"Dunărea de Jos" University of Galaţi

Doctoral School of Fundamental Sciences and Engineering

PhD thesis

The development of value-added foods by exploiting the biologically active potential of purple maize
(PhD Thesis Summary)

PhD student,

Mioara Gabriela SLAVU (URSU-SLAVU)

Scientific coordinator,

Prof. univ. dr. eng. habil. Nicoleta STĂNCIUC

Series I 7: FOOD ENGINEERING Nr. 16

Galaţi

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Content

Introduction

In recent years, the increase in the prevalence of allergies, celiac disease, metabolic diseases, and so many more has led to the need to develop new nutritional approaches that will improve the quality of life, with a focus on preventing various diseases. On the other hand, consumers are becoming increasingly aware of the need for a balanced diet, rich in natural compounds, with a significant impact on maintaining or improving their health. In this context, the food industry is facing an increased demand for functional foods and beverages. Among a wide range of bioactive compounds, polyphenolic compounds have gained significant interest among researchers and within the industry. This class of compounds is represented mostly by the secondary metabolites of plants, with the help of which plants protect themselves from harmful elements in the environment. Numerous studies in the literature have shown the important biological activities of this class of compounds, for the human body to be allowed to fight against diseases such as cancer, cardiovascular disease, atherosclerosis, diabetes etc. (Güneş Bayir et al., 2019).

The doctoral thesis entitled **"The development of value-added foods by exploiting the biologically active potential of purple corn"** followed the opportunity to exploit the functional potential of purple corn (*Zea mays* L.) in the food industry and on the growing demand for functional products, given the awareness of consumers regarding the importance of a healthy diet, the growth rate of metabolic diseases, celiac and not only, but also on the progress of processing technologies. In addition, the topic of the doctoral thesis is in line with the need to develop innovative purple corn food products, imposed by an economic operator, which grows purple corn on an important land in Brăila County. In this context, the topic of the doctoral thesis is aligned to, on one hand, the need to scientifically substantiate the use of purple corn as a new source of biologically active compounds, and, on the other hand, to the development of recovery technologies that meet the economic operators' requirements, the potential for patents and technology transfer, which should contribute alsp to the supplementation of a diet with new sources of polyphenolic compounds, that have an impact in limiting or inhibiting the oxidative processes at a cellular level, with the role of prevention and amelioration.

The doctoral thesis entitled **"The development of value-added foods by exploiting the biologically active potential of purple corn"** aimed at scientifically substantiating the phytochemical profile of purple corn extracts, by extracting, optimizing, quantifying and evaluating the *in vitro* active biological potential and establishing the guidelines for the recovery of biologically active compounds from purple corn (*Zea mays* L.) by developing new technologies to obtain functional foods with benefits on the consumers' health.

The research carried out during the doctoral studies aimed at the following **scientific objectives**:

- phytochemical profiling of purple corn extracts by extraction, optimization, identification and characterization of the polyphenolic compounds from the flour, in correlation to their antioxidant properties and stability to heat processing, in order to establish the optimal conditions to obtain and to store products that are rich in biologically active compounds;
- *in vitro* evaluation of the active biological potential of the optimized extract, by testing the ability to stimulate the metabolic activity of yeasts, from the perspective of using purple corn flour as a raw material in the food industry;

- *in vitro* evaluation of the active biological potential of the optimized extract, by determining the inhibition potential against some enzymes involved in the metabolic syndrome;
- the development of technological variants based on purple corn flour as a raw material for obtaining *gluten-free products*, with a high functionality, and the characterization of the obtained products from a phytochemical, sensory and storage stability point of view;
- the development of technological variants based on purple corn flour as a raw material for obtaining *products with gluten*, with a high functionality, and the characterization of the obtained products from a phytochemical, sensory and storage stability point of view.

The doctoral thesis is structured in two parts, as follows:

I. DOCUMENTARY STUDY, entitled **"Fundamental aspects regarding the technological and nutritional functionality of biologically active compounds"** is divided into 2 chapters, which summarizes the theoretical considerations from the scientific literature on the bioactive compounds from purple corn (*Zea mays* L.), thus emphasizing the health benefits of these types of compounds. It also presents the structure of gluten, associated diseases and the technological challenges in making gluten-free products.

II. THE EXPERIMENTAL STUDY includes 3 chapters in which the results of the research studies carried out during the doctoral period are highlighted and presented briefly as follows:

CHAPTER 3, entitled **"Solid-liquid extraction of biologically active compounds from purple corn flour"** presents the results obtained following the extraction, separation, identification and quantification of the biologically active compounds from purple corn flour (anthocyanins, polyphenols, flavonoids); the optimization and validation of the extraction of anthocyanins from purple corn flour using spectrophotometric methods and high performance liquid chromatography (HPLC) techniques and the functionality evaluation of the optimized extract on the metabolic activity of selected yeasts and its inhibition potential on enzymes involved in the metabolic syndrome. Also, the chapter includes the results obtained from testing the thermal stability of the biologically active compounds and the antioxidant activity at different temperature-time combinations, modeling and estimating the degradation kinetics using degradation kinetic models, including here the kinetic parameters, from the perspective of using the purple corn flour in different applications that involve the use of heat treatment.

CHAPTER 4, entitled **"Technological aspects for the development of gluten-free products with added-value by exploiting the biologically active potential of purple corn"** presents the results obtained by developing a technology that exploits the functional potential of purple corn flour, respectively a technology for obtaining gluten-free biscuits composed of purple corn flour and rice flour.

CHAPTER 5, entitled **"Technological aspects regarding the development of food products with gluten, with added-value by exploiting the biologically active potential of purple corn"** presents the results obtained by developing a technology that highlights the functional potential of purple corn flour, respectively a technology to obtain buns based on purple maize flour and wheat flour type 650.

Each chapter of the experimental study includes the following subchapters: Introduction, Objectives, Materials and Methods, Results and Discussions, Partial Conclusions and References.

CHAPTER 6, General conclusions, presents the main conclusions resulting from the performed experiments.

The doctoral thesis comprises 126 pages, which includes **23** figures and **24** tables. The documentary study represents 25% and the experimental part 75%.

Finally, ***the original contributions*** of the doctoral thesis are presented, with an impact on the development of knowledge in the field and the future research perspectives, as well as the dissemination of the results obtained in the researched field. Thus, the research results were highlighted through the elaboration of **3 scientific articles, 2 articles** published in WOS-indexed journals (*Foods, International Journal of Food Science and Technology*) and **1 article submitted for publication** (*Food Chemistry X*), as well as **6 national communications** at representative scientific events for the field of food engineering.

The PhD research activities were conducted using the modern research infrastructure of the Integrated Research Centre, Expertise and Technology Transfer (BIOALIMENT-TehnlA) (www.bioaliment.ugal.ro) of the Faculty of Food Science and Engineering, "Dunarea de Jos" University.

The thesis was conducted under the coordination of **Prof. dr. eng. habil. Nicoleta STĂNCIUC**, as the doctoral supervisor and of the scientific committee with the following members: **Prof. dr. eng. habil. Gabriela RÂPEANU**, **Prof. dr. eng. habil**. **Iuliana BANU** and **Prof. dr. eng. habil. Iuliana APRODU**.

CHAPTER 3: Solid-liquid extraction of biologically active compounds from purple corn flour

3.1. Introduction

Purple corn or purple maize (*Zea mays* L) has recently been highlighted as a "superfood" due to its remarkable polyphenols content, with potential health benefits (Lee et al. 2021). Purple maize presents a series of anthocyanins such as cyanidin-3-*O*-dimalonylglucoside, cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, compounds distributed in leaves, cobs and seeds (Cuevas Montilla et al. 2011). In addition to polyphenols, and especially anthocyanins, purple corn is a rich source of phenolic acids, carotenoids, flavonoids, resistant starch, dietary fiber, minerals (phosphorus, potassium, and magnesium), vitamins (A, B, E and K), phytosterols (Siyuan et al. 2018). For example, relevant compounds with positive health effects have been identified by Lee et al. (2021), such as two alkaloids (synomenin and codeine), responsible for a wide range of biological activities, including anti-inflammatory and antispasmodic properties. Moreover, Lee et al. (2021) reported the presence of cilandelate, linoleic and docosahexaenoic acids, tiotropium, bisoprolol, glycocholic acid, 6-gingerol, 4-methylumbelliferone. The health benefits associated with these compounds include neuroprotective effect, anti-inflammatory, anti-nausea, antioxidant, antidiabetic, anticancer, promoting blood circulation, regulating blood pressure, controlling cholesterol levels etc..

Due to the abundance of bioactive substances, purple corn can be used in various food, pharmaceutical, nutraceutical and biotechnological applications and thus opens up the prospects for the development of functional products that can help alleviate lifestyle diseases such as obesity, diabetes, hyperglycemia, high blood pressure and cardiovascular disease. A critical step for the recovery of anthocyanins from purple maize in various applications is the extraction efficiency and stability of the compounds. Therefore, during extraction it is essential to optimize the solid-liquid ratio, extraction time and temperature, in order to maximize the extraction yield, while minimizing the extraction time. Secondly, the prolonged extraction time (Zhang et al. 2014) or high temperatures (Piyapanrungrueang et al. 2016) may increase the extraction yield of anthocyanins, but these conditions also can reduce the stability of anthocyanins. However, several factors can affect the extraction efficiency of polyphenols, such as the type of solvent, the particle size of the raw material, the liquid/material ratio, the solvent's concentration, the stirring speed, the extraction time, the extraction temperature etc..

3.2. Objectives of the study

The objectives of the study related to this chapter were:

▪ Testing several extraction techniques of polyphenolic compounds from purple corn flour from the perspective of obtaining high quality extracts, in terms of extraction yields, concentration and phytochemical profile of biologically active compounds;

▪ Qualitative and quantitative evaluation of the phytochemical and antioxidant profile of the obtained extracts by high performance liquid chromatography techniques;

▪ Optimization and validation of anthocyanin extraction from purple corn flour (PCF), by testing the influence of the following factors: extraction time, extraction temperature, liquid/solid ratio and ethanol concentration, in order to optimize anthocyanin extraction conditions from PCF. The Box-Behnken matrix (BBD) with four factors and three levels of significance was used, with three replicas in the central points. Consequently, the validated extraction model was analyzed and

could provide a reference point for the large-scale application for the polyphenols' extraction from PCF;

▪ Qualitative and quantitative evaluation of the phytochemical and antioxidant profile of the optimized and validated extract, using spectrophotometric techniques and high-performance liquid chromatography. The optimized extract was analyzed for the content of total polyphenols, total flavonoids, and the antioxidant activity, while the advanced profile was determined by chromatographic analysis.

▪ Thermal degradation kinetics study of anthocyanins in the extract correlated to the antioxidant activity, from the perspective of evaluating the processing behavior and the use of extracts as ingredients in the food industry

▪ Evaluation of its functionality by testing the efficiency of the validated extract on the metabolic activity of a selected yeast, from the perspective of using PCF as a raw material for the production of bioethanol and/or as adjuvants in food applications; the effect of different concentrations of PCF extract on specific parameters such as multiplication, yeast cell viability and alcoholic fermentation dynamics was also monitored;

▪ Evaluation of some biological properties of the optimized extract by determining the inhibition potential of some enzymes involved in the metabolic syndrome.

3.3. Materials and methods

Plant material

Purple corn flour (*Zea mays L.*) was purchased from a local producer (Brăila County, Romania). The flour, with a water content <12%, was stored in dark paper bags at 4°C.

Yeast stalk

The freeze-dried bakery yeast, *Saccharomyces cerevisiae* (Puratos, Andenne, Belgium) was reactivated through a stationary cultivation in sterile malt must, for 24 hours, at room temperature. An activated culture concentration of $5x10^7$ CFU/mL fermentative medium was used as the vegetative inoculum in both the multiplication and alcoholic fermentation samples.

3.4. Comparative analysis of different extraction techniques

Extraction is a separation technique of biologically active compounds from plants using selective solvents by standard procedures (Handa et al. 2008). The purpose of all extractions is to separate the soluble metabolic compounds of the plant from the insoluble cell parts (residue). Before extraction, some matrices need a **preliminary preparation** (Azwanida, 2015). Purple corn belongs to this category, its preliminary preparation being represented by grinding followed by a fine crushing in order to increase the contact surface between the matrix and the solvents, aiming at a higher extraction yield of polyphenolic compounds. For the extraction of antioxidant compounds and for the determination of the antioxidant activity from the purple corn flour prepared in advance, two conventional extraction techniques were chosen: extraction using water as a solvent and extraction with organic solvents; but also unconventional extraction techniques: ultrasound-assisted extraction.

Solvent extraction

The solvent extraction is part of an extraction process known as **solid-liquid extraction**. The following variants were used in the performed experiments:

Experimental extraction variant 1 represented by an extraction in distilled water. An amount of 1 g of purple maize flour, previously prepared by grinding, was solubilized in 10 mL of distilled water at pH 4.5. The homogenized sample was centrifuged at 6000 rpm, for 15 min. From the resulting supernatant, the content of monomeric anthocyanins, using the pH differential method, the content of total flavonoids, the content of total polyphenols by Folin-Ciocalteu colorimetric method and the antioxidant activity were determined.

Experimental extraction variant 2 represented by an ethanol extraction. A quantity of 1 g of purple corn flour, previously prepared by grinding in a kitchen grinder, was mixed with 10 mL (1:10) of ethanol (70%). The homogenized sample was centrifuged at 6000 rpm/15 min. From the resulting supernatant, the content of monomeric anthocyanins, using the pH differential method, the content of total flavonoids, the content of total polyphenols by Folin-Ciocalteu colorimetric method and the antioxidant activity were determined.

Experimental extraction variant 3 represented by an extraction in organic solvents, ethanol (70%) and 1N HCl. This is the classic method of extracting anthocyanins from plant materials. This procedure involves macerating or soaking the plant material in ethanol containing a low concentration of a mineral acid (eg HCl) (Rodriguez-Saona, 2001). The acid breaks the plant walls and releases the compounds. 1 g of purple corn flour, prepared in advance, was solubilized in 9 mL of ethanol (70%) and 1 mL of 1N HCl. The sample was homogenized and centrifuged at 6000 rpm/15 min. From the resulting supernatant, the content of monomeric anthocyanins, using the pH differential method, the content of total flavonoids, the content of total polyphenols by Folin-Ciocalteu colorimetric method and the antioxidant activity were determined.

