TAC, TFC, TPC and AA values did not vary significantly ($p<0.05$) upon storage.

4.5. Partial conclusions

The study presented in this chapter aimed at exploiting the extract of red GS (*Băbească neagră variety*), rich in bioactive compounds, to obtain ingredients with a functional potential through microencapsulation. The obtained results allowed the elaboration of the following partial conclusions:

- The combination of various microencapsulation techniques (coacervation - lyophilization) aimed at the comparative use of the biopolymeric matrices, based on whey protein isolate, maltodextrin, carboxymethylcellulose, pectin and gum arabic, in different concentrations and proportions for the microencapsulation of red grape GS extract (*Băbească neagră variety*) (in six experimental variants).
- Regardless of the used encapsulation medium, fine, purple powders with microparticles of various sizes resulted.
- The obtained powdered composites were characterized in terms of their composition in the biologically active compounds and encapsulation efficiency. The results showed

an increased encapsulation efficiency of the anthocyanins.

- The microencapsulation efficiency for the tested variants varied depending on the encapsulation material, between 57% and 93%. The mixture consisting of 1% whey protein isolate and 1% carboxymethyl cellulose (1:1) (V1-2) solutions resulted in the most efficient binding rate of the bioactive compounds compared to the other tested variants. The obtained powder showed the highest total anthocyanins content of 10.35 ± 0.57 mg C_3G/g d.w. and an antioxidant activity of 46.19 ± 0.61 mMol Trolox/g d.w..
- The stability of the bioactive potential was maintained after 28 days of storage, at room temperature, with a limited access to oxygen and light.
- The results showed remarkable microencapsulation properties of the biopolymer combination obtained from whey protein isolate and carboxymethyl cellulose, both in terms of their encapsulation efficiency and retention potential of the bioactive compounds in the microparticles upon a controlled storage.
- These data supported the hypothesis that the microencapsulation of the extract from red GS (*Băbească neagră variety*) allowed the obtainment of functional composites (powders) that can be recommended as value-added bioingredients with beneficial technological and functional properties for their use in the food industry but also in other fields, for obtaining feed, nutraceuticals, cosmetics, etc.

CHAPTER 5.

THE OBTAINMENT OF VARIOUS VALUE-ADDED FOOD PRODUCTS THROUGH THE VALORISATION OF THE BIOLOGICALLY ACTIVE COMPOUNDS EXTRACTED FROM THE EPICARP OF RED GRAPES (BĂBEASCĂ NEAGRĂ VARIETY)

5.1. General aspects

The food industry is currently facing problems related to the management of by-products resulting from the industrial processing of fruit and vegetables (Bertagnolli et al., 2014). The fruits and vegetables' processing industry has evolved considerably over the last 25 years, this has also been driven by the increased demand for the pre-processed and packaged foods, especially ready-to-eat (RTE) preparations, and consequently the amount of waste products has increased. The by-products from the fruit and vegetable processing were often disposed relatively cheaply through composting or used as animal feed. However, since the Kyoto agreement in 1997, the issue of by-products in modern society has become more prominent as it contributes to many of the environmental sustainability problems. Large quantities of by-products from food processing are generated worldwide. In Europe, about 100 million tonnes of waste and by-products are generated every year, with the beverage industry (26%) with fruit and vegetable processing (14.80%) accounting for the largest share (Marić et al, 2018). In terms of the food categories, the highest level of loss from post-harvest to the distribution is linked to the roots, tubers and oilseed crops (25%), cereals (9%), fruit and vegetables (30%), meat and other animal products (12%) (Socas-Rodríguez et al., 2021).

5.2. Objectives of the study

The studies presented in this chapter have had the following specific objectives:

- To develop new technologies for the production of two value-added products (gluten-free biscuits, beer) by exploiting the functional potential of the bioactive compounds from the skins of the red (GS) grape berries (*Băbească neagră* variety).

- The obtainment of the technological recipes of the targeted products (biscuits and beer) with the addition of the GS derivatives, powder and extract.

- The evaluation of the phytochemical and sensory characteristics of the products and the storage bioactive potential stability.
- The evaluation of the sensory characteristics of the obtained products.

5.3. Materials and methods

5.3.1. Materials, reagents, and equipment

The commercial ingredients, rice flour, corn flour, coconut butter, green sugar, lemon essence, baking powder, egg and salt, were purchased from a supermarket in Galati.

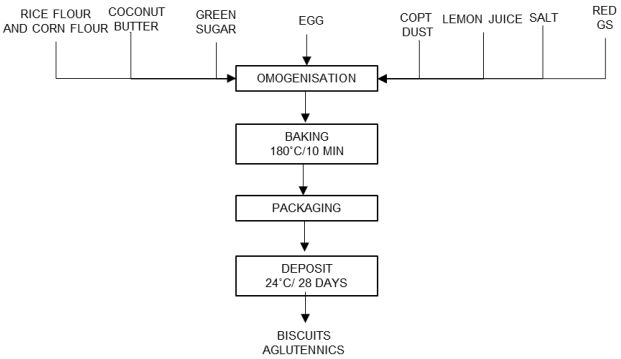


Figure 5.1. Block diagram of the unit operations emplyed to obtain the biscuits with the addition of the freeze-dried powder from the red GS (*Băbească neagră variety*)

The raw and auxiliary materials used to make the biscuits were: 250 g agglutinous flour mix (188 g rice flour and 62 g corn flour), 70 mL coconut butter, 100 g green sugar, 1 egg, 2.50 g baking powder, 1 mL fresh lemon juice, 0.1 g salt. After the preliminary preparation, the raw materials were blended with a household blender for 10 minutes. During the blending, the powder obtained from the red GS was added at a concentration of 1% (w/w)(P1), and 2% (w/w)(P2). The control sample was made from the same ingredients without the addition of the powder. The samples were assessed in duplicate. The biscuits were baked at 180˚C, for 10 minutes. After the baking, the biscuits were packed in an airtight container and stored at room temperature (24˚C) for 28 days of storage.