Experimental extraction variant 4 represented by an extraction in organic solvents, ethanol and 1N HCl, followed by an ultrasonic assisted extraction. A quantity of 1 g of purple corn flour, prepared in advance, was solubilized in 9 mL of ethanol and 1 mL of 1N HCl. The sample was homogenized and subsequently subjected to an ultrasound extraction at 30 min/30°C and then centrifuged at 6000 rpm/15 min. From the resulting supernatant, the content of monomeric anthocyanins, using the pH differential method, the content of total flavonoids, the content of total polyphenols by Folin-Ciocalteu colorimetric method and the antioxidant activity were determined.

Experimental extraction variant 5 represented by an extraction in organic solvents, ethanol and 1N HCl, followed by an ultrasound-assisted extraction. A quantity of 1 g of purple corn flour, prepared in advance, was solubilized in 9 mL of ethanol and 1 mL of 1N HCl. The sample was subsequently subjected to an ultrasound extraction at 60 min / 30 ˚C and then centrifuged at 6000 rpm / 15 min. From the resulting supernatant, the content of monomeric anthocyanins, using the pH differential method, the content of total flavonoids, the content of total polyphenols by Folin-Ciocalteu colorimetric method and the antioxidant activity were determined. All extraction experiments were performed in triplicate.

3.4.1. Phytochemical characterization of the extracts

Determination of monomeric anthocyanin content by pH differential method

The content of monomeric anthocyanins was determined through a method based on the property of anthocyanins to change their color according to different pH values, process that can be assessed by validated spectrophotometric methods, namely the pH differential method, which is a fast method that does not require prior hydrolysis of the prime materials. Monomeric anthocyanin pigments can reversibly change color depending on pH, so at pH value of 1.0 the predominant form is colored, while at the pH value of 4.5, the colorless form predominates. The difference in absorbance at 520 and at 700 nm is proportional to the concentration of these

pigments. The degraded anthocyanins found in polymeric forms are not influenced by the change of the pH and are not taken into account because they absorb both at 4.5 and at 1.0. The concentration of the pigments was expressed as cyanidin-3-glucoside equivalents (C3G) (equation 3.1.):

$$\text{Antocyanins (mg C3G/g)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l} \quad (3.1.)$$

where:

$A = (A_{520\ nm} - A_{700\ nm})_{pH\ 1.0} - (A_{520\ nm} - A_{700\ nm})_{pH\ 4.5}$; MW (molecular weight) = 449.2 g/mol for C3G; DF = dilution factor; l = cuvette length, in cm; ε = 26 900, molar extinction coefficient for cyanidin-3-glucoside L/mol·cm, 10^3 = conversion factor from g to mg.

Determination of total flavonoids content

The modified colorimetric method was used to determine the total flavonoids content. Over 0.25 mL of diluted purple corn extract, 1.25 mL of distilled water and 0.075 mL of 5% sodium nitrite solution were added. The mixture was left to rest, at room temperature, for 5 minutes, after which a volume of 0.15 mL of 10% aluminum chloride solution was added. After a 6-minute rest, 0.5 mL of 1M sodium hydroxide and distilled water were further added to reach the volume of 3 mL. The absorbance of the mixture was measured immediately at 510 nm, the total flavonoids content being expressed in mg catechin equivalents (CE)/g lyophilised powder, therefore a standard curve was depicted using catechin.

Determination of the total polyphenols content by the Folin-Ciocâlteu colorimetric method

The modified Folin-Ciocâlteu method was used to determine the content of phenolic compounds. 200 µL of extract were diluted with distilled water (15.8 mL), over which 1 mL of Folin-Ciocalteu reagent was added. After 10 minutes, a volume of 3 mL of 20% sodium carbonate solution was added, and after another 60 minutes, in which the mixture was kept in the dark, the absorbance was determined at a wavelength of 765 nm. The content of phenolic compounds was expressed as mg gallic acid/g lyophilized powder, using a standard curve of gallic acid.

Determination of antioxidant activity

To determine the antioxidant activity, the protocol for measuring the antiradical activity against DPPH (2,2-diphenyl-1-picrilhydrazyl) described by Ursu et al. (2020) was used. A volume of 3.9 mL of DPPH solution reacts with 100 µL of the diluted sample for 90 minutes at room temperature in the dark. The absorbance of the solution was measured at 515 nm. The antioxidant activity of the extracts was expressed as mMol Trolox/g DW by reference to a standard curve.

Identification of the biologically active compounds from purple corn flour extracts by liquid chromatography techniques

The purple corn flour extracts were analyzed using a Thermo Finnigan Surveyor HPLC system, controlled by the Xcalibur software system (Finnigan Surveyor LC, Thermo Scientific, USA), according to the method described by Turturica et al. (2016). The extract was filtered through a C18 Sep-Pack cartridge (Cartridge-Waters, USA) to separate the anthocyanins. The chromatographic elution profile was performed with a Synergi 4u Fusion-RP 80A stationary phase column (150 x 4.6 mm, 4 pm) at an optimum column temperature of 25°C. The mobile phase consisted of 100% methanol (A) and 10% formic acid (B). A volume of 10 µL of sample was injected at a flow rate of 1 mL/min, while elution was performed under the following gradient conditions: 0-20 min, 9-35% (A); 20-30 min, 35% (A); 30-40 min, 35-50% (A); and 40-55 min, 50-

9% (A). All samples were filtered through a 0.22 µm syringe filter (Bio Basic Canada Inc., ON, Canada) prior to injection into the apparatus.

3.4.2. Methods used in the ultrasonic-assisted solid-liquid extraction optimization and validation experiments

The optimal extraction conditions of TAC were estimated as reported previously by Dumitrașcu et al. (2019), using the response optimizer function available in the Minitab 18 software. The validation experiments were conducted in triplicate under the optimized conditions, the experimental results being compared to the model predicted values based on the coefficient of variation (CV, %) and the percentage errors. The phytochemical characterization of the optimized extract aimed to determine the total content of monomeric anthocyanins, total polyphenols content and the antioxidant activity.

Determination of total anthocyanins content followed the protocol used by Lao and Giusti, (2016) for the quantification of anthocyanins. Briefly, anthocyanins were extracted from the purple corn powders using a 9:1 (*v*/*v*) mixture of 70% ethanol and 1N HCl. After the extraction, the supernatant obtained by centrifugation at 6000 rpm/15 minutes was subjected to the absorbance measurement operation using a spectrophotometer (UV-VIS double beam spectrophotometer with data analysis software, JENWAY) at the wavelength of 535 nm. The experiments were performed in triplicate. The TAC was calculated with Equation 3.2.:

$$\text{TAC}\left(\frac{\text{mg}}{\text{g}}\right) = \left(\frac{A\,x DF x V}{98{,}2x\,X}\right) \qquad (3.2)$$

where:

98.2 - unit conversions into consideration, A – the absorbance of the sample at 535 nm, DF - dilution factor, V - the known volume of the anthocyanins extract after the extraction (mL); X - the weight of purple corn powder used for the extraction (g)

Determination of antioxidant activity The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was performed as reported previously Ursu et al. (2020). Briefly, 0.1 mL of extract solution (1 mg/mL) were mixed with 2.9 mL of 0.1 mM DPPH in methanol solution and allowed to react for 60 min in the dark. The absorbance was measured at 515 nm using a spectrophotometer (Jenway, UK). The DPPH radical-scavenging activity was expressed as mMol Trolox equivalents/g DW according to the equation obtained from the standard graph using Trolox as standard.

Determination of total flavonoids content (TFC). A colorimetric method was used to determine the total flavonoids content. The total flavonoids content was determined by the catechin standard curve which is expressed in mg catechin equivalents/g lyophilised powder.

Determination of the total polyphenols content by the Folin-Ciocalteu colorimetric method (TPC). The modified Folin-Ciocâlteu method was used to determine the content of phenolic compounds. The method is based on the chemical reduction of Folin-Ciocalteu reagent, a mixture of tungsten and molybdenum oxides.

3.4.3. Chromatographic analysis of the biologically active compounds from the optimized extract

The separation and identification of the bioactive compounds from the optimized PCF extract was carried out with an Agilent 1200 HPLC system equipped with an autosampler, a degasser, a quaternary pump system, a multi-wavelength detector (MWD) and a column

thermostat (Agilent Technologies, Santa Clara, CA, USA). A Synergi Max-RP-80 Å column (250 × 4.6 mm, 4 µm particle size, Phenomenex, Torrance, CA, USA) was used for the polyphenolic compounds' separation. For the separation of the flavonoids and polyphenolic compounds from PCF extract, solvent A contained ultrapure water:acetonitrile:formic acid in a ratio of 87:3:10 (v/v/v), whereas solvent B contained ultrapure water:acetonitrile:formic acid in a ratio of 40:50:10 (v/v/v). These solvents were flushed into the system at a flow rate of 0.5 mL/min at 30℃, an injection volume of 20 µL, using the following gradient: 0 min – 94% A; 20 min – 80% A; 35 min – 60% A, 40 min – 40% A, 45 min – 10% A. The method runtime was 80 min, with the detection wavelengths being set at 280 nm and 320 nm. For anthocyanins' detection the wavelength was set at 520 nm under the previously mentioned conditions, using a flow rate of 1.0 mL/min and a method runtime of 50 min. To identify the bioactive compounds from the PCF extract, a comparison of the retention times of each peak to those obtained for the standard solutions was performed, whereas the quantification was determined through external calibration curves using the peak area. The data acquisition was done by using the Chemstation software, version B.04.03 (Agilent Technologies, Santa Clara, CA, USA). The results were expressed as mg/100g DW extract.

3.4.4. Mathematical models used to optimize the extraction process

In order to optimize the extraction of the anthocyanins from PCF, the Response Surface Methodology (RSM) was applied. A four-factor, three-level Box-Behnken design (BBD) with three replicates in the central points was employed, where time (X1), temperature (X2), liquid/solid ratio (X3) and ethanol concentration (X4) were chosen as the independent variables as shown in Table 3.1.

Table 3.1. Coded level of the independent variables considered in the Box-Behnken design

Factor level	Independent variable			
	Time (h) X_1	Temperature (°C) X_2	Liquid/solid ratio (mL/g) X_3	Ethanol concentration (%) X_4
-1	1	20	10	20
0	3	30	20	50
+1	5	40	30	80

About 1 g of purple corn flour was weighed and mixed with organic solvents in the concentrations presented above. Afterwards, the samples were centrifuged at 6000 rpm for 15 minutes and the supernatant was collected and further analyzed for the total anthocyanins content (TAC). A total of 27 experiments were carried out, the experimental data for the response were fitted using a second-order polynomial model (Equation 3.3.):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_j \sum_{i=2}^{k} \beta_{ij} x_i x_j \quad (3.3.)$$

where: Y is the response, x_i, x_j are the independent variables (with i and j ranging between 1 to k), β_0 is a constant, whereas β_i, β_{ii}, and β_{ij} are the regression coefficients of the linear, quadratic and interaction terms, k is the number of independent variables.

3.4.5. Heat treatment

Compared to the synthetic dyes, anthocyanins pigments are more susceptible to degradation by heat treatment. For the heat treatment stability study, an amount of 8 g of sample was homogenized with 80 mL of solvent (70% ethanol and 1N HCl in a ratio of 9:1). The obtained extract was subjected to an advanced ultrasonic extraction for 60min at 30^0C and then centrifuged

at 4^0C, 5000rpm/20 min. The supernatant was concentrated using a CHRIST 2-18 AVC vacuum concentrator. The obtained concentrate was solubilized with acidified water at pH 4.5, thus performing a new extraction assisted by ultrasounds, followed centrifugation under the same parameters. From the obtained supernatant, the following analyzes were performed: kinetic degradation and identification of biologically active compounds by liquid chromatography techniques. The heat treatment was performed in the following temperature and time ranges: 80^0C (0, 10, 20, 30 40 min), 90^0C (0, 10, 20, 30 min) , 100^0C - 110^0C (0, 5, 10, 15 min), 120^0C - 180^0C (0, 2, 5, 7 min). The total anthocyanins content and antioxidant activity were determined and all tests were performed in triplicate.

3.4.6. First order degradation kinetics of polyphenolic compounds

The degradation kinetics of many compounds in food systems can be described using the first order kinetic model, described in equation (3.4.) (Villota and Hawkes, 2007) :

$$-\frac{dC}{dt} = k \cdot C \qquad (3.4.)$$

where k is the constant degradation rate (min^{-1}). By integration, equation (3.4.) becomes (3.5.):

$$-\ln\left(\frac{C_0}{C}\right) = k \cdot t \qquad (3.5.)$$

The half-life ($t_{1/2}$) is given by equations 3.6. and 3.7.:

$$k \cdot t_{1/2} = -\ln(1/2) \qquad (3.6.)$$

$$t_{1/2} = \frac{\ln 2}{k} \qquad (3.7.)$$

The above mathematical expressions indicate that the half-life and rate constant of first-order reactions are independent of the initial concentration. The effect of temperature for first order reactions is given by the Arrhenius' equation (3.8.):

$$\ln k = \ln k_0 - \frac{E_a}{R \cdot T} \qquad (3.8.)$$

where k_0 - collision factor, E_a - activation energy, R - universal gas constant ($R = 8{,}314\ \mathrm{J/mol \cdot K}$), T - absolute temperature (°K).

The decimal reduction time (D) is the time required to achieve a 90% degradation of the initial compound's concentration. For the first order reactions, the value of D is calculated according to the rate constant equation (3.9.):

$$D = \frac{\ln(10)}{k} \qquad (3.9.)$$

The parameter z describes the dependence of D as a function of temperature and represents the temperature required for the value of D to be reduced by 10%.

3.4.7. The multiplication and viability of yeast cells

In order to assess the multiplication yeast cells rate, three experiments were performed, in which the PCF concentrated extract was added to 100 mL of sterile medium (liquid malt must, LMM), in different concentrations, as follows: 0.3% (V1), 0.6% (V2) and 1.2% (V3), respectively. The yeast population density was analyzed by a direct method of cell counting using the Thoma chamber for 72 h (0 h, 8 h, 12 h, 24 h, 48 h, 72 h) at 25°C, under stationary cultivation conditions. The yeast viability assay was examined by microscopy in the presence of blue methylene as an indicator, based on the capacity of viable cells of reducing the redox indicator from the blue

oxidated form (blue) to the reduced form of a leuco-derivative (colorless). A control test without the extract was performed (MV).

3.4.8. Dynamics of alcoholic fermentation

The fermentation dynamics of the yeast cells was determined using the same concentration of the PCF extract as for the multiplication study, the samples being coded with F. The fermentation process was analyzed for 144 hours (0h, 8h, 12h, 24h, 48h, 72h, 144 h) at 25°C under anaerobic conditions and on an stationary cultivation. The inoculum concentration added for both determinations was 0.32 mL for each sample. A control test without the extract was performed (MF).

3.4.9. Enzymatic inhibition activity of the optimized extract

The optimized extract was dissolved in 0.1 M PBS at pH 6.9 at a concentration of 5 mg/mL, and from this concentration, serial dilutions were performed. The results for the enzymes inhibitory assays are expressed as a mean of three replicates and given as 50% inhibition concentration (IC_{50}) calculated from the linear regression of the inhibitory activities (%) versus the extract concentrations, using the following equation for inhibitory activity (Equation 3.10.):

$$Inhibitory\ effect\ (\%) = \frac{A_c - A_s}{A_c} x100 \qquad (3.10.)$$

where: Ac = Absorbance of the control, As = Absorbance of the sample. In all the experiments, the blank sample absorbance's (the mixture containing extract and substrate solution without the enzyme was replaced by the buffer) were recorded and subtracted from the absorbance.

3.4.10. Tyrosinase inhibitory activity

The tyrosinase (from *Agaricus bisporus*, ≥1000 units/mg solid) inhibitory ability of the optimized extract was performed according to the protocol described by Meziant et al. (2021), by mixing a volume of 0.5 mL of extract solution with 0.8 mL of tyrosinase solution (46 U/mL), 2.0 mL of PBS (0.1 M, pH 6.9) and incubated for 15 min at 37°C. Then, 1 mL of L-DOPA (2.5 mM in 0.1 M PBS at pH 6.9) was added to initiate the reaction, followed by incubation for 10 min at 37°C and absorbance reading at 492 nm (Jenway, UK). The tyrosinase inhibitory activity was expressed as IC_{50} mg/mL, using kojic acid as the standard.

3.4.11. α-amylase inhibitory activity

For the α-amylase inhibitory assay, a volume of 100 µL of samples was added over 100 µL of α-amylase enzyme solution (1 mg/mL PBS) and left at room temperature for 10 minutes. Further, a volume of 100 µL of 1% starch solution was added and incubated at 37°C for 20 minutes. Finally, 0.2 mL of dinitro-salicylic acid was added and the samples were kept in a water bath at 98°C for 10 minutes. The final mixture was diluted with 2 mL of distilled water and the absorbance was read at 540 nm. The α-amylase inhibitory activity was expressed as IC_{50} mg/mL, using acarbose as the standard.

3.4.12. α-glucosidase inhibitory activity

The method described by Meziant et al. (2021) was used to evaluate the potential inhibitory effect of the extract on α-glucosidase, with minor changes. 100 µL of the extract were mixed with 20 µL of the enzyme solution (1 mg/mL in PBS buffer 0.1 M, pH 6.9), then incubated for 10 min at 37°C. After incubation, a volume of 40 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside in 0.1 M PBS (pH 6.9) was added to the mixture, and the mixture was incubated

at 37°C for 20 min, followed by the reading of the absorbance at 405 nm. The α-glucosidase inhibitory activity was expressed as IC_{50} mg/mL, using acarbose as the standard.

3.4.13. Lipase inhibitory activity

The method that follows the hydrolysis of p-nitrophenyl palmitate to p-nitrophenol, at 400 nm, was used to evaluate the effect of PCF extract on the lipase activity. Briefly, the lipase solution (1.0 mg/mL in PBS, pH 7.0) was mixed with the PCF extract at concentrations ranging from 0.9 to 7.2 mg/mL and pre-incubated on ice for 5 min. The reaction mixture contained 330 µL of PBS 0.1 M (pH 7.0) supplemented with 0.6% (v/v) Triton X-100 and 0.15% (w/v) arabic gum, and 20 µL of 10 mM p-nitrophenyl palmitate. The enzymatic reaction started by adding 50 µL of the lipase/PCF extract solution into the substrate mixture, and incubating at 37°C for 20 min. The lipase inhibitory activity was expressed as IC 50 mg/mL, using orlistat as a standard.

3.4.14. Statistical analysis

The results were expressed as mean values. The stepwise regression analysis was performed using Minitab 18 Software. The statistical significance of the polynomial equation (based on the p value) was evaluated by using ANOVA, as well as the regression coefficients of the individual linear and quadratic terms. The accuracy and validity of the model were evaluated in terms of the coefficient of determination (R^2), whereas the lack of fit test and the F-test were considered significant at p-value < 0.05.

3.5. RESULTS AND DISCUSSIONS

3.5.1. Comparative analysis between the biologically active compounds extraction techniques from purple corn flour

In Table 3.2. the global phytochemical profile of the 5 extract variants is presented.

Table 3.2. Content of total anthocyanins, flavonoids, polyphenols and the antioxidant activity of purple corn flour extracts

Phytochemicals	Variant 1	Variant 2	Variant 3	Variant 4	Variant 5
Anthocyanins, C3G, mg/g DW	1.48 ± 0.16 ^{b}	2.96 ± 0.07 ^{a}	2.38 ± 0.38 ^{ab}	2.33 ± 0.65 ^{ab}	2.09 ± 0.24 ^{ab}
Flavonoids, mg EC/g DW	0.77 ± 0.03 ^{a}	0.67 ± 0.10 ^{ab}	0.66 ± 0.05 ^{ab}	0.58 ± 0.05 ^{b}	0.51 ± 0.06 ^{b}
Polyphenols, mg EAG/g DW	0.93 ± 0.06 ^{c}	1.48 ± 0.02 ^{a}	1.12 ± 0.70 ^{bc}	1.22 ± 0.07 ^{b}	1.22 ± 0.11 ^{b}
Antioxidant activity, mM Trolox/g DW	3.03 ± 0.12 ^{b}	5.35 ± 0.41 ^{a}	4.76 ± 0.13 ^{a}	4.92 ± 0.78 ^{a}	4.82 ± 0.01 ^{a}

*values in the same row that do not share a letter (a, b) are statistically different (p <0.05) according to the Anova method, Tukey test (95% confidence level)

From table 3.2., it can be noted that the overall phytochemical profile of the 5 extract variants varies. For example, the total content of anthocyanins (TAC) varies between 1.48 mg C3G/g (variant 1) and 2.96 mg C3G/g (variant 2), and the total content of polyphenols (TPC) varies between 0.93 mg EAG/g and 1.48 mg EAG/g. In the present study, the total flavonoids content (TFC) varied between 0.51 mg EC/g (variant 5) and 0.77 mg EC/g (variant 1), while the antioxidant activity varied between 3.03 ± 0.12 mM Trolox/g DW and 5.35 ± 0.41 mMol Trolox/g DW. To further quantify the antioxidant activity, the IC_{50} was calculated. When measuring the antioxidant activity using different extraction systems, it was observed that the addition of methanol in different percentages had a significant influence on the antioxidant activity. The best response, taking into account the extraction system applied for the DPPH test, was 80:20, methanol:water acidified with 1% HCl (1 N), which showed a better response with an IC_{50} of 66.3

μg/mL. For the DPPH, the IC_{50} values observed for the purple corn extracts were significantly lower compared to those of other plant species.

From the experimental data presented in Table 3.2. it can be concluded that the hydroalcoholic extraction (70% ethanol) allowed us to obtain an extract with a higher overall phytochemical profile.

3.5.2. Identification and chromatographic separation of the antioxidant compounds from the purple corn flour extract

The HPLC-DAD profile of purple corn flour extract obtained by ultrasound-assisted extraction at 30°C/60 min allowed the identification of six main compounds (Figure 3.1.) as follows: cyanidin-3- *O* -glucoside, pelargonidine -3-*O*-glucoside, peonidin-3-*O*-glucoside, cyanidin-3-*O*-(6''- malonylglucoside), pelargonidin-3-*O*-(6''-malonylglucoside) and peonidin-3-*O*-(6''-malonyl glucoside). The two major compounds were cyanidin-3-*O*-glucoside and its acylated form cyanidin-3-*O*-(6''-malonylglucoside). The compound with the lowest content was the acylated form of pelargonidin-3-*O*-glucoside.

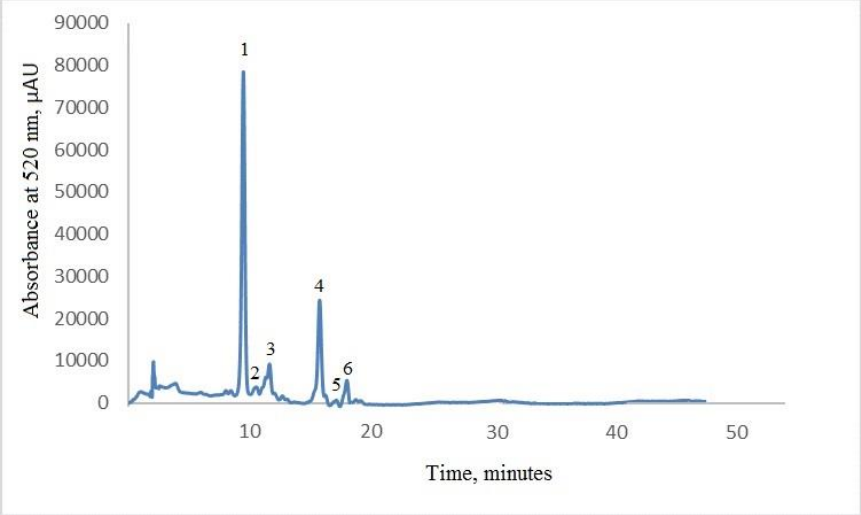


Figure 3.1. Chromatographic profile of purple corn flour extract - Peak 1 - cyanidin-3-*O* -glucoside; Peak 2-pelargonidin-3-*O*-glucoside; Peak 3 - peonidin-3-*O*-glucoside; Peak 4 - cyanidin-3-*O*-(6”-malonyl glucoside); Peak 5 - pelargonidin-3-*O*-(6''- malonylglucoside) and Peak 6 - peonidin-3-*O*-(6'' - malonylglucoside)

Yang et al. (2010) reported that the HPLC profile of purple maize was represented by anthocyanins such as cyanidin-3-glucoside, pelargonidine-3-glucoside and peonidine-3-glucoside. Moreover, Pascual-Teresa et al. (2002) and Aoki et al. (2002) also determined the presence of cyanidin-3-*O* - (6” -malonyl glucoside), pelargonidine-3-*O*-(6”-malonylglucoside), peonidin-3-*O*-(6”-malonyl glucoside), cyanidin-3-*O*-(6”-ethylmalonylglucoside), pelargonidine-3-*O*-(6”-ethylmalonylglucoside) and peonidin-3-*O*-(6”-ethylmalonylglucoside). Based on the results, it can be confirmed that purple corn has a significant fraction of acylated anthocyanins, the acylated form being represented by malonic acid mainly.

3.5.3. Optimization of anthocyanins’ extraction conditions from purple corn flour to solid-liquid extraction

In this study, a three-level, four-factor BBD was performed to evaluate the effect of various independent variables on the recovery of TAC from PCF. The experimental data were collected in Table 3.3. The ANOVA parameters presented in Table 3.4. indicated that the second-order regression was significant ($p < 0.05$) and the quadratic polynomial model fitted the experimental

results. The lack of fit test did not show a significant influence, indicating a high accuracy of the model for the prediction of TAC. The determination coefficient R^2 suggested that the model is able to explain 79% of TAC variation.

Table 3.3. Box–Behnken design (BBD) design matrix with the independent variables and experimental dataa for the response of total anthocyanins content (TAC) from purple corn flour

Run Order	X_1	X_2	X_3	X_4	TAC (mg/100 g)
1	3	30	10	50	14.14
2	1	30	20	20	6.25
3	1	40	20	50	15.1
4	5	30	20	20	7.77
5	3	20	20	80	11.74
6	1	30	30	50	11.75
7	3	40	20	80	11.81
8	5	40	20	50	15.87
9	1	30	10	50	15.61
10	5	30	20	80	13.50
11	3	40	10	50	19.02
12	3	30	20	50	13.60
13	3	30	10	20	8.95
14	3	30	20	50	13.46
15	3	20	20	20	7.52
16	3	30	30	20	8.29
17	5	30	30	50	13.18
18	3	40	20	20	11.32
19	3	20	30	50	9.54
20	3	30	30	80	13.60
21	3	30	10	80	14.40
22	3	40	30	50	10.84
23	5	20	20	50	12.48
24	3	30	20	50	15.09
25	3	20	10	50	13.72
26	1	30	20	80	15.60
27	1	20	20	50	11.23

[a]values are expressed as the mean value of three determinations

From Table 3.4., it can be seen that in linear terms, that the temperature, liquid/solid ratio, and ethanol concentration presented a significant effect on TAC, whereas in quadratic terms only the liquid/solid ratio displayed a significant effect. The regression model resulted after eliminating the insignificant terms was expressed was expressed using equation 3.11.:

$$TAC = 0.45 + 0.14\ X_2 - 0.16\ X_3 + 0.39\ X_4 - 0.003\ X_4^2 \quad (3.11)$$

where: X_2 is the temperature (°C), X_3 is the liquid/solid ratio (mL/g) and X_4 is the ethanol concentration (%).

From the regression equation we can observe a positive effect exerted by temperature and ethanol concentration on the recovery of TAC and a negative effect exerted by the liquid/ solid ratio both in linear and quadratic terms. In order to obtain high extraction yields, increased attention has to be given to the parameters interval chosen for the extraction. Liquid-solid ratio plays a significant effect on the yield of bioactive compounds extraction. For example, in a recent review, Cristianini and Sanchez (2020) reported that two of the most significant variables in the extraction of anthocyanins from purple corn cob are solid/liquid ratio and pH. In a recent study

conducted by Rafael (2017), the author used a liquid/solid ratio of 20 and reported an anthocyanins content of 7.43 mg C3G/g.