5.3.4. The obtainment and characterisation of a beer assortment with grape skin extract

A white beer (made from wheat) and a lager beer were purchased on the local market in Galati, Romania, and were used as the basis for the supplementation with red GS extract (GSE) to obtain value-added products. Three different concentrations of the GSE, namely 1, 5 and 10 mg/mL, were added to the beer samples and the resulting experimental variants were coded as follows: P1 + 1 mg GSE/mL, P1 + 5 mg GSE/mL, P1 + 10 mg GSE/mL and P2 + 1 mg GSE/mL, P2 + 5 mg GSE/mL, P2 + 10 mg GSE/mL, respectively. The GSE concentration was determined based on the results of a preliminary sensory test aimed at identifying the GSE concentration without impacting the taste of the final product. The control sample consisted of beer without GSE. The samples were stored at 4°C and their quality was assessed during 21 days of storage, in the dark.

5.4. Results and discussion

5.4.1.2. Characterisation of the bioactive potential of the value-added gluten-free biscuits

The phytochemical composition and the antioxidant activity of the obtained biscuits are presented in Table 5.2. The results confirmed the bioactive potential of the powder from the red GS (Băbească neagră variety), the content of bioactive compounds in the biscuits increasing proportionally with the concentration of the added powder. Thus, the addition of the GS powder in the composition of the biscuit dough, at a concentration of 1% (g/g) (P1) and 2% (g/g) (P2), respectively, led to a clear increase in the concentration of anthocyanins, from 6, 80 ± 0.01 mg C_3G/100 g biscuits (P1) to 9.90 ± 1.70 mg C_3G/100 g biscuits (P2) and the polyphenol content from 142.50 ± 14.30 mg GAE/100 g biscuits to 235.70 ± 14.40 mg GAE/100 g biscuits. The antioxidant activity varied depending on the used method, which was correlated concentration of the compounds.

Table 5.2. Phytochemical composition of gluten-free biscuits

Parameter	Sample		
	M	P1	P2
TAC, mg C_3G/100 g product	-	6.80 ± 0.01^a	9.90 ± 1.70^b
TFC, mg EC/100 g product	92.80 ± 12.00^a	92.90 ± 15.40^a	105.20 ± 1.30^a
TPC, mg EAG/100 g product	142.50 ± 14.30^c	188.90 ± 14.30^b	235.70 ± 14.40^a
AA (DPPH), mM Trolox/100 g product	1693.70 ± 104.60^b	2006.30 ± 19.10^a	1943.40 ± 21.30^a
AA (Inhibition DPPH), %	82.31± 5.04^a	82.62 ± 0.52^a	83.72 ± 0.54^a
AA (ABTS) mM Trolox/100 g product	2079.00 ± 15.00^c	2220.00 ± 3.00^b	2490.00 ± 8.00^a
AA (Inhibition ABTS, %)	79.12 ± 0.61^c	83.13 ± 0.13^b	94.77 ± 0.31^a

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row highlight significant differences (p<0.05).

5.4.1.3. Stability of the biologically active compounds in biscuits during storage

The biscuits were stored in an airtight packaging at room temperature, for 28 days, after which the content of the bioactive compounds (TAC, TFC, TPC) and the antioxidant activity were determined. As it can be seen from the data presented in **Table 5.3**, no significant changes of the concentration of the biologically active compounds were observed during storage. In both of the formulated technological variants, several changes in the antioxidant activity were observed, probably due to the structural transformations of the phenolic compounds. Thus, in the P1 sample, the concentration of anthocyanins increased by 10%, while in the P2 sample, it decreased by 5%. On the other hand, after 28 days of storage, the obtained biscuits with the P2 technological variant underwent an accelerated 22% reduction of the antioxidant activity, correlated to a reduction of the total polyphenols content.

Table 5.3. Stability of the functional potential of biscuits after 28 days of storage at room temperature.

Biscuits samples	Storage time,days	TAC, mg C_3G/100 g	TFC, mg EC/100g	TPC, mg EAG/100g	AA (DPPH), mM Trolox/100g	AA (ABTS), mM Trolox/100g
M	0	nd	92.79 ± 12.01^a	142.51 ± 14.28^a	1693.70 ± 104.61^a	2079.51 ± 15.97^a
	28	nd	82.27 ± 1.65^a	126.22 ± 3.87^a	1679.26 ± 3.39^a	1945.03 ± 16.35^b
P1 (1%)	0	6.82 ± 0.01^a	92.85 ± 15.40^a	188.87 ± 14.26^a	2006.38 ±19.13^a	2220.03 ± 3.57^a
	28	6.72 ± 0.66^a	83.35 ± 1 .15^a	147.14 ± 2.14^b	1722.13 ± 5.65^b	1860.70 ± 0.15^b
P2 (2%)	0	9.97 ± 1.76^a	105.19± 1.26^a	235.65 ± 14.36^a	1946.29 ± 16.68^a	2490.65 ± 8.09^a
	28	9.46 ± 0.03^a	82.54 ± 7.73^b	139.15 ± 18.23^b	2006.11 ± 6.32^a	1938.19 ± 3.83^b

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row highlight significant differences (p<0.05).

5.4.1.4. Physico-chemical characterisation of the value-added biscuits

The results of the physico-chemical analysis for the biscuits obtained is shown in **Table 5.4.**

Table 5.4. Physico-chemical characterisation of the biscuits with added red GS powder (*Băbească neagră variety*)

Characteristics	M	P1	P2
Proteins, g/100 g	11.87 ± 0.08^a	11.29 ± 0.04^a	13.39 ± 2.64^a
Fats, g/100 g	15.52 ± 0.19^a	15.37 ± 0.06^a	15.45 ± 0.20^a
Fibers, g/100 g	4.53 ± 0.02^a	5.06 ± 0.07^c	4.85 ± 0.04^b
Umidity, g/100 g	3.34 ± 0.09^a	3.48 ± 0.03^a	3.43 ± 0.03^a
Ash, g/100 g	1.52 ± 0.09^a	1.59 ± 0.10^a	1.55 ± 0.08^a
Carbohydrate g/100 g	58.68 ± 0.34^a	58.15 ± 0.38^a	58.47 ± 0.15^a
Energy value, %			
kcal	289.27^c	427.71^b	438.35^a
kJ	1210.32^c	1789.54^b	1834.08^a

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row highlight significant differences (p<0.05).

The obtained biscuits had an important nutritional and energy value, so that they can be recommended for consumption by the people with special nutritional needs (diabetes, gluten intolerance).