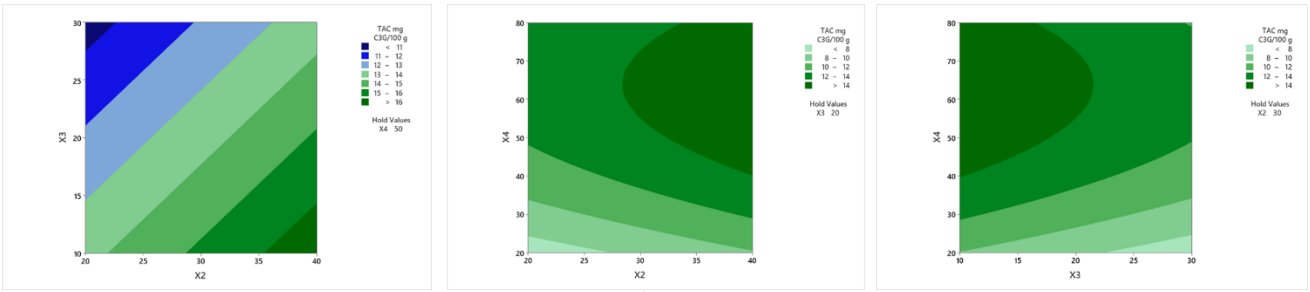
Table 3.4. Analysis of variance and coefficients estimate of the second order polynomial model for total anthocyanins content (TAC) from purple corn flour

Source	DF	Adj SS	Adj MS	F-value	*p* -value
Model	4	183.20	45.80	20.75	0.000
Linear	3	132.91	44.30	20.07	0.000
X_2	1	26.19	26.19	11.87	0.002
X_3	1	28.97	28.97	13.12	0.002
X_4	1	77.74	77.74	35.22	0.000
Square	1	50.29	50.29	22.78	0.000
X_4^2	1	50.29	50.29	22.78	0.000
Error	22	48.56	2.20		
inadequacy	20	46.93	2.34	2.87	0.290
Pure error	2	1.63	0.81		
Total	26	231.77			
R^2			0.79		
Adj R^2			0.75		

Besides solid-liquid ratio, temperature is another important variable that can affect the extraction procedure. The diffusion of bioactive compounds, mainly anthocyanins can be accelerated at a higher temperature, however a temperature above 40°C can result in the anthocyanins degradation as revealed previously by Timberlake, 2009.

The plots were obtained by plotting the response of TAC using the z-axis against two independent variables while maintaining the other independent variable at a constant level. The effect of the solid-liquid ratio and temperature on TAC at constant ethanol concentration of 50% is shown in (Figure 3.2.a). The anthocyanin content increased with increasing temperature and decreasing the liquid/solid ratio. Blackhall et al. (2018) showed that the anthocyanins content increased when using a liquid/solid ratio of up to 10 mL/g, similar results being reported by Dumitrașcu et al. (2019), a further increase resulted with the decrease of the anthocyanins recovery.

In Figure 3.2.b., it is depicted the effect of temperature and ethanol concentration on the TAC extraction at a constant liquid/solid ratio of 10 mL/g. It can be seen that the TAC increased with the increasing of both the temperature and ethanol concentration and reached a maximum when using an ethanol concentration of 80% and an extraction temperature of 40°C.



a b c

Figure 3.2. Contour plots of anthocyanins from purple corn flour with respect temperature and liquid/solid ratio (a); temperature and ethanol concentration (b); liquid/solid ratio and ethanol concentration (c)

On the other hand, when considering the effect of solid/liquid ratio and ethanol concentration on the anthocyanin extraction (Figure 3.2.c), it can be seen that higher values of TAC are obtained when the ethanol concentration ranged between 40 to 80%, whereas the liquid/solid ratio ranged between 10 to 20 mL/g. Ali et al. (2018) reported that using solvents with similar characteristics as the matrix favors the solubilization of compounds from the matrix.

3.5.4. Model validation

The optimum extraction conditions proposed by the model, based on the desired function method, were performed to validate the model equation. The optimum conditions for the maximum recovery of TAC were: extraction time of 5 hours, temperature of 39˚C, liquid/solid ratio of 30 mL/g and ethanol concentration of 73%. Under these conditions, the maximum response predicted for TAC was of 13.77 mg C3G/g dry weight (DW). The experimental results obtained under the optimum extraction conditions predicted for TAC confirmed the validation extraction model (Table 3.5.). The validation is also supported by the percentage of errors which was lower than 10% and by a low variation coefficient (CV).

Table 3.5. Experimental data of the validation of predicted values for total anthocyanins content (TAC) at optimal extraction conditions

Dependent variable	Predicted value	95 % Confidence intervals	Experimental value	CV (%)	Error (%)
TAC (mg C3G/g DW)	13.77	12.19 - 15.36	14.04 ± 0.02	1.08	1.95

Fan et al. (2008) studied the extraction of the bioactive compounds from purple corn (*Zea mays* L.), and found that the highest recovery of anthocyanins was obtained when using the following extraction conditions: a temperature of 40°C, extraction time of 8 hours, a solid-liquid ratio of 33% and a solvent volume of 1:15. These authors suggested an extraction yield for TAC of 42.28 mg C3G/100 g. Gorriti-Gutierrez et al. (2009a and 2009b) reported the highest anthocyanin content in purple corn at 75°C, for an extraction time of 120 min and 240 min. The results obtained by these authors suggested a negative correlation between the temperature and time applied for extraction, showing that a higher temperature results in a better diffusion coefficient and a higher solubility of the compounds.

3.5.5. Phytochemical profile of the optimized extract

The validated extract was characterized in terms of flavonoids content and antioxidant activity. The TFC content of the extract was 1.37± 0.05 mg CE/g DW, yielding an antioxidant activity of 55.61± 0.25 mM Trolox/g DW. Another study aimed to optimize the extraction of anthocyanins from purple corn revealed a maximum anthocyanin extraction yield of 5.90 mg/g DW was obtained (Yang et al. 2007). In a previous study, Slavu (Ursu) et al. (2021) followed the phytochemical profile of PCF and reported a total monomeric anthocyanin content of 14.94 ± 0.68 mg C3G g DW, TFC of 0.35 ± 0.03 mg CE g DW and an activity antioxidant of 15.40 ± 0.16 M Trolox/g DW.

3.5.6. Chromatographic profile of the optimized extract

The chromatographic profile of the optimized extract is presented in Figure 3.3. and summarised in Table 3.6.

From Figure 3.3., it can be observed that, using different wavelengths, 34 compounds were separated, but only gallic acid (349.00 ± 4.39 mg/100 g DW), *p -coumaric acid* (260.01 ± 1, 90 mg/100 g DW), caffeic acid (19.73 ± 1.89 mg/100 g DW) and vanillic acid (245.46 ± 8.00 mg/100 g DW) were identified and quantified. The main flavonoids identified were myricetin (722.89 ± 2.70 mg/100 g DW), quercetin 3-β-D-glucoside (688.16 ± 5.00 mg/100 g DW) and kaempferol (512.72 ± 9.99 mg/100 g DW). Six anthocyanins (Figure 3.4.) were separated, namely cyanidin 3- *O* -glucoside (395.66 ± 1.26 mg/100 g DW) and malvidin chloride (159.30 ± 0.04 mg/100 g DW) .

a.

b.

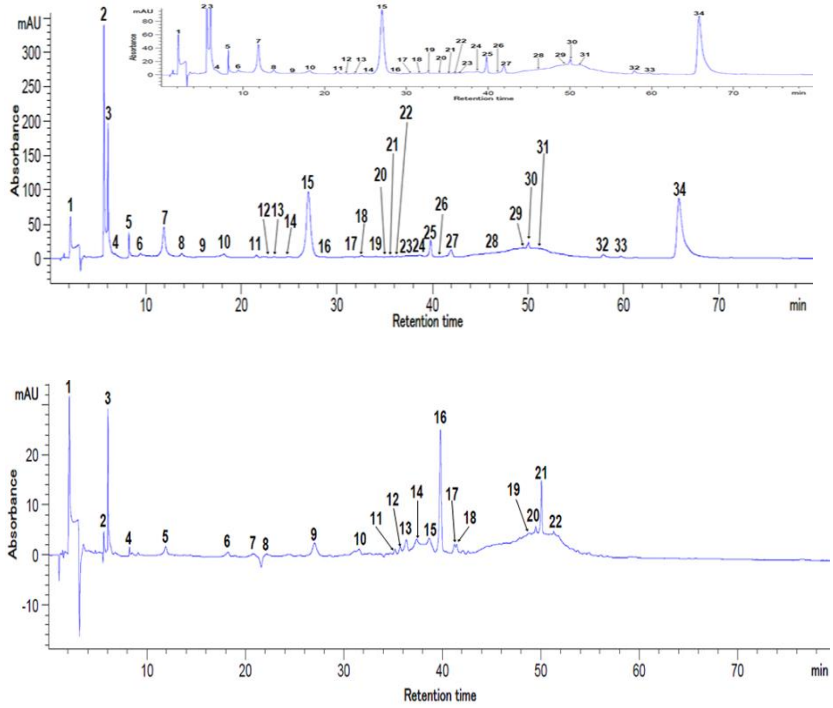


Figure 3.3. HPLC chromatograms for the flavonoids and polyphenols from purple corn flour extract at 280 nm (a) and 320 nm (b). Peaks' identification: (a) 2 – Gallic acid, 4 – Caffeic acid, 8 – Vanillic acid, 25 – Myricetin, 28 – Quercetin 3-β-D-glucoside, 31 – Kaempferol, 1, 3, 5 – 7, 9 – 24, 26, 27, 29, 30, 32 – 34 – unidentified peaks; (b) 2 – Gallic acid, 7 – p-coumaric acid, 16 – Myricetin, 22 – Kaempferol, 1, 3 – 6, 8 – 15, 17 – 21– unidentified peaks.

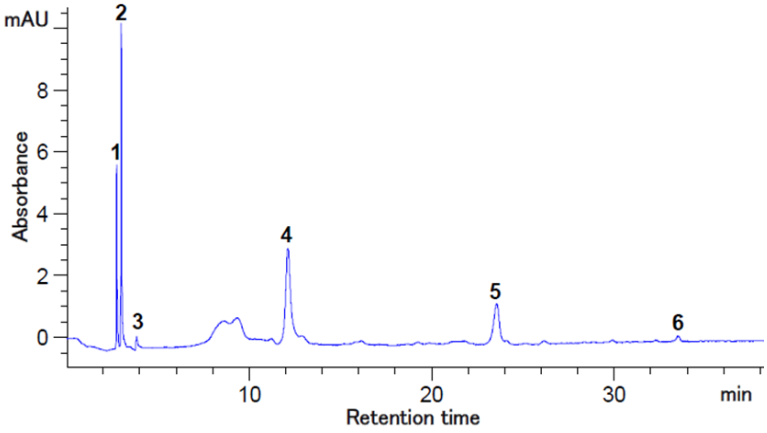


Figure 3.4. HPLC chromatogram for the anthocyanins from purple corn extract at 520 nm. Peaks' identification: 4 – Cyanidin 3-O-glucoside, 6 – Malvidin chloride, 1 – 3, 5 – unidentified peaks.

Table 3.6. Quantification of the bioactive compounds from purple corn flour extract

Compounds	**Concentration, mg/100 g DW**	
	280 nm	**320 nm**
Gallic acid	349.00 ± 4.39	6.83 ± 0.00
Acid *p* -cumaric	ND	260.01 ± 1.90
Caffeic acid	19.73 ± 1.89	ND
Vanilla acid	245.46 ± 8.00	ND
Myricetin	722.89 ± 2.70	698.18 ± 0.62
quercetin	688.16 ± 5.00	ND
Kaempferol	512.72 ± 9.99	15.81 ± 4.91
anthocyanins	**520 nm**	
Cyanidin 3- *O* -glucoside	395.66 ± 1.26	
Malvidin chloride	159.30 ± 0.04	

Hong et al. (2020) identified eighteen different anthocyanins compounds, consisting of cyanidin-, peonidin- and pelargonidin-glucoside, in sweet corn and pigmented corn. For example, these authors reported a cyanidin 3- *O* -glucoside content ranging from 72.4 to 137.1 mg in purple corn.

3.5.7. Thermal degradation kinetics of extracted anthocyanins

Following the heat treatment, from the TAC analysis by the pH differential method, the results from Table 3.7 were obtained, in the temperature range 80-110°C. The heat treatment between 80-110°C did not cause a significant influence on the content of monomeric anthocyanins.

Table 3.7. Anthocyanins content (mg G3G/g DW) of purple corn flour extract after a heat treatment between 80-110^{0}C

Hold time (min)	Temperature (° C)			
	80	90	100	110
0	2.24 ± 0.10	2.68 ± 0.04	2.44 ± 0.05	2.39 ± 0.25
2	ND	ND	ND	ND
5	ND	ND	2.46 ± 0.20	2.40 ± 0.01
7	ND	ND	ND	ND
10	2.37 ± 0.15	2.44 ± 0.05	2.47 ± 0.11	2.42 ± 0.07
15	ND	ND	2.47 ± 0.04	2.46 ± 0.11
20	2.37 ± 0.15	2.48 ± 0.20	ND	ND
30	2.44 ± 0.04	2.43 ± 0.02	ND	ND
40	2.60 ± 0.08	ND	ND	ND

Next, a detailed study of the anthocyanins' degradation kinetics, as a function of temperature and time was performed. The thermal degradation kinetics of the monomeric anthocyanins from the extract was studied for temperatures between 120 and 180°C. The results were linearized by a logarithm function and the fit of the first order kinetic model was verified using the linear regression method (Figure 3.5.).

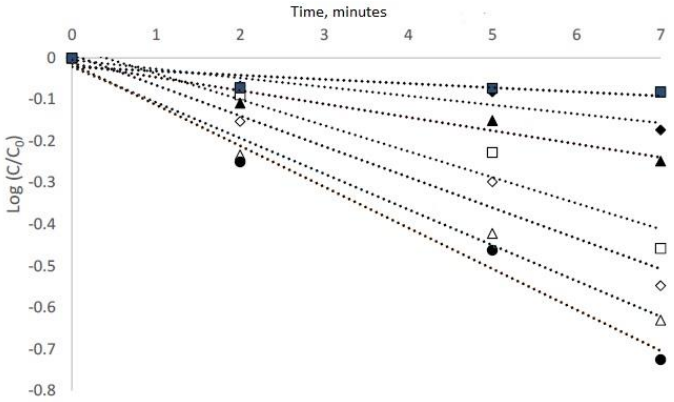


Figure 3.5. Thermal degradation kinetics of total monomeric anthocyanins in acidified water at pH 4.5 at temperatures between 120 and 180°C (■ 120°C, ◆130°C, ▲ 140°C, □150°C, ◊160°C, Δ170°C, ● 180°C)

The results presented in Figure 3.5. showed a linear decrease of the TAC, at a constant temperature and different time periods, results which support the hypothesis of a thermal degradation based on the first order degree kinetic model. The linear regression applied for equation 2 led to the estimation of D values, the decimal reduction times being estimated as seen in Table 3.8. As expected, the decimal reduction time decreased with the increasing temperature. The values of the degradation rate constants increased with the increasing temperature. At the

temperature of180 °C, the decimal reduction time was shorter, about 10 minutes, while it took more than 100 minutes to degrade the anthocyanins at 120°C.

The values of the kinetic parameters obtained and presented in Table 3.8. and Figure 3.5., showed that the anthocyanins from PCF, responsible for the color and some biological properties of this matrix, had a higher thermostability compared to that registered for the spores or vegetative cells (**z = 5–12°C**).

Table 3.8. Degradation rate constants and *D values* for the thermal degradation of the purple corn flour anthocyanins

Temperature (°C)	*k* x 10 $^{-2}$ (1 / min)	*D* values (min)	t $_{1/2}$ (min)
120	2.28 ± 0.024 *	101.01 ± 2.66	30.40 ± 1.24
130	4.99 ± 0.035	46.08 ± 3.72	13.86 ± 1.98
140	7.39 ± 0.125	31.15 ± 0.61	9.37 ± 0.97
150	14.37 ± 0.17	16.02 ± 0.39	4.82 ± 0.67
160	16.95 ± 0.23	13.58 ± 0.19	4.08 ± 0.45
170	19.29 ± 0.12	11.93 ± 0.25	3.59 ± 0.22
180	22.68 ± 0.20	10.15 ± 0.14	3.05 ± 0.12
E_a = 55.75 kJ / mol ± 6.83 (R^2 0.93)		z_T = 61.72 ± 2.28 (R^2 0.90) °C	

Therefore, the constant rates of the thermal degradation of anthocyanins are much less dependent on temperature (Holdsworth, 1985). Regarding the mechanism, it is known that the thermal degradation of anthocyanins begins with the opening of the central ring followed by the hydrolysis of the molecule, and the formation of colorless compounds. Yang et al. (2008) studied the thermal degradation of anthocyanins from purple corn at 70, 80 and 90°C. They showed that the rate of degradation of anthocyanins increased with the increasing temperature. The same results were recorded by Kırca et al. (2006). The half-life values ranged from 11.6 to 7.5 for the aqueous extracts at pH 4.0 at 70, 80 and 90°C, respectively.

The temperature dependence of the thermal degradation rate constants is present in Figure 3.6.

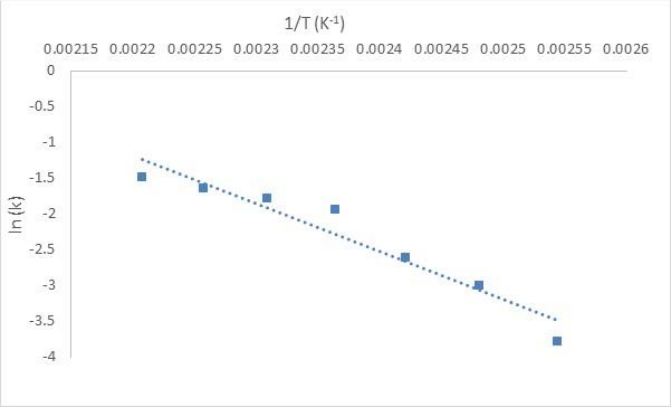


Figure 3.6. Arrhenius correlation describing the temperature dependence of the of thermal degradation of anthocyanins constant rates in the temperature range 120-180°C

Peron et al. (2017) suggested activation energy values of 99.77 ± 0.87 kJ/mol in a juçara extract (a palm species) and 93.62 ± 0.44 kJ/mol for the anthocyanins degradation in "Italy" grape extract, while Heldman (2011) suggested that the activation energy for the anthocyanins degradation ranged from 35 to 125 kJ/mol.

3.5.8. Thermal degradation kinetics of the antioxidant activity of purple corn flour extract

The antioxidant activity of purple corn is given by all the polyphenolic compounds (anthocyanins, phenolic acids, flavonoids) that usually are thermolabile. This fact was demonstrated by evaluating the heat treatment behavior (Figure 3.7.). Throughout the studied

temperature range, the antioxidant activity registered a slight decrease, suggesting the involvement of other compounds with different thermostability. Thus, after a heat treatment at 120 °C, for 30 minutes there was a decrease in the antioxidant activity by 20%, while after 7 minutes of maintenance at 180°C, the antioxidant activity decreased by about 30%. The decrease in the antioxidant activity of the heat-treated purple corn extract could be attributed to the synergistic combinations or interactions of several types of chemical reactions, the diffusion of water-soluble compounds and their formation or degradation, as explained by Harakotr et al. (2014).

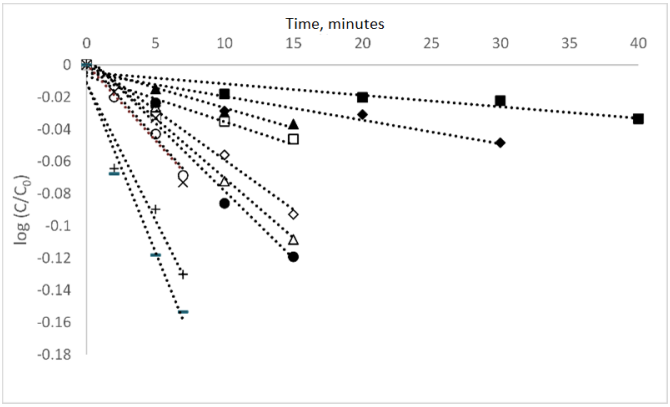


Figure 3.7. Thermal degradation kinetics of antioxidant activity in acidified water at pH 4.5 at temperatures between 80 and 180 °C (■ 80°C, ◆90°C, ▲ 100°C□110°C, ◊120°C, Δ130°C, ● 140°C, ○ 150°C, x160°C, + 170°C, -180°C)

The thermal degradation of the antioxidant activity of the purple corn flour extract followed in this case a first-order kinetic model (Figure 3.7.). Therefore, the *k values* ranged from 1.61 ± 0.01 · 10^{-3} 1/ min at 80°C, increasing significantly to 4.85 ± 0.29 · 10^{-2}1/ min at 180°C (Table 3.9.). Significant differences can be seen in table 3.8. when analyzing the *D* values that demonstrate clear differences in thermal sensitivity at different temperatures. By increasing the temperature with 10°C from 80 to 90°C, the *D value* was 2 times lower, while the increase to 120°C caused a reduction of almost 10 times. The temperature dependence of the thermal degradation rate constants is presented in Figure 3.8.

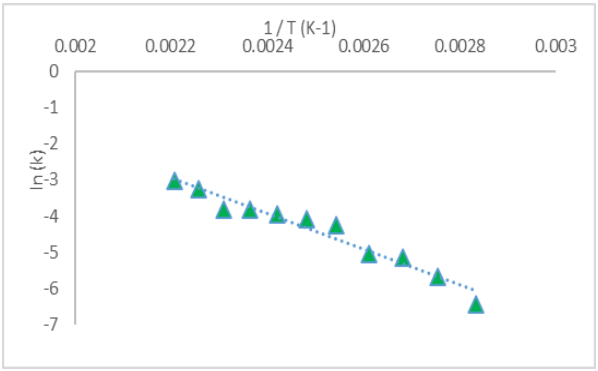


Figure 3.8. Arrhenius correlation describing the temperature dependence constant rates of the thermal degradation of antioxidant activity in the purple corn flower extract in the 80-180°C temperature range

The estimated *E values* for the degradation of the antioxidant activity was 41.12 ± 3.00 kJ/mol, suggesting that a much higher energy is required to thermally degrade the antioxidant activity, which can be explained through the different thermostability of the compounds in the extract, compounds responsible for the antioxidant activity.

Table 3.9. Degradation rate constants and *D values* for the thermal degradation of anthocyanins in the purple corn flour extract

Temperature (° C)	*k* x 10 $^{-2}$ (1 / min)	D values (min)	t $_{1/2}$ (min)
80	0.16 ± 0.01 *	1428.57 ± 25.67	429.96 ± 13.45
90	0.34 ± 0.02	666.66 ± 17.59	200.65 ± 11.26
100	0.57 ± 0.10	400 ± 19.35	120.39 ± 16.78
110	0.64 ± 0.09	357.14 ± 8.89	107.49 ± 11.27
120	1.42 ± 0.28	161.29 ± 7.66	48.54 ± 2.24
130	1.70 ± 0.26	135.13 ± 3.82	40.67 ± 2.87
140	1.93 ± 0.25	119.04 ± 4.61	35.83 ± 2.98
150	2.18 ± 0.27	105.26 ± 2.39	31.68 ± 1.97
160	2.23 ± 0.13	103.09 ± 2.19	31.02 ± 2.67
170	3.91 ± 0.18	58.82 ± 1.12	17.70 ± 1.43
180	4.85 ± 0.29	47.39 ± 1.14	14.26 ± 1.82
E_a = 41.12 kJ/mol ± 3.00 (0.95)		**z_T = 75.75 ± 2.87 (0.93)°C**	

As expected, the *z -value* indicated that the thermal resistance of the compounds responsible for the antioxidant activity (75.75 ± 2.87°C) was higher than that for spores or vegetative cells, suggesting that the rates of thermal degradation rates of the biologically active compounds are much less dependent on the temperature. A comparative analysis of the degradation kinetics of anthocyanins versus the antioxidant activity is shown in Figure 3.9., in the 150-180°C temperature range.

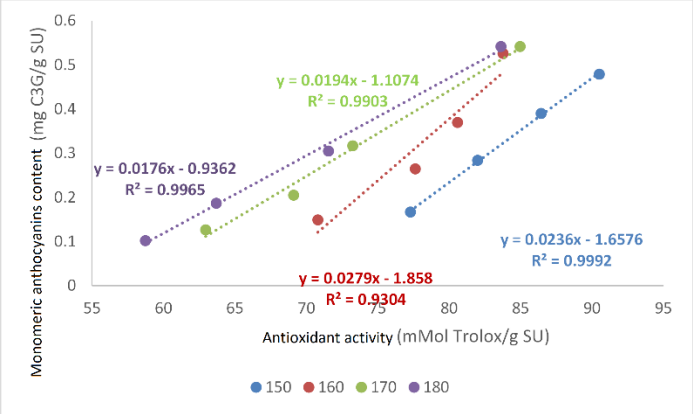


Figure 3.9. Correlation between the antioxidant activity and monomeric anthocyanins content at different temperatures, for holding times between 0-7 minutes

The antioxidant activity decreased with the decrease of the anthocyanins' concentration (Figura3.9.), regardless of the temperature and the maintenance time, there was a linear dependence between the two variables.

3.5.9. The influence of the biologically active compounds from the extracts of PCF on the yeasts' metabolic activity

In the present study, PCF extract was tested as an adjuvant in the fermentation culture medium of baking yeasts, to study the effect on the yeast's metabolic activity, in terms of multiplication, cell viability and alcoholic fermentation dynamics. Figure 3.10. shows that the highest multiplication rate was obtained after 48 hours of cultivation, being correlated to the highest amount of added PCF extract (1.2 mg/100 mL), compared to the control sample. The generation number (*n*) of yeast cells increased significantly from 2.88 ± 0.76 for the control samples to 6.72 ± 0.88 for V1, 7.52 ± 0.16 and 7.68 ± 0 , 56 in the case of V2 and V3, respectively.

The growth rate also increased significantly from 5.23 ± 0.97 for MV to 1.91 ± 0.11 for V1 and 1.44 and 1.40 for V2 and V3, respectively.

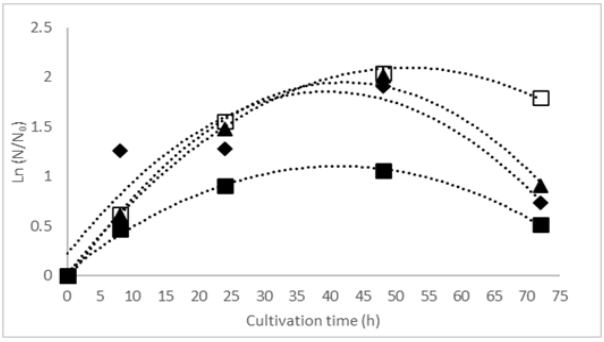


Figure 3.10. Dynamics of yeast multiplication by cultivation in a liquid medium supplemented with purple corn flour extract: (■) control sample - MV; (◆) sample with 0.3% extract (▲) sample with 0.6% extract; (□) sample with 1,2% extract.

Therefore, it can be appreciated that no significant differences were observed when considering different concentrations of the optimized PCF extract between 0.6 and 1.2%. The significant effect of the added extract was observed when the generation time was taken into account, the time interval for the yeast cells to double their population was 1.81 ± 0.23 h for the MV sample and decreased significantly to 0.28 ± 0.01 h for V1, 0.19 ± 0.01 h for V2 and 0.18 ± 0.01 h for V3. From Figure 3.10, it can be seen that the cell autolysis began after 48 hours of culture, reaching a maximum after 72 hours. The results showed that in the absence of the purple corn flour extract, the autolysis was faster, the percentage of autolysed cells compared to the total number of cells in the culture was visibly reduced after 72 hours of cultivation in the control samples ($8.4x10^7$ CFU/mL), compared to V1 ($10.4x10^7$ CFU/mL), V2 ($12.4x10^7$ CFU/mL), while the highest number of cells was determined in V3 ($30x10^7$ CFU/mL). The results support the hypothesis of the protective effect of the anthocyanin extract against the yeast cell, depending on the dose. The obtained results are in agreement to Râpeanu et al. (2008), who used polyphenolic extracts from red grapes to study the influence on the yeast multiplication kinetics as well as on the alcoholic fermentation capacity.