5.4.1.5. Color analysis of the value-added biscuits

Colour is an important sensory characteristic that influences the consumer's acceptability of food (Spence et al., 2015). The biscuit samples were analyzed for the CIELAB colorimetric parameters using a handheld colorimeter with a C-illuminator, standardized with a white reference plate before each measurement. The values of the colour parameters including L* (lightness), a* (tendency to red for an a* "+" or green for an a* "-"), b* (tendency to yellow for b* "+" or blue for b* "-") are shown in Table 5.5 and Figure 5.2. The value of the lightness (L) decreased with the supplementation with the red GS powder. The red colour component (a) was significantly reduced by the addition of GS, with no influence on the degree of enrichment. The blue-yellowish intensity was represented by the b* parameter, with a negative value, hence indicating a tendency towards the blue shades of the samples. The b* values decreased significantly ($p<0.05$) for all the samples after the storage at room temperature, for 4 weeks.

M P1 P2

Figure 5.2. Biscuits samples with added red GS (Băbească neagră variety): P1- biscuits with 1% GS; P2- biscuits with 2% GS; M- control sample (without GS)

Biscuits without the GS (control sample) showed a reddish (a* 4.30) and yellowish (b* of 22.52) hue, suggesting the absence of anthocyanins in the sample. As a result of the increased amount of GS added to the biscuit samples, which supplied significant amounts of anthocyanins from the grape skins, the a* value increased to 5.37 for the sample supplemented with 2% powder, compared to the first sample. In contrast, compared to the control sample, the yellow colour faded and b* reached a value of 13.70.

Table 5.5. CIELAB colour analysis of the biscuit samples

Characteristics	M	P1	P2
L	56.37 ±0.02[a]	51.87±0.04[b]	45.00 ± 0.43[c]
a*	4.30 ± 0.11[b]	3.04 ± 0.007[c]	5.37 ± 0.14[a]
b*	22.52 ± 0.26[a]	13.35 ± 0.02[b]	13.7 ± 0.29[b]

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row highlight significant differences (p<0.05).

5.4.1.6. Sensory analysis of the dietetics value-added gluten-free biscuits

The sensory evaluation of the resulting biscuits was performed using a nine-point hedonic scale. The sensory analysis was carried out following the sensory characteristics: external appearance, appearance in the section, texture, aftertaste, aroma, colour and taste. As the concentration of the added powder to the dough increased, the red-brown colour intensified due to the presence of the anthocyanins (**Figure 5.3**). The hedonic scale of appreciation by sensory attributes was used, on a scale from 1 to 9 (1 - minimum attribute intensity, 9 - maximum attribute intensity).

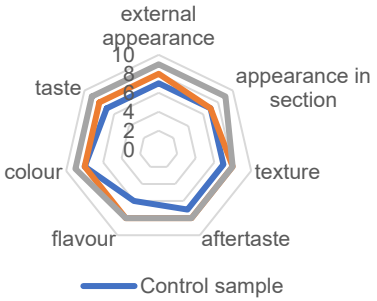


Figure 5.3. The comparative diagram of the specific sensory attributes of the biscuits: control sample - biscuits without powder; P1 and P2 - biscuits with added 1% and 2% freeze-dried powder from red GS (*Băbească neagră variety)*

Figure 5.3 shows the mean values of the characteristics analysed for the obtained biscuits and it can be seen that in terms of the colour uniformity, the most appreciated sample was P2. For the colour intensity, as expected, the biscuits with 2% GS were preferred by the panelists, while the control sample had the lowest score. Regarding the flavour, the control and P1 samples were rated similarly, while P2 had the lowest score. In terms of the overall assessment, it was shown that the addition of

red GS powder did not substantially influence the basic sensory characteristics (colour, taste, flavour).

5.4.2.2. Physico-chemical characterisation of the beer samples without the extract

The physico-chemical characteristics of the beer samples used as the base beverage for the GSE enrichment were determined using a FermentoFlash (Funke-Dr.N. Gerber Labortechnik GmbH, Berlin, Germany) and involved the determination of the alcohol content, extract, CO_2 content and pH (**Table 5.7**).

Table 5.7. Physico-chemical parameters of the control beer samples (without the extract)

Physico-chemical characteristics	Beer samples	
	P1 White beer	P2 Pils beer
Alcohol by mass (%)	4.29 ± 0.01[a]	3.86 ± 0.03[b]
Alcohol by volume (% vol)	5.39 ± 0.02[a]	4.90 ± 0.04[b]
Real Extras (°P)	4.25 ± 0.01[a]	3.89 ± 0.02[b]
Primitive Extras (°P)	12.48 ± 0.02[a]	11.34 ± 0.05[b]
Extract apparent (°P)	2.05 ± 0.01[a]	1.88 ± 0.02[b]
CO_2 (g/100 mL)	0.68 ± 0.02[a]	0.62 ± 0.01[b]
pH	4.88 ± 0.02[a]	4.74 ± 0.04[b]

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row highlight significant differences ($p<0.05$).

5.4.2.3. Phytochemical characterisation of the value-added beer and the evaluation of the phytochemical compounds' stability during storage

The increase of the nutritional value of the food and beverages can be achieved by enriching the content of bioactive compounds through the addition of extracts, fruits or fruit juices. To enhance the phytochemical profile of the beer samples, the GSE (*Băbească neagră variety*) was used in three concentrations 1, 5 and 10 mg GSE/mL. The phytochemical composition of the beer samples was monitored during 21 days of storage, at 4°C.

Table 5.8. Phytochemical characterisation and storage stability (21 days, 4°C) of the white beer with added red GSE (Băbească neagră variety)

Sample	Bioactive compounds*	Storage time, days			
		0	7	14	21
Control sample (P1)	TAC, mg C_3G/mL	Nd[c]	Nd	Nd	Nd
	TFC, mg CE/mL	0.84 ± 0.00^{aD}	0.81 ± 0.01^{aC}	0.79 ± 0.08^{aC}	0.79 ± 0.06^{aB}
	TPC, mgGAE/mL	3.17 ± 0.06^{aC}	3.12 ± 0.23^{aC}	3.08 ± 0.07^{aC}	2.98 ± 0.06^{aC}
P1	TAC, mg C_3G/mL	0.01 ± 0.00^{aC}	0.001 ± 0.00^{aC}	0.01 ± 0.00^{aC}	0.001 ± 0.00^{aC}
+	TFC, mg CE/mL	0.964 ± 0.05^{aC}	0.96 ± 0.11^{aBC}	0.96 ± 0.11^{aBC}	0.92 ± 0.12^{aAB}
1mg GSE/mL	TPC, mgGAE/mL	3.64 ± 0.30^{aB}	3.64 ± 0.06^{aB}	3.53 ±0.23^{aBC}	3.38 ± 0.17^{aB}
P1	TAC, mg C_3G/mL	0.02 ± 0.00^{aB}	0.02 ± 0.00^{bB}	0.02 ± 0.00^{cB}	0.02 ± 0.00^{cB}
+	TFC, mg CE/mL	1.10 ± 0.04^{aB}	1.07 ± 0.09^{aAB}	1.06 ± 0.09^{aAB}	1.01 ± 0.02^{aAB}
5 mg GSE/mL	TPC, mgGAE/mL	4.00 ± 0.10^{aA}	3.98 ± 0.19^{aB}	3.80 ± 0.44^{aAB}	3.61 ± 0.15^{aB}
P1	TAC, mg C_3G/mL	0.03 ± 0.00^{aA}	0.03 ± 0.00^{aA}	0.02 ± 0.00^{bA}	0.02 ± 0.00^{bA}
+	TFC, mg CE/mL	1.23 ± 0.03^{aA}	1.21 ± 0.04^{aA}	1.18 ± 0.05^{aA}	1.10 ± 0.14^{aA}
10 mg GSE/mL	TPC, mgGAE/mL	4.48 ± 0.10^{aA}	4.47 ± 0.06^{aA}	4.21 ± 0.11^{aA}	4.06 ± 0.07^{bA}

** Nd.- undetected concentration*
The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row and different upper case letters per column indicate significant differences ($p<0.05$).

The addition of GSE to the beer helped to enrich the product in bioactive compounds. The results presented in **Table 5.8** certify the high anthocyanins content, so that the concentration of TAC increased with the amount of GSE added to the beer. In the beer samples obtained through the enrichment with the three concentrations of GSE, a TAC content of 0.03 mg C_3G/mL was found in the sample after the addition of 10 mL GSE. Regarding the stability of the TAC content during storage, a slight decrease ($p<0.05$) was observed in the case of the beer samples fortified with higher amounts of GSE (P1+5 mg GSE/mL and P1+10 mg GSE/mL). In the control beer sample, the TFC was 0.84 mg CE/mL and TPC was 3.17 mgGAE/mL, compounds that showed a high stability during storage. The high content of total polyphenols was due to the fact that a type of white beer was used in the present study for the supplementation with biologically active compounds. A broad spectrum of phenolic compounds with antioxidant activity has clearly been identified in the obtained beer.

The results on the improvement of the bioactive composition of the lager beer by adding GSE are shown in **Table 5.9.** From Table 5.9, it can be seen that the GSE addition to the composition of the lager beer resulted in an improvement of the bioactive composition and functional potential of the beer compared to the control sample. Thus, by incorporating the GSE into the beer, the concentrations of anthocyanins, flavonoids and total polyphenols increased in regards to the amount of

extract that was added. Furthermore, the sensory characteristics were also improved, in particular the colour, with shades towards red.

The beer contained varied amounts of flavonoids, depending on the barley and hop varieties, growing conditions, brewing parameters and type of beer. These compounds influenced the colour, taste, aroma, stability and shelf-life of beer (Pai et al., 2015). The phenolic content of the beer depended on the quality and quantity of the raw materials and brewing parameters and has been shown to strongly influence the flavour and colloidal stability of the beer.

Table 5.9. Phytochemical characterisation and storage stability (21 days, 4°C) of the white beer with added red GSE (*Băbească neagră variety*)

Sample	Bioactive compounds*	Storage time, days			
		0	7	14	21
Control sample (P2)	**TAC, mg C_3G/mL**	Nd*	Nd	Nd	Nd
	TFC, mg CE/mL	0.68 ± 0.04^{aD}	0.67 ± 0.03^{aC}	0.65 ± 0.03^{aC}	0.65 ± 0.03^{aB}
	TPC, mg GAE/mL	3.33 ± 0.07^{aC}	3.33 ± 0.05^{aC}	3.24 ± 0.34^{aC}	3.00 ± 0.17^{aC}
P2 + 1 mg GSE /mL	**TAC, mg C_3G/mL**	0.01 ± 0.00^{aC}	0.01 ± 0.00^{aC}	0.01 ± 0.00^{aC}	0.01 ± 0.00^{aC}
	TFC, mg CE/mL	0.84 ± 0.03^{aC}	0.82 ± 0.02^{aBC}	0.81 ± 0.06^{aBC}	0.79 ± 0.05^{aAB}
	TPC, mg GAE/mL	3.34 ± 0.09^{aB}	3.21 ± 0.57^{aB}	3.20 ± 0.10^{aBC}	3.10 ± 0.22^{aB}
P2 + 5 mg GSE /mL	**TAC, mg C_3G/mL**	0.01 ± 0.00^{aB}	0.01 ± 0.00^{bB}	0.01 ± 0.01^{cB}	0.01 ± 0.01^{cB}
	TFC, mg CE/mL	0.96 ± 0.03^{aB}	0.96 ± 0.04^{aAB}	0.96 ± 0.01^{aAB}	0.87 ± 0.027^{aAB}
	TPC, mg GAE/mL	3.64 ± 0.17^{aA}	3.58 ± 0.24^{aB}	3.530 ± 0.129^{aAB}	3.41 ± 0.11^{aB}
P2 + 10 mg GSE /mL	**TAC, mg C_3G/mL**	0.03 ± 0.00^{aA}	0.03 ± 0.00^{aA}	0.03 ± 0.00^{BA}	0.03 ± 0.00^{bA}
	TFC, mg CE/mL	1.47 ± 0.04^{aA}	1.43 ± 0.05^{aA}	1.43 ± 0.05^{aA}	1.43 ± 0.01^{aA}
	TPC, mg GAE/mL	4.35 ± 0.31^{aA}	3.94 ± 0.04^{aA}	3.91 ± 0.24^{aA}	3.76 ± 0.71^{bA}

** Nd.- undetected concentration*

The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row and different upper case letters per column indicate significant differences ($p<0.05$).