3.5.10. The effect of purple corn polyphenols on the dynamics of alcoholic fermentation

The fermentation dynamics was evaluated according to the CO_2 released during 6 days. The highest total loss of CO_2 was observed in V2 when compared to the control sample VM (Figure 3.11.). Li et al. (2020) showed that grape-derived proanthocyanidins could act as a protector against various environmental requirements for *Saccharomyces cerevisiae* during wine fermentation, resulting in an increased physiological activity, fermentation efficiency and an improved wine quality.

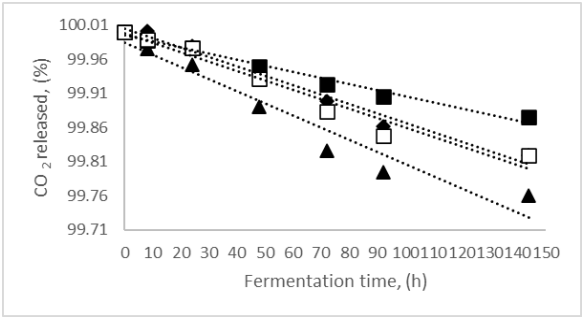


Figure 3.11. The rate of CO_2 release in the alcoholic fermentations of liquid medium supplemented with purple corn flour extract: (■) control sample – MV; (◆) sample with 0.3% extract (▲) sample with 0.6% extract; (□) sample with 1.2% extract

3.5.11. Evaluation of some biological properties of the optimized extract by determining the inhibition potential of some enzymes involved in the metabolic syndrome

Tyrosinase inhibition activity

Tyrosinase activity is associated with diseases such as hyperpigmentation, melanoma and Parkinson. Therefore, the potential inhibitors of tyrosinase activity may be a treatment alternative for these diseases, such as phenolic acids, flavonoids and anthocyanins (Koyu et al. 2018). In the study, the inhibition ability of PCF extract was compared to the one of kojic acid as a reference drug. The IC_{50} for the PCF extract, obtained under optimum extraction conditions, was 1.15 ± 0.01 mg/mL. The reference value for kojic acid was 4.8 µg/mL, suggesting, therefore, that the extract was less effective in inhibiting tyrosinase.

Inhibitory activity of α-amylase, α-glucosidase and lipase

Currently, various strategies are used to inhibit the enzymes involved in the digestion of carbohydrates, to control hyperglycemia, diabetes and other disorders, by inhibiting α-glucosidase and α-amylase with acarbose. In our study, we also followed the potential of the PCF extract to inhibit the α-glucosidase and α-amylase, and therefore to test the potential use as an alternative for drug administration. The IC_{50} values for α-glucosidase and α-amylase were 23.00±1.41 mg/mL and 6.51±0.14 mg/mL, whereas the corresponding values for acarbose were 298.57 µg/mL and 207.34 µg/mL. At a concentration of 3.6 mg/mL, the PCF extract reduced the activity of α-glucosidase by 95%, whereas at the same concentration of the extract, α-amylase was inhibited by 41%. The corresponding IC_{50} value for the lipase inhibition was 2.97±0.38 mg/mL, with a corresponding value for orlistat of 13.24 µg/mL. It has been suggested that the flavonoid-rich extracts were more potent inhibitors of digestive enzymes than the anthocyanins-rich extracts (Kim, 2020; Siegień et al. 202). Therefore, it is fair to consider that the content of flavonoids in PCF extract may be responsible for the inhibitory activity, althought a joint effect is also possible. Previous studied suggested that flavonoids possessed excellent inhibitory effects on α-glucosidase (Semaan et al. 2018). Quercetin derivatives and kaempferol are the most predominant flavonoids in the PCF extract, showing an excellent inhibitory effect on α-glucosidase, α-amylase and lipase, in good agreement with the findings of Hamed et al. (2021). From the obtained results, it seems that the PCF extract is more effective in inhibiting the lipase, followed by tyrosinase, α-amylase and last the α-glucosidase.

3.6. Partial conclusions

The objectives of the study were met as follows:

- Comparatively, 5 extraction techniques were tested in terms of their biologically active compounds content and antioxidant activity. The extraction techniques applied were both conventional (water extraction, organic solvent extraction) and assisted extraction (ultrasounds-assisted extraction). Each extract was analyzed comparatively for the total content of anthocyanins, polyphenols, flavonoids and antioxidant activity.
- The extract obtained by ultrasounds-assisted extraction at 30˚C/60 min was considered superior in terms of the biologically active compounds concentration and used in the subsequent analyzes to chromatographically identify and separate the antioxidant compounds and to determine the behavior of anthocyanins at a heat treatment.
- Following the identification and chromatographic separation of the purple corn extract, the two major compounds were cyanidin-3-*O*-glucoside and its acylated form cyanidin 3-*O*-(6

"-malonylglucoside). The compound with the lowest content was the acylated form of pelargonidin-3-*O* -glucoside.

- The optimum extraction conditions for the maximum recovery of anthocyanins from the PCF were: extraction time 5 hours, at a temperature of 39°C, liquid/solid ratio of 30 mL/g and ethanol concentration of 73%, while obtaining a maximum predicted response and experimental values of 13.77 and 14.04 ± 0.02 mg C3G/g DW, respectively. The chromatographic analysis of the optimized extract showed a high concentration of flavonoids, the major compounds being myricetin and quercitin, while the major anthocyanins were cyanidin 3- *O* -glycoside and malvidin chloride.
- The results highlighted the significant effect of temperature, liquid/solid ratio and ethanol concentration on the anthocyanin extraction in linear terms, while in quadratic terms only the liquid/solid ratio had a significant effect. The obtained regression equation showed a positive effect exerted by temperature and ethanol concentration on the anthocyanin recovery yield and a negative effect exerted by the liquid/solid ratio both in linear and polynomial terms.
- The thermal degradation kinetic studies in the 80-180°C temperature range, highlighted a high thermostability of the anthocyanins.
- In the 120-180°C temperature range, the thermal degradation of anthocyanins in the purple corn extract followed a first-order degradation kinetics, which allowed the estimation of the thermal degradation parameters, such as degradation rate constants, decimal reduction time, half-life, *z -value* and activation energy;
- The thermal degradation parameters related to the antioxidant activity were significantly lower compared to those estimated for the thermal degradation of anthocyanins, which indicates a different thermostability of the biologically active compounds in the extract;
- A positive correlation was established between the degradation of anthocyanins in the extract and the decrease in the antioxidant activity;
- The extract obtained under the optimum extraction conditions (5 hours, at 39°C, liquid/solid ratio of 30 mL/g and ethanol concentration of 73%) was used to test the effect on the metabolic activity of *Saccharomyces cerevisiae*. Different concentrations of the optimized PCF extract were added to the fermentation culture medium. The highest multiplication rate was observed for the highest amount of PCF extract added (1.2 mg/100 mL), while the effects of autolysis were observed after 72 hours. The results showed a continuous fermentation process, due to the release of CO_2;
- The extract was effective in inhibiting the activity of the selected metabolic enzymes, such as tyrosinase, α-amylase, α-glucosidase and lipase, at higher levels than the recommended drugs. The inhibitory effect was mainly correlated to the flavonoids content of the extract; however, a common cumulative effect of the compounds on the enzymes associated with the metabolic syndrome has also been suggested. Therefore, the extract showed an inhibitory effect, hence suggesting potential antidiabetic, hypocholesterolemic and preventive effects against Parkinson's disease and melanoma.
- The obtained results confirm the potential use of purple corn flour for the development of functional and nutraceutical foods, with the potential to inhibit some enzymes involved in the metabolic syndrome.

CHAPTER 4: Technological aspects for the development of gluten-free products with added-value by exploiting the biologically active potential of purple corn

4.1. Introduction

There is currently a growing demand for gluten-free products, as reported by the Transparency Market Research, with a global market valued at $ 2.84 billion in 2014 and $ 4.89 billion in 2021 (http://www.transparencymarketresearch.com/GF-products-market.html).The need for a global market for gluten-free products is given by the fact that a significant part of the global population must avoid the consumption of cereals, because they do not tolerate gluten proteins (Drabińska et al. 2016). The gluten-free diet is the only effective treatment for celiac disease (Midhagen and Hallert, 2003) and wheat allergy, thus requiring new perspectives in identifying gluten-free flours as an alternative to traditional wheat flours, with a similar functionality and efficiency. The need for the gluten-free products market is due to the significant increase in celiac disease, which has increased two to four times in the last 50 years, as reported by Rubio-Tapia et al. (2009). However, the use of gluten-free substitutes has been associated to some nutritional deficiencies, which can be attributed to several eating habits and to a decreased nutritional quality of derived foods compared to the gluten-containing ones (Giuberti and Gallo, 2018). Purple corn flour can be used for the development of gluten-free foods because it brings significant benefits by increasing the functional properties in terms of the phytochemicals content, such as polyphenols and anthocyanins. A diet rich in anthocyanins has protective effects against cancer, cardiovascular disease and other elderly diseases. The beneficial health effects of anthocyanins, including the anti-inflammatory, anti-cancer and antioxidant effects, have been reported in both *in vivo* and *in vitro studies* (Yang and Shin, 2017).

Therefore, the aim of this study was to exploit the results obtained previously as to develop a technology for obtaining gluten-free new products, enriched in anthocyanins and polyphenols, with a significant added-value, in terms of antioxidant activity through the use of purple corn flour. The thermo-mechanical properties of the composite flours composed of rice flour and purple corn flour were tested on a firsthand. The composite flours that included different contents of purple corn flour were eventually used to develop the biscuits. These products were characterized in terms of sensory analysis, phytochemicals content and antioxidant activity, structural and morphological texture, but also in terms of microbial load and stability of phytochemicals under storage conditiones.

4.2. Objectives of the study

The study had the following specific objectives:

- Assessing the thermos-mechanical properties of some composite flours, consisting of purple corn flour and rice flour, in order to establish the optimal ratio, from a technological point of view, to obtain a functional food product for a special purpose, which has biologically active properties;
- Phytochemical and structural-morphological analysis of the product;
- Evaluation of the gluten-free character;
- Assessing the microbial load and storage stability in regard to the phytochemical characteristics.
- Establishing the acceptability criteria of consumers;

- Developing a general technological scheme for the gluten-free product.

4.3. Materials

Plant material

Purple corn flour (*Zea mays L.*) was purchased from a local producer (Brăila County, Romania). Commercial whole wheat flour (Solaris Plant, Bucharest, Romania) was used as a basis for the production of gluten-free biscuits. The flours with a water content <12% were sealed in dark bags and kept at 4°C until the following analysis. The purple corn flour used for the phytochemicals' extraction was pre-sieved through a 10 mm sieve.

4.4. Methods

4.4.1 Thermo-mechanical properties of rice flour, purple corn flour and composite flours

The Mixolab equipment (Chopin Technology, Villeneuve La Garenne, France) was used to determine the thermo-mechanical properties of the rice and purple corn flours and the obtained composite flour consisting of 75% rice flour and 25% purple corn flour. The Chopin+ protocol with a dough weight modified from 75 to 90 g was used. The following parameters of the Mixolab curves were registered: maximum consistency of the dough during mixing at a constant temperature of 30^{o}C (C1), protein destabilization during mixing and heating (C2), starch gelatinization (C3), hot gel stability (C4) and starch retrogradation (C5). These torque values were used to calculate the starch gelatinization rate (C3-C2), breakdown (C3-C4) and starch retrogradation rate (C5-C4). The thermo-mechanical properties of dough samples were measured at different water absorption (WA) levels which also included the level at which the standard maximum consistency of 1.1 Nm would be reached. In the case of purple corn flour (PCF) the maximum consistency was registered at a WA of 66%, while in the case of rice flour (RF) at 68%.

4.4.2 Technological variants for obtaining the biscuits

For the design of the gluten-free biscuits, two types of composite flours were used, namely a combination of 25% PCF and 75% RF (C1), 75% PCF and 25% RF (C2). The control sample (C) consisted of 100% RF. The other ingredients were identical for all the types of biscuits.

4.4.3 Extraction and phytochemical analysis of the purple corn flour

A quantity of 10 g of biscuits (crushed) was subjected to the extraction process with 90 mL of 70% ethanol and 10 mL of 1N HCl. The extraction with ultrasounds was performed at 40°C for 30 minutes, followed by a centrifugation at 5000x*g* for 10 minutes, at 4°C. The supernatant was characterized in terms of its phytochemical content and antioxidant capacity by using different techniques. The pH differential method was used to determine the total monomeric anthocyanin content (TAC). The absorbance of the samples was measured at the wavelengths of 520 and 700 nm, for both pH values of 1.0 and 4.5, using a spectrophotometer (Jenway, UK). TAC was expressed as mg of cyanidin 3-O-glucoside (C3G)/g dry weight (DW).

The antioxidant activity was measured according to the slightly modified protocol for measuring the antiradical activity on DPPH (2,2-diphenyl-1-picrihydrazyl) as described by Ursu et al. (2020). The results were expressed as mMol Trolox per g DW (mgTE/g DW). For the total flavonoids content (TFC), a modified colorimetric method was used, based on the property of aluminum chloride to form stable acid complexes with keto C-4 groups and C-3 or C-5 hydroxyl groups of flavones or flavonols. The results were expressed as milligrams of catechin equivalents

(EC)/g DW. The modified Folin-Ciocalteu method was used to determine the total polyphenolic content (TPC) and it was expressed as milligrams of gallic acid equivalents (GAE)/g DW.

4.4.4 The structural and morphological analysis of the biscuits

To determine the microstructure of the samples, a laser scanning microscopic analysis was performed with a Zeiss Confocal Laser scanning system (LSM 710). The system is equipped with several types of lasers, a diode laser (405 nm), Ar laser (458, 488, 514 nm), DPSS laser (solid state pumped diode - 561 nm) and HeNe laser (633 nm). The images were evaluated with the Black Edition of the ZEN 2012 SP1 software. The samples were observed both in their native state for their natural autofluorescence and also stained with the Red Congo fluorophore.

4.4.5 Microbial shelf-life examination of the biscuits

The Enterobacteriaceae, yeasts and molds were determined and counted by employing the ISO 21528-2: 2017 and ISO 21527-2: 2008 standards.