The influence of the chemical composition on the antioxidant capacity of the beer, as well as the synergies of different biologically active compounds, should also be taken into account (Aprodu, 2020). **Figure 5.4** and **Figure 5.5** show the results on the antioxidant activity of the studied beer samples.

5.4.2.4. Evaluation of the beer color with the added bioactive compounds and the study of its stability during storage

The colour characteristics of the beers with the added GSE in different concentrations were determined using the EBC method and the CIELAB colour system. The visual sense contains three types of receptors, for red, green and blue colour, existing in unequal amounts, so that colours can be perceived differently (Pai et al., 2015).

The impact of the GSE addition on the beer colour was monitored during the 21-day storage period, at 4°C (**Table 5.10** and **Table 5.11**), and the CIELAB test was found to be more effective in differentiating the beer samples compared to the

EBC method. A significant decrease of the sample's brightness and an increase of the a* values were observed with the increase of GSE concentration ($p<0.05$). The yellowish-blue intensity was represented by the b* parameter, with a negative value indicating a tendency towards blue hues. The b* values increased significantly ($p<0.05$) after the storage at 4°C, for 21 days, for all the samples.

Table 5.10. Evolution of the colour parameters of white beer enriched with red grape skin extract of the Băbească neagră variety for 21 days of storage, at 4°C

Sample	Colour parameters	Storage time, days			
		0	7	14	21
Control sample (P1)	L	68.05 ± 0.02^{cA}	68.10 ± 0.04^{cA}	68.94 ± 0.17^{bA}	69.83 ± 0.07^{aA}
	a	1.20 ± 0.03^{bD}	1.07 ± 0.11^{bD}	1.20 ± 0.09^{bD}	1.50 ± 0.11^{aD}
	b	7.67 ± 0.02^{aA}	7.85 ± 0.11^{aA}	7.70 ± 0.10^{aA}	7.60 ± 0.33^{aA}
	EBC	$18.41 \pm 2.^{57aC}$	17.05 ± 0.28^{aD}	17.08 ± 0.33^{aD}	17.04 ± 0.34^{aD}
P1 + 1 mg GSE/mL	L	61.34 ± 0.17^{aB}	61.78 ± 0.03^{aB}	61.91 ± 0.06^{aB}	63.57 ± 1.99^{aB}
	a	6.75 ± 0.02^{aC}	6.96 ± 0.22^{aC}	6.77 ± 0.04^{aC}	6.71 ± 0.12^{aC}
	b	4.54 ± 0.04^{aC}	4.43 ± 0.08^{aB}	4.48 ± 0.22^{aB}	4.88 ± 0.30^{aB}
	EBC	22.52 ± 0.04^{aB}	22.55 ± 0.07^{aC}	22.54 ± 0.04^{aC}	22.52 ± 0.04^{aC}
P1 + 5 mg GSE/mL	L	52.59 ± 0.01^{bC}	52.54 ± 0.08^{bC}	52.81 ± 0.22^{bC}	57.10 ± 0.74^{aC}
	a	12.09 ± 0.21^{aB}	12.43 ± 0.04^{aB}	12.41 ± 0.27^{aB}	12.27 ± 0.73^{aB}
	b	4.82 ± 0.01^{aB}	4.00 ± 0.14^{aC}	4.03 ± 0.10^{aC}	3.81 ± 1.42^{aBC}
	EBC	28.5 ± 0.26^{aA}	28.48 ± 0.28^{aB}	28.32 ± 0.03^{aB}	28.5 ± 0.26^{aB}
P1 + 10 mg GSE/mL	L	44.04 ± 0.61^{aD}	43.66 ± 0.50^{aD}	43.65 ± 0.37^{aD}	43.66 ± 1.44^{aD}
	a	24.54 ± 0.96^{aA}	24.88 ± 0.67^{aA}	24.26 ± 0.29^{aA}	23.50 ± 2.20^{aA}
	b	2.27 ± 0.02^{aD}	2.21 ± 0.23^{aD}	2.40 ± 0.18^{aD}	2.49 ± 0.78^{aC}
	EBC	30.90 ± 0.34^{aA}	30.80 ± 0.20^{aA}	31.02 ± 0.06^{aA}	30.91 ± 0.34^{aA}

**L-brightness; a-green to red; b-blue to yellow; For each colour parameter,*
The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row and different upper case letters per column highlight significant differences ($p<0.05$).

Table 5.11. Evolution of the colour parameters of the lager beer enriched with red grape skin extract for 21 days of storage at 4°C

Sample	Colour parameters	Storage time, days			
		0	7	14	21
Control sample (P2)	L	66.15 ± 0.11^{bA}	66.39 ± 0.27^{bA}	66.88 ± 0.36^{abA}	67.83 ± 0.81^{aA}
	a	0.89 ± 0.07^{aD}	0.92 ± 0.03^{aD}	0.88 ± 0.07^{aD}	0.88 ± 0.01^{aD}
	b	10.36 ± 0.47^{aA}	11.46 ± 0.53^{aA}	11.01 ± 0.13^{aA}	10.00 ± 1.02^{aAB}
	EBC	8.28 ± 0.26^{aD}	8.29 ± 0.25^{aD}	8.28 ± 0.27^{aD}	8.28 ± 0.27^{aD}
P2 + 1 mg GSE/mL	L	62.93 ± 0.20^{aB}	63.65 ± 0.48^{aB}	63.35 ± 0.14^{aB}	63.15 ± 0.76^{aB}
	a	4.78 ± 0.09^{aC}	4.63 ± 0.33^{aC}	4.71 ± 0.20^{aC}	4.92 ± 0.39^{aC}
	b	10.66 ± 0.24^{aA}	9.52 ± 0.32^{abB}	9.32 ± 0.49^{bC}	9.63 ± 0.74^{abAB}
	EBC	11.27 ± 0.22^{aC}	11.23 ± 0.22^{aC}	11.19 ± 0.28^{aC}	11.27 ± 0.22^{aC}
P2 + 5 mg GSE/mL	L	47.87 ± 0.02^{bC}	49.76 ± 0.78^{aC}	48.22 ± 0.06^{bC}	48.01 ± 0.33^{bC}
	a	17.41 ± 0.01^{aB}	15.67 ± 0.41^{cB}	16.13 ± 0.14^{bcB}	16.50 ± 0.24^{bB}
	b	10.48 ± 0.05^{bA}	10.09 ± 0.16^{cB}	10.33 ± 0.04^{bcB}	11.34 ± 0.17^{aA}
	EBC	14.44 ± 0.20^{aB}	14.42 ± 0.23^{aB}	14.42 ± 0.26^{aB}	14.44 ± 0.20^{aB}
P2 + 10 mg GSE/mL	L	40.28 ± 0.01^{bD}	42.53 ± 0.27^{bD}	42.94 ± 0.09^{abD}	45.89 ± 2.52^{aC}
	a	25.81 ± 0.05^{aA}	23.86 ± 0.07^{bA}	23.83 ± 0.11^{bA}	21.48 ± 1.27^{cA}
	b	6.91 ± 0.03^{bB}	8.44 ± 0.04^{aC}	8.57 ± 0.04^{aD}	8.65 ± 0.53^{aB}
	EBC	22.65 ± 0.20^{aA}	22.66 ± 0.18^{aA}	22.66 ± 0.16^{aA}	22.65 ± 0.20^{aA}

**L-brightness; a-green to red; b-blue to yellow; For each colour parameter,*
The results are the mean of 3 independent determinations ± standard deviation. Different lower case letters per row and different upper case letters per column highlight significant differences ($p<0.05$).

5.5. Partial conclusions

- The bioactive potential of the phytochemical compounds from the skins of red grape (Băbească neagră variety) was exploited to obtain value-added food products with aan enriched functional potential, gluten-free biscuits and beer.

- The use of the freeze-dried powder, obtained from the red grape skin extract, at a concentration of 2% in the dough for the production of gluten-free biscuits, improved the bioactive composition of the final product and its functional potential. At the same time, the biscuits showed sensory characteristics accepted by consumers and were characterized by a stability of bioactive properties for 28 days of storage, at room temperature (ca. 25°C).

- The supplementation of the samples, white and lager beer, with red grape skin extract (Băbească neagră variety) resulted in derived beverages with an increased bioactive potential, which retained their functional properties after 21 days of storage, at 4°C. The obtained beverages had an attractive colour with various shades derived from red-brownish colour in correlation to the added extract concentration.

- The obtained results certified the quality of the natural ingredients with the bioactive potential of the freeze-dried extract and pulp obtained from the skin of red grape berries of the *Băbească neagră variety*, for multiple uses in the food industry, in order to obtain functional products with a high stability during storage and to promote the principles of circular economy.

CHAPTER 6.

FINAL CONCLUSIONS

The PhD thesis**,** entitled ***The valorization of various biologically active compounds from red grapes´ processing by-products and the obtainment of certain value-added ingredients*****,** had as main objective to highlight the bioactive potential of the epicarp (skin) of red grape berries (GS), the native Băbească neagră variety, in order to extract, characterize and valorise them in new products and added-value food ingredients.

The identification and the establishment of the scientifically based strategies to exploit the nutritional and functional potential of by-products resulting from the industrial processing of grapes is part of the global strategies for the valorisation of agro-food by-products based on the principles of a circular economy, with impact in the strategic areas of Bioeconomy, Health, Environment. Thus, the fundamental idea on which the research was carried out during the doctoral studies took into account the current trend of consumers' reorientation towards food products with beneficial effects on their health, in conjunction with the international policies to reduce the impact of the industry on the environment and to make better use of our natural resources.

Grape skins (GS) are obtained as a by-product of the grape processing and can generally account for up to 65% of the grape pomace, the main by-product of the winemaking. This by-product had, in the past, been mainly used for compost, to obtain bio-fertilisers and to extract biologically active compounds such as lecithin or seed oil. However, the high amounts of polyphenolic compounds present in the grape skins make them a valuable source of biologically active phytochemicals. In terms of the phytochemical profile, grape skins offer the opportunity to use properly the biologically active substances that have well-defined functions for the human body and which can also lead to successful business ideas for the intelligent and sustainable use of agri-food resources, especially by-products.

Through its subject matter, the PhD thesis aimed at the comparative study of the different extraction techniques efficiency on increasing the extraction yield and composition of biologically active compounds with a functional potential (total polyphenols, anthocyanins, flavonoids), correlated to the increase of the antioxidant activity of the extracts and the biochemical and functional stability of the extracted compounds.

Thus, different extraction techniques were tested, such as conventional solvent and ultrasound-assisted extraction, microwave and enzymatic preparations, and through the mathematical modelling and statistical analysis of the results, the optimum extraction conditions were determined to obtain extracts rich in valuable compounds with a highly functional potential and antioxidant activity. The extraction yield and the phytochemical composition of the extracts depended on a number of parameters, the most important being the method of matrix processing (microwave, ultrasonic, enzyme), the type and concentration of the solvents and the extraction time.

The phytochemical composition of the obtained extracts and the stability of the bioactive compounds under different physico-chemical conditions and the storage stability were also analysed. As such, advanced methods of investigation such as high-performance liquid chromatography, inactivation kinetics and thermodynamic behaviour modelling were used for this purpose. Seven main anthocyanins, derivatives of delphinidin, cyanidin and petunidin, were detected as part of the chromatographic profile of GS red grapes (*Băbească neagră variety*) extracts, with cyanidin 3-*O*-glucoside compound being predominant in the extracts obtained by the different extraction techniques.

At the same time, the thermal degradation kinetics of the bioactive compounds were studied by kinetic modelling in the temperature range 80-140°C, at different processing times. It was shown that anthocyanins exhibited a high thermal stability, with the degradation kinetic pattern following the first order.

In vitro molecular docking studies have demonstrated the effect of the bioactive compounds from red GS (*Băbească neagră variety*) to inhibit the activity of several enzymes involved in metabolic disorders (hyperglycemia, dyslipidemia and oxidative stress), namely α-amylase, α-glucosidase, lipase and lipoxygenase. These results are of great importance for the valorisation of grape GS (*Băbească neagră variety*) to obtain ingredients and food products with an added functional value.

The possibility of the bioactive compounds' microencapsulation by binding them within different matrices (whey protein isolate, carboxymethyl cellulose, pectin, gum arabic and maltodextrin), in different combinations, was studied to obtain a chemically stable, functional potential composites as bioingredients for their potential use in the food industry.