4.4.6 Enzyme-linked immunosorbent assay to determine the allergenic potential

The presence of prolamins recognized by the R5 antibodies was checked using the RIDASCREEN® Gliadin (Biopharm AG, Darmstadt, Germany) which is a kit for running the enzyme-linked immunosorbent assay (ELISA). In agreement to the recommendations of the producer, the biscuit samples (0.25 g) were subjected to an extraction for 40 min at 50°C using the patented mixture (2.5 mL), followed by the addition of 80% ethanol solution (7.5 mL) and stirred for 60 min at 25°C. The supernatant (100 μL) obtained by centrifugation for 10 min at 2500xg was used to test the specific binding by the R5 antibodies coated on the wells. The second specific R5 antibody conjugated to peroxidase was then added to allow the obtaining of the antibody-antigen-antibody complexes. The peroxidase assisted conversion of the substrate was finally used to quantify the prolamins recognized by the R5 antibodies. The gliadin standard was used for the calibration and the Stat Fax 4200 microplate reader (Awareness Technology, USA) for measuring the absorbance of the samples.

4.4.7 Sensory analysis of the biscuits

The sensory evaluation of the biscuits was performed through the scoring method, by assessing the intensity of perception for each attribute on a scale ranging from 1 (very low) to 7 (very high). The participants of the testing panel were informed about the general purpose of the study and the procedures required for the handling of personal data. All participants gave their written consent before participating.

Ten trained experienced members were selected (they received specific additional training on the sensory attributes relevant to the biscuits' production developed in this study). The samples were presented to the panelists in coded plates, made of transparent plastic and they were asked to rinse their mouth before the first sample and between samples by using water. The analyzed sensory attributes were color uniformity and intensity, smell, appearance, taste, flavor, consistency, mouthfeel, and overall impression.

4.4.8 Stability of the phytochemical compounds in biscuits during storage

The biscuits were stored for 21 days, at room temperature, in dark conditions. The biscuits were analyzed for the stability of the phytochemicals and microbiological properties.

4.4.9 Statistical analysis

The results of this study represent the mean values ± standard deviation, representing the arithmetic mean of the analyzes performed in triplicate. The statistical analysis of the data was performed with the Minitab software.

4.5. RESULTS AND DISCUSSIONS

4.5.1 Evaluation of the thermo-mechanical properties of composite flours

The modified Chopin+ protocol and the Mixolab device were used to measure and compare the thermo-mechanical profile of the rice flour, purple corn flour and the composite flour obtained by mixing 75% rice flour and 25% purple corn flour. Preliminary tests were performed on the purple corn flour at WA levels higher than the value of 66%, which ensured the reaching of the standard consistency of 1.1 Nm.

The tests performed at a WA of 100% were intented in order to gather the preliminary useful information on the starch behavior. At such a high hydration, C1 and C2 samples of the purple corn dough were close to zero, most likely due to the lack of cohesion within the protein network. At the same time, it should not be neglected the fact that the water in the system is in excess, hence resulting low starch gelatinization values (C3 of 0.97 Nm), hot gel stability (C4 of 0.80 Nm) and starch retrogradation (C5 of 1.24 Nm). Dubat and Boinot (2012) studied the thermo-mechanical properties of corn flour under different condition of WA and reported values between 2.50-3.00 Nm for C3 and C4, and 4.00 Nm for C5, when lower hydration value of flour (WA of 60%, flour moisture of 14%) was used, compared to C3 of 1.2 Nm, C4 in the 0.80-1.00 Nm range and C5 over 1.2 Nm, when a higher hydration was deployed (WA 115%, flour moisture of 14%).

Higher flour hydration levels are often used when baking gluten-free products compared to the wheat flour-based ones. Therefore, in view of these preliminary observations, further tests on the investigated flours were carried out at a higher WA compared to the levels required for obtaining the C1 of 1.1 Nm.

In particular, the WA of 76% was considered, since it does not significantly affect the dough cohesiveness, as indicated by the first zone of the Mixolab curve, and, at the same time, provides enough water for the starch gelatinization. The Mixolab curves of PCF and rice flour at different levels of water absorption are shown in Figure 4.1.

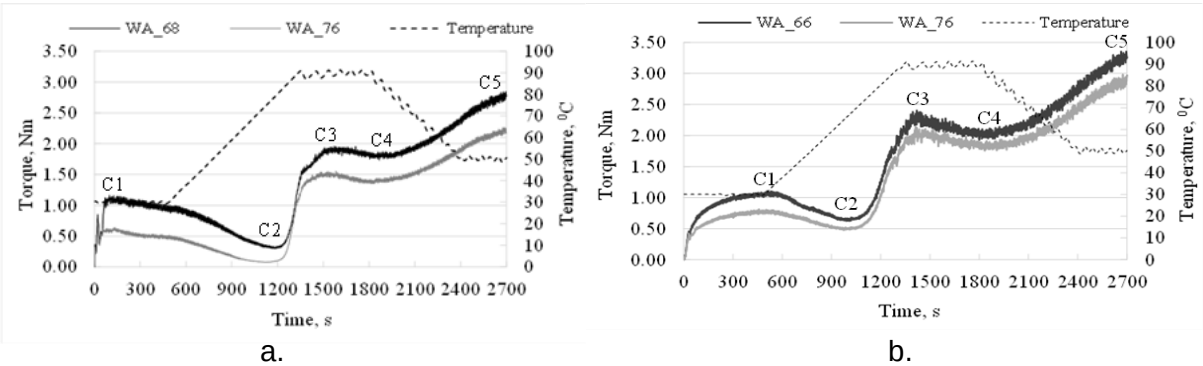


Figure 4.1. Mixolab curves of purple corn flour (a) and rice flour (b) at different water absorption (WA) levels

In Figure 4.1. (a), the Mixolab FPM curves at different CH levels are shown. The typical profile of the Mixolab curves was observed for the samples with a WA of 66% and 76%. During the mixing at 30ºC, the torque values increased up to a maximum C1 value of 1.11 Nm, and 0.61

Nm, respectively. After a period of stability of 8.3 min, and 4.5 min, respectively, the dough's consistency decreased. At a further increase of the temperature from 30 to about 60ºC, the torques registered a minimum C2 value of 0.31 Nm and 0.08 Nm. The starch behavior during the heating and cooling were influenced by the WA level. As such, the starch gelatinization rate and the starch retrogradation rate decreased from 1.60 to 1.43 Nm and from 0.98 to 0.84 Nm, respectively, with the increase of the WA level, while the breakdown increased from 0.11 to 0.13 Nm. However, the peak gelatinization and starch retrogradation of the PCF at the WA of 66% (1.91 and 2.78 Nm) were higher than those at a WA of 76% (1.51 and 2.22 Nm, respectively).

In Figure 4.1. (b) are shown the Mixolab curves of the RF registered for a WA of 68 and 76%. The two curves presented similar profiles, but the decrease of the breakdown from 0.28 to 0.25 Nm, and of the starch retrogradation rate from 1.27 to 1.09 Nm were registered with the increase of the WA levels, while the starch gelatinization rate did not change (1.64 Nm).

According to Cappa et al. (2013), in the case of gluten-free formulas it is not necessary to reach the consistency of 1.1 Nm during the mixing at 30°C, a lower dough consistency being preferable. An explanation for this fact would be the presence in the gluten-free formulas of ingredients with a high affinity for water whereas another explanation would be the properties of proteins. Some proteins from a gluten-free flour, such as rice flour, present a lower water absorption level than the gluten proteins. Therefore, the C1 torque during the mixing at 30˚C is less than 1.1 Nm, the water from the system being mainly used by the starch during the gelatinization stage. To compare the thermo-mechanical properties of the dough samples prepared with the composite flour (75RF + 25PCF) and the RF (control) at a 76% WA are presented in Table 4.1.. The addition of PCF to the RF modified both the proteins' functionality and also the starch behavior. The proteins from RF retained more water than the proteins from PCF and thus formed a dough with a higher C1 (Table 4.1.). An explanation for these results could be the differences in particle size between the two flours. Moreira et al. (2015) reported a decrease of C1 from 1.05 to 0.25 Nm for the purple corn dough when the average particle diameter increased from 93 to 184 Nm.

Table 4.1. Thermo-mechanical properties of the dough prepared with rice flour (RF) and composite flour (75 RF + 25 PCF) at CH of 76%

Mixolab parameters	Samples	
	RF	75 RF + 25 PCF
C1, Nm	0.77 ± 0.01 ^{a*}	0.67 ± 0.02 ^{b}
C2, Nm	0.50 ± 0.01 ^{a}	0.34 ± 0.01 ^{b}
C3, Nm	2.14 ± 0.03 ^{a}	1.68 ± 0.01 ^{b}
C4, Nm	1.89 ± 0.02 ^{a}	1.67 ± 0.01 ^{b}
C5, Nm	2.98 ± 0.02 ^{a}	2.82 ± 0.02 ^{b}
C3-C2, Nm	1.64 ± 0.03 ^{a}	1.34 ± 0.01 ^{b}
C3-C4, Nm	0.25 ± 0.04 ^{a}	0.01 ± 0.00 ^{b}
C5-C4, Nm	1.09 ± 0.03 ^{b}	1.15 ± 0.02 ^{a}

*mean values on the same line that do not have the same letter are significantly different at p <0.05

The decrease of C2 from 0.50 to 0.34 Nm was observed when adding PCF to the RF (Table 4.1.). These results suggested that the network formed by the RF proteins was more resistant during kneading and heating, compared to the network obtained by mixing PCF and RF proteins. Mixing the two flours determined the change of the ratio between the main protein fractions, resulting in a weaker network while kneading and heating the dough. Rice flours were reported to be particularly rich in glutelins and albumins, whereas the protein fractions prevailing in corn are glutelins and prolamins. Therefore, the replacement of 25% RF by PCF caused the reduction of the content of water-soluble albumins and the concomitant increase of the soluble

prolamins in alcohol solutions. These changes in the protein solubility profile might explain the variation of the thermo-mechanical parameters corresponding to the first zones of the Mixolab curve, in respect to the RF based dough.

Regarding the behavior of starch, the results from Table 4.1. indicated the decrease of the starch gelatinization, hot gel stability and starch retrogradation when PCF was added to the RF. The results can be explained by the differences between the starch properties of rice and corn.

Moreover, according to the results in Table 4.1, the addition of PCF to the RF resulted in a decrease of the gelatinization rate of starch (C3-C2) and an increase in the rate of starch retrogradation (C5-C4). On the other hand, the breakdown (C3-C4) has been significantly reduced, which according to Dubat and Boinot (2012) is considered to improve temperature stability, thus competing with the effect induced by the butter introduced into the dough system to obtain the biscuits.

4.5.2 Formulation of products derived from the composite flours

The composite flour consisting of RF and PCF was used to prepare the gluten-free biscuits. The three samples were tested to determine their phytochemical content, in terms of anthocyanins, polyphenols, flavonoids and antioxidant activity. The TAC content varied from 0 in the C sample to 2.01±0.19 mg C3G/100 g DW in the C1 sample and 6.99±0.20 mg C3G/100 g DW in the C2 sample. Due to the higher anthocyanin content, C2 showed the highest antioxidant activity of 18.46±0.18 mM Trolox/g DW. The TPC varied from 74.32±5.81 mg GAE/100 g DW in C, 79.24±1.30 mg GAE/100 g DW in C1 to 100.99±1.97 mg GAE/100 g DW in C2. The higher content of PCF in C2 had no significant influence on the TFC (Table 4.2.).

The manufacture of gluten-free muffins from white, yellow, and purple corn is described Trehan et al. (2018), which suggested an initial TPC that varied between 1223 µg GAE/g and 1843 µg GAE/g.

Table 4.2. Phytochemical profile and antioxidant activity of the biscuits samples during storage

Phytochemical profile and antioxidant activity	TECHNOLOGICAL VARIANTS					
	C		C1		C2	
	T0	T21	T0	T21	T0	T21
Total anthocyanin content, C3G, mg/100 g SU	-	-	2.01 ± 0.19 ^{a*}	2.52 ± 0.28 ^{a}	6.99 ± 0.20 ^{b}	8.53 ± 0.82 ^{a}
Total flavonoids content, mg CE/100 g SU	33.51 ± 0.75 ^{a}	33.99 ± 0.96 ^{a}	33.15 ± 0.20 ^{a}	34.36 ± 2.25 ^{a}	33.87 ± 1.36 ^{a}	35.30 ± 1.67 ^{a}
Total polyphenols content, mg GAE/100 g SU	74.32 ± 5.82 ^{a}	82.52 ± 1.42 ^{a}	79.24 ± 7.70 ^{b}	105.91 ± 4.62 ^{a}	100.99 ± 1.97 ^{b}	111.46 ± 4.19 ^{a}
Antioxidant activity, mM Trolox/g SU	18.09 ± 0.13 ^{a}	16.48 ± 0.58 ^{b}	18.33 ± 0.11 ^{a}	16.79 ± 0.26 ^{b}	18.46 ± 0.18 ^{a}	17.16 ± 0.04 ^{b}

* within a line, the mean values corresponding to the same samples that do not have the same letter are significantly different at p <0.05

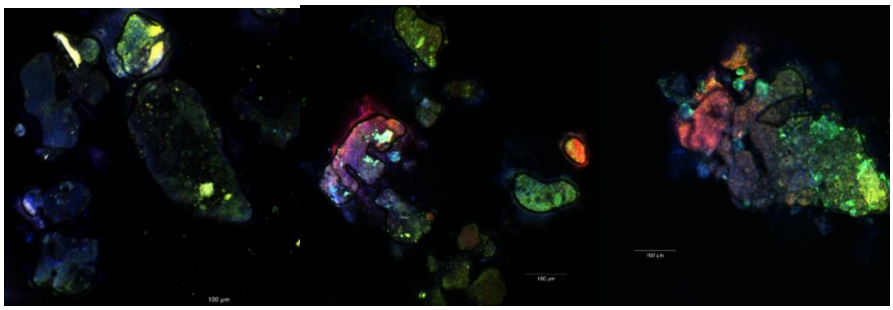
4.5.3 Determination of gluten absence by ELISA

In the present study, ELISA was used to confirm the absence of prolamins recognized by the R5 monoclonal antibody in biscuits. The obtained results highlighted the absence of prolamins in the composition of biscuits.