At the same time, in the applied research stage, different variants of value-added products (biscuits and beer) were obtained by exploiting the freeze-dried powder and extracts from red GS (Băbească neagră variety). The phytochemical composition, sensory characteristics and stability of the functional potential were important features in the choice of the percentage of the extract or powder used to increase the functional value of the final products.

CHAPTER 7

PERSONAL CONTRIBUTIONS AND FUTURE PERSPECTIVES

The PhD thesis**,** entitled **The valorization of various biologically active compounds from red grapes´ processing by-products and the obtainment of certain value-added ingredients** is an original work, carried out during the doctoral studies by going through several stages of fundamental and applied research, with the aim of establishing the right strategies for the intelligent valorisation of the by-products resulted from the vinification of red grapes (*Băbească neagră variety*), in order to reintegrate into the food chain some bioactive compounds (total polyphenols, anthocyanins and flavonoids), well-recognised for their beneficial effects on the consumer's health.

The studies were carried out using an integrated research, development and innovation strategy that aimed to:

- Test different extraction techniques, from the perspective of optimizing the conditions for the biologically active compounds (total polyphenols, flavonoids and anthocyanins) recovery from the red grape berries skins of the *Băbească neagră variety*.

- Obtain an advanced characterisation of the obtained complex extracts, from a phytochemical point of view and of their functional potential (inhibition activity of some enzymes involved in metabolic diseases), as well as to evaluate the stability of these compounds and the antioxidant potential of the extracts.

- Study the thermodynamic behaviour and thermal inactivation kinetics of the bioactive compounds in the obtained extracts, from the perspective of their preservation through the processing of their functional potential and antioxidant activity.

- Formulate bioingredients with a highly functional potential through microencapsulation techniques within different matrices and to characterize the bioactive composition and stability of the obtained composites.

- Incorporate the bioactive compounds from red grape skins (*Băbească neagră variety*), in the form of freeze-dried powder or extracts, into food products (gluten-free biscuits, beer), with valuable contributions to the improvement of their functional potential, especially their antioxidant activity.

- Provide the scientific basis of a standard, integrated approach for the valorisation of the by-products of red grape biologically active compounds from processing into functional foods, thus contributing to the application of the Emerging bioeconomy principles in Romania.

From the perspective of further studies, the approach of this PhD thesis can be extrapolated to other by-products resulting from the processing of vegetables and fruits, for the separation, characterization and valorisation of their biologically active composition, in order to obtain highly enhanced food products and supplements, feed, nutraceuticals, cosmetics, etc., with a positive impact on the quality of life, the circular economy, for the valorisation of agro-food resources and for the environmental protection.

CHAPTER 8.

DISSEMINATION OF RESEARCH RESULTS

The dissemination of the research results throughout the doctoral studies resulted in the following scientific contributions published or communicated at relevant national and international conferences in the field of Biotechnology, patent applications and awards, as follows:

A. Articles published in WOS-indexed journals with impact factor

1. **Daniela Serea**, Condurache, N. N., Aprodu, I., Constantin, O. E., Bahrim, G. E., Stănciuc, N., Rapeanu, G. 2022. Thermal Stability and Inhibitory Action of Red Grape Skin Phytochemicals against Enzymes Associated with Metabolic Syndrome. *Antioxidants*, 11(1), 118, https://doi.org/10.3390/antiox11010118 **(Q1, IF – 7.675**).

2. **Daniela Serea**, Horincar, G., Constantin, O. E., Aprodu, I., Stănciuc, N., Bahrim, G. E., Rapeanu, G. 2022. Value-Added White Beer: Influence of Red Grape Skin Extract on the Chemical Composition, Sensory and Antioxidant Properties. *Sustainability*, 14(15),9040,https://doi.org/10.3390/su14159040 (**Q2, IF – 3.889**).

B. Articles published in WOS-indexed journals

1. **Daniela Serea**, Constantin, O.E., Horincar, G., Stănciuc, N., Aprodu, I., Bahrim, G.E., Râpeanu, G. 2023. Optimization of extraction parameters of anthocyanin compounds and antioxidant properties from red grape (Băbească neagră) peels. *Inventions*, 8 (2), 59, https://doi.org/10.3390/inventions8020059.

2. **Daniela Serea**, Râpeanu, G., Constantin, O. E., Bahrim, G. E., Stănciuc, N., Croitoru, C. 2021. Ultrasound and enzymatic assisted extractions of bioactive compounds found in red grape skins băbească neagră (vitis vinifera) variety. *The Annals of the University of Dunarea de Jos of Galati. Fascicle VI. Food Technology*, 45(1), 9-25, https://doi.org/10.35219/foodtechnology.2021.1.01.

C. Patent applications

1. **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu, Iuliana Aprodu, Gabriela-Elena Bahrim, Nicoleta Stanciuc, *Bere cu valoare adaugată obținută prin adaos de extract de pieliță de struguri roșii*, A/0006/21.01.2022.

2. **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc, *Biscuiți aglutenici pentru diabetici cu valoare adăugată obținuți prin adaos de pieliță liofilizată de struguri roșii*, A/00297/02.06.2022.

D. Papers presented at national and international conferences

1. **Daniela Serea,** Nicoleta Stănciuc, Gabriela Bahrim, Gabriela Râpeanu, 2020. Extraction and characterization of bioactive compounds from red grape skins. *8th Edition of Scientific Conference of Doctoral Schools SCDS-UDJG*, "Dunarea de Jos" University of Galați.

2. **Daniela Serea**, Nicoleta Stănciuc, Gabriela Râpeanu , Gabriela Bahrim, Luminita Georgescu, 2020. Red grape skins by-products as a functional ingredients used in food industry. *8th Edition of Scientific Conference of Doctoral Schools SCDS-UDJG*,, "Dunărea de Jos" University of Galați.

3. **Daniela Serea**, Gabriela-Elena Bahrim, Gabriela Rapeanu, Nicoleta Stanciuc, 2020. Red grape skins as a sustainable source of bioactive compounds: extraction and characterization. *9th Edition of the Intrenational Conference Agriculture for Life, Life for Agriculture*, University of Agronomic Sciences and Veterinary Medicine, București.

4. **Daniela Serea**, Gabriela Bahrim, Gabriela Râpeanu, Oana-Viorela Nistor, Nicoleta Stănciuc, 2020.Microwave-assisted extraction of phenolic compounds from red grape skins (Babeasca neagra variety). *Scientific Symposium "Young people and multidisciplinary research in applied life sciences", 7th Edition,* Section: Food Engineering, Banat`s University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" Timisoara.

5. **Daniela Serea**, Gabriela Râpeanu, Nicoleta Stănciuc, Iuliana Aprodu, Gabriela- Elena Bahrim, 2021.Thermostability and biological activity of bioactive compounds recovered from red grape skins (*Băbească neagră* variety). *EuroAliment Symposium,* "Dunărea de Jos" University of Galați.

6. **Daniela Serea**, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc, Oana Constantin, Gabriela Rapeanu, 2021. Encapsulation of phenolic compounds from grape skin extract in whey protein isolate and pectin. *Multidisciplinary Conference on Sustainable, Development, Section: Food Chemistry, Engineering & Technology*, Faculty of Food Engineering Timişoara.

7. **Daniela Serea**, GabrielaElena Bahrim, Iuliana Aprodu, Gabriela Râpeanu, 2021. Thermal degradation kinetics of antocyanins extracted from red grape skin extract *of Babeasca neagra* variety. *9th Edition of Scientific Conference*

of Doctoral Schools SCDS-UDJG, "Dunarea de Jos" University of Galați.

8. **Daniela Serea**, Gabriela- Elena Bahrim, Iuliana Aprodu, Nicoleta Stănciuc, Oana-Emilia Constantin, Gabriela Râpeanu, 2021 Potential anti-diabetic proprieties of red grape skin extract: an in vitro study of α-amilase and α-glucosidase inhibition,poster, *9th Edition of Scientific Conference of Doctoral Schools SCDS-UDJG*, "Dunarea de Jos" University of Galați.

9. **Daniela Serea**, Nicoleta Stanciuc Iuliana Aprodu, Gabriela Elena Bahrim, Gabriela Rapeanu, 2021.Thermal Stability Of Bioactive Compounds Extracted From Red Grape Skins. *20th International Conference „Life sciences for sustainable development''*, University of Agricultural Sciences and Veterinary Medicine, Cluj- Napoca.

10. **Daniela Serea**, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc, Oana Constantin, Gabriela Rapeanu, 2021. Encapsulation of phenolic compounds from ared grape skin extract in whey protein isolate and pectin. *International scientific symposium "Young researchers and scientific research in life sciences"*, Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" Timişoara.

11. **Daniela Serea**, Georgiana Horincar, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stănciuc, Gabriela Râpeanu, 2022. Effect of the addition of grape skin extract (*Băbească neagră* variety) on bioactive compounds and antioxidant activity of white beer.*10th Edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University Galați.

12. **Daniela Serea**, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stănciuc, Gabriela Râpeanu, 2022. Encapsulation of grape skin extract phenolics (Băbească neagră variety) using whey protein isolate, carboximetilcelulose and gum arabica blends. *10th Edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University Galați.

13. **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu, Iuliana Aprodu, Gabriela-Elena Bahrim, Nicoleta Stanciuc, 2022. Bere cu valoare adaugată obținută prin adaos de extract de pieliță de struguri roșii. *Scientific Research, Innovation And Invention Exhibition Pro Invent, XX Edition,* Technical University of Cluj-Napoca.

14. **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc, 2022. Biscuiți aglutenici pentru diabetici cu valoare adăugată obținuți prin adaos de pieliță liofilizată de struguri roșii. *Scientific Research, Innovation And Invention Exhibition Pro Invent, XX Edition,* Technical University of Cluj-Napoca.

E. Awards

1. **Honorable Mention, Poster**, *Extraction and characterization of bioactive compounds from red grape skins,* **Daniela Serea**, Nicoleta Stănciuc, Gabriela Bahrim, Gabriela Râpeanu, *8th edition of Scientific Conference of Doctoral Schools SCDS-UDJG,* "Dunarea de Jos" University of Galati, 2020.

2. **3rd Award, Poster**, *Microwave-assisted extraction of phenolic compounds from red grape skins (Babeasca neagra variety),* **Daniela Serea**, Gabriela Bahrim, Gabriela Râpeanu, Oana-Viorela Nistor, Nicoleta Stănciuc. *Scientific Symposium "Young people and multidisciplinary research in applied life sciences", 7th edition, Section: Food Engineering,* Banat`s University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania", Timisoara, 2020.

3. **Honorable Mention, Poster,** *Thermal degradation kinetics of antocyanins extracted from red grape skin extract of Babeasca neagra variety,* **Daniela Serea**, Gabriela-Elena Bahrim, Iuliana Aprodu, Gabriela Râpeanu. *9th Edition of Scientific Conference of Doctoral Schools SCDS-UDJG,* "Dunarea de Jos" University of Galati, 2021.

4. **3rd Award, Poster,** *Encapsulation of phenolic compounds from ared grape skin extract in whey protein isolate and pectin*, **Daniela Serea**, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc, Oana Constantin, Gabriela Rapeanu, *International scientific symposium "Young researchers and scientific research in life sciences* " Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" Timişoara, 2021.

5. **Honorable Mention, Poster**, *Effect of the addition of grape skin extract (Băbească neagră variety) on bioactive compounds and antioxidant activity of white beer*, **Daniela Serea**, Georgiana Horincar, Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stănciuc, Gabriela Râpeanu, *10th edition of Scientific Conference of Doctoral Schools SCDS-UDJG*, "Dunarea de Jos" University of Galati, 2022.

6. **Gold medal, silver medal and excellence diploma, Poster**, *Bere cu valoare adaugată obținută prin adaos de extract de pieliță de struguri roșii*, **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu, Iuliana Aprodu, Gabriela-Elena Bahrim, Nicoleta Stanciuc. *Scientific Research, Innovation And Invention Exhibition Pro Invent, XX Edition*, Technical University of Cluj-Napoca, 2022.

7. **Gold medal and excellence diploma, Poster,** *Biscuiți aglutenici pentru diabetici cu valoare adăugată obținuți prin adaos de pieliță liofilizată de struguri roșii*, **Daniela Serea**, Georgiana Horincar, Gabriela Rapeanu,

Gabriela-Elena Bahrim, Iuliana Aprodu, Nicoleta Stanciuc. *Scientific Research, Innovation And Invention Exhibition Pro Invent, XX Edition*, Technical University of Cluj-Napoca, 2022.