4.5.4 Structural and morphological analysis of gluten-free products

The micro-structure of the designed cookies was observed by CLSM. In the native, non-colored samples (Figure 4.2.), large (> 100 µm), complex aggregates were observed, in which

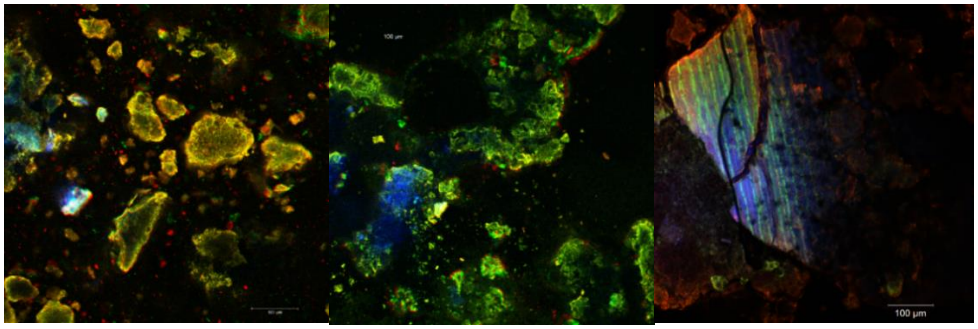
small, polygonal, irregular, starch granules (5-15 µm) were also found, trapped in the protein matrix together with the anthocyanins that presented a predominant emission in the green field.



a b c

Figure 4.2. Structure of the biscuits' variants ((a - control, b - biscuits with 75% rice flour and 25% purple corn flour and c - biscuits with 25% rice flour and 75% purple corn flour)

The dehydration during the baking process enhanced the aggregation process. The black rice starch granules (Figure 4.2, a), as it is known, are small (most having 3-6 µm), often with an invisible hilum, without concentric streaks and with a higher tendency of aggregation. Instead, the corn starch granules are larger (10-20 µm), with less sharp edges between the facets, and often with visible hilum, stellate or punctate. The higher the proportion of PCF, the larger and richer in anthocyanins the aggregates are (Figure 4.2., c).



a b c

Figure 4.3. Confocal laser scanning microscopy (CLSM) images of fluorophore-colored biscuit variants (a - control, b - biscuits with 75% rice flour and 25% purple corn flour and c - biscuits with 25% rice flour and 75% purple corn flour)

The stained samples (Figure 4.3.) highlighted much better the ultrastructure of the aggregates. In the control sample based of RF (Figure 3, a), average aggregates were observed, with an anthocyanins' emission range at values between 560-580 nm, results similar to those obtained by Croitoru et al. (2018). The addition of PCF (Figure 4.3., b) in the composition of the cookies enriched the product in anthocyanins and shifted the emission range to green (520-550 nm). With the addition of 75% PCF (Figure 4.3., c), the cookies presented a textured structure, in the form of successive layers of aggregate starch granules (in blue) and anthocyanins (in green). The complex formula for preparing the cookies, brought the flour mix in a complex matrix along with proteins, various carbohydrates, and lipids from the other ingredients (butter, sugar, eggs according to the recipe), therefore it was difficult to distinguish among the compounds. Nevertheless, although the baking process itself causes complex interactions between the gelatinized or the expanded starch and denatured proteins, the abundance of biologically active compounds in the flour mix is obvious and adds a higher nutritional value to the product.

4.5.5 Sensory analysis

The acceptance test remains one of the most used methods to evaluate a final product by the food industry in order to test new formulations, as it reflects the preference degree of consumers concerning the product. Therefore, the sensorial analysis was performed following the different sensorial characteristics such as: color uniformity and intensity, smell, taste, flavor, consistency, mouthfeel, and overall impression. From Table 4.3., it can be observed that for the color uniformity, the most appreciated sample was C. The PCF based cookies showed similar values.

Table 4.3. Average attributes values of the evaluated biscuit samples

samples	Characteristics							
	Uniformity of color	Intensity of color	Appearance	Taste	Smell	Consistency	Mouthfeel	Overall impression
C	6.50 ± 0.97 ^{a*}	1.70 ± 1.25 ^{b}	6.00 ± 0.66 ^{a}	5.90 ± 0.87 ^{a}	5.30 ± 0.67 ^{a}	5.70 ± 1.15 ^{a}	5.50 ± 1.35 ^{a}	5.90 ± 0.73 ^{a}
C1	5.30 ± 2.00 ^{a}	3.20 ± 1.22 ^{b}	5.80 ± 0.91 ^{a}	5.70 ± 0.82 ^{a}	5.00 ± 0.94 ^{a}	4.70 ± 1.49 ^{a}	4.80 ± 1.39 ^{a}	5.40 ± 0.69 ^{a}
C2	5.50 ± 1.71 ^{a}	5.60 ± 1.07 ^{a}	6.10 ± 0.73 ^{a}	5.40 ± 1.17 ^{a}	4.70 ± 1.33 ^{a}	4.70 ± 1.56 ^{a}	4.70 ± 1.56 ^{a}	5.20 ± 1.31 ^{a}

* average values on the same column that do not have the same letter are significantly different at p <0.05

In terms of color intensity, as expected, the cookies with the highest addition of PCF (C2) were evaluated as being more appreciated, while the control had the lowest result. For the odor, the C and C2 were similar assessed, while C1 had the lowest result. The PCF added cookies were appreciated especially for their color, but downgraded most likely because of the PCF granularity, thus adding more hardness and fragility to the cookies. When assessing the overall impression, it was demonstrated that the substitution of RF with PCF had no significant influence on the acceptability results, generally reflecting the profile of the color, taste, and flavor. As a result, these sensorial attributes became the most influential attributes in the results.

4.5.6 Stability under an accelerated storage of the biscuits

The results of the shelf-life tests performed on the biscuit samples, kept for 21 days at room temperature, were presented in next tables. No significant variation of TAC values was found for C1, while an increase of approximately 18% was observed for C2. A slight increase of TFC was observed for both types of gluten-free biscuits, while TPC showed a significant increase of 25% for C1 and 9% for C2. These variations of phytochemicals affected the antioxidant activity, which was found to slightly decrease for both samples, up to about 9%. The results are similar to Milea et al. (2020), which reported an increase of the biologically active compounds values during the storage for 14 days of biscuits with microencapsulated anthocyanins from black rice and lavender essential oils.

In Table 4.4. and 4.5. the values obtained from the microbiological test of the biscuits are presented.

Table 4.4. Yeasts and molds counted during storage, CFU/g

Samples	Storage period, days			
	0	**7**	**14**	**21**
C	<100	<100	<100	<100
C1	<100	<100	<100	<100
C2	<100	<100	<100	<100

Table 4.5. *Enterobacteriaceae* counted during storage, CFU/g

Samples	Storage period, days			
	0	7	14	21
C	<10	<10	<10	<10
C1	<10	<10	<10	<10
C2	<10	<10	<10	<10

The microbiological test displayed that the biscuits showed microbiologically satisfactory values during the storage period (Tables 4.4. and 4.5.).

4.5.7 Development of a general technological scheme for obtaining gluten-free biscuits with the addition of purple corn flour

Following the presented experimental results, two technological schemes for obtaining gluten-free biscuits were developed, which are presented in Figure 4.4. In order to obtain gluten-free biscuits, it is recommended to use composite flours, in a ratio of 25% PCF and 75% RF, 2% baking powder, 30% sugar, acidifier (5%), butter and egg. The obtained dough must be cooled to 4°C for 30 minutes, and the heat treatment should involve a temperature of 160°C for 10 minutes. It is also recommended that after cooling, the biscuits to be packed in dark paper bags to preserve their biologically active properties.

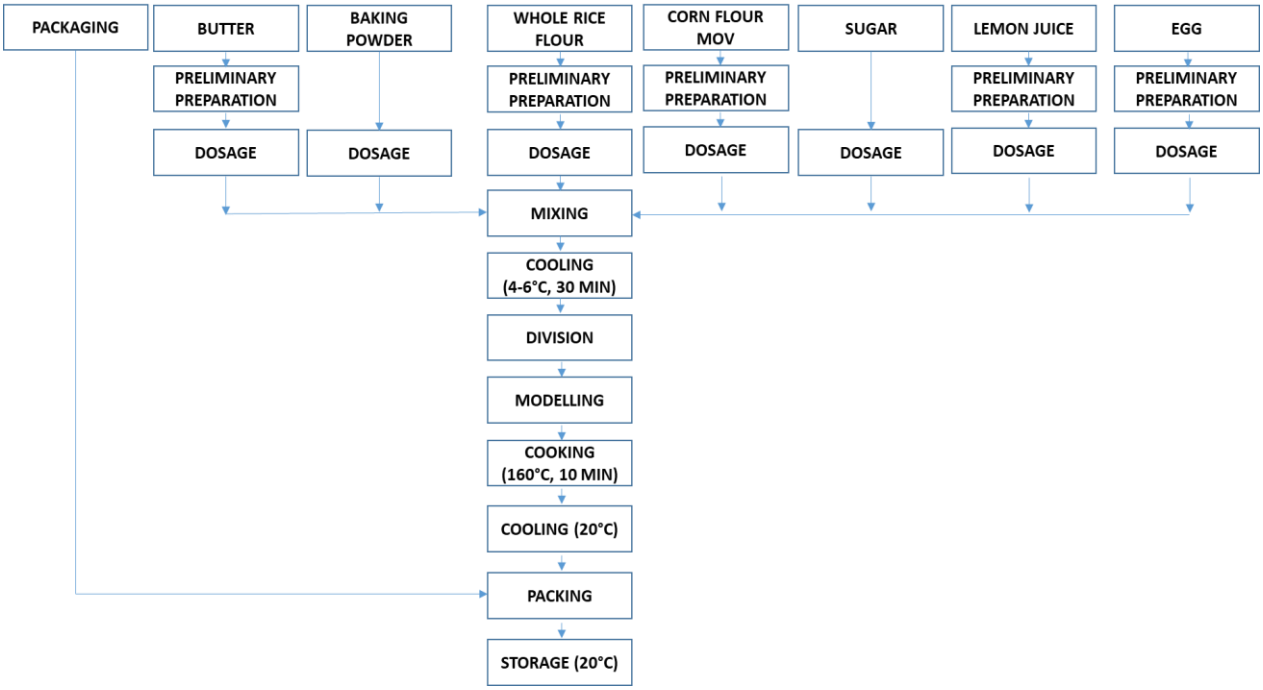


Figure 4.4. Proposed technological scheme for obtaining the gluten-free biscuits (through the use of composite flour - 25% purple corn flour and 75% rice flour)

4.6. Partial conclusions

In this chapter, the thermo-mechanical properties of the composite flours, rice flour and purple corn flour were tested in order to develop technologies to obtain gluten-free food products with a high functionality.

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

- As a result of the thermo-mechanical profile analysis of the purple corn flour, rice flour and some composite flours, three biscuit variants were obtained (25% purple corn flour and 75% rice flour (C1), 75% purple corn flour and 25% rice flour (C2) and 100% rice flour).

- The sensory analysis showed that the replacement of rice flour and wheat flour with purple corn flour had no significant influence on the overall acceptability.
- The C1 and C2 biscuit variants showed a higher anthocyanins and polyphenols content compared to the control sample C, due to the addition of purple corn flour as follows: the anthocyanins values varied from 0 in the control sample, 2.01 ± 0.19 mg C3G/100 g DW for the biscuit variant which used 25% purple corn flour (C1) and 6.99 ± 0.20 mg C3G/100 g DW for the biscuit variant which used 75% purple corn flour (C2); polyphenols content ranged from 74.32 ± 5.81 mg GAE/100 g DW for the control sample C, 79.24 ± 1.30 mg GAE/100 g DW for C1 to 100.99 ± 1.97 mg GAE/100 g DW for C2.
- The content of purple corn flour in both biscuit variants, C1 and C2, did not have a significant influence on the total flavonoids content.
- The antioxidant activity of the C2 biscuits variant was higher (18.46 ± 0.18 mM Trolox/g DW) due to the addition of higher content of purple corn flour.
- The ELISA test indicated the absence of the antigenic prolamins, from the obtained biscuit variants, so they can be considered gluten-free products.
- The analysis of the storage stability of the biscuits indicated that they presented a good storage stability while the phytochemical profile varied in terms of an increase in the content of polyphenols (25% in C1 and 9% in C2) and flavonoids, while for the content of anthocyanins no major changes for both types of gluten-free biscuits were detected. These changes in the gluten-free biscuits phytochemicals' profile led to a slight decrease of their antioxidant activity for both of the samples with purple corn flour addition of 9%.
- The morphological and functional analysis highlighted the microstructure of the biscuits. In the non-stained samples, it was observed that the higher the percentage of purple corn flour, the larger and richer the anthocyanins appear in the aggregates, a fact emphasized by the predominant green color. The stained samples better highlighted the structure of the aggregates; for the biscuit variants with 75% purple corn flour, a textured structure was observed, in the form of DWccessive layers of aggregated starch granules and anthocyanins.
- The microbiological test of the biscuits during storage showed satisfactory values.
- A technological scheme to obtain gluten-free biscuits based on purple corn flour has been developed, which can contribute to the diet diversification of celiac patients.

CHAPTER 5: Technological aspects regarding the development of food products with gluten, with added-value by exploiting the biologically active potential of purple corn

5.1. Introduction

Traditionally, most people prefer white corn for food and especially for bread. However, purple corn contains polyphenolic compounds that are of a particular importance in the development of various value-added with a high biological activity bakery products (Kean et al. 2008; Perichart-Pereira et al. 2010). Purple corn is considered aa a health-promoting *superfood*, with antibacterial, anti-aging, anticancer, neuro-protective, cardiovascular and antidiabetic properties (Bento-Silva, 2018).

From previous experimental results, the positive influence of the polyphenolic compounds in the optimized purple corn extract on the metabolic activity of yeasts (*Saccharomyces cerevisae*) was observed. Therefore, this study aimed to exploit these results in the technological process for obtaining buns as bakery products. In addition, this study meets the requirements of the economic operator that cultivates purple corn and obtains the flour and its desire to develop technologies for the exploitation of purple corn flour.

5.2. Objectives of the study

Taking into account the results obtained and presented in the previous chapters, the study presented in this chapter meets the requirements of the economic agent, which provided the purple corn flour and who expressed the intention to develop technologies to valorise the purple corn flour. Therefore, this chapter had as a main objective the valorifisation of the bioactive potential of purple corn through the development of bakery products, enriched in anthocyanins and polyphenols, with a significant added value. Purple corn flour was used in combination with the wheat flour to obtain bakery products, namely buns. These products were analyzed to assess the influence of fermentation time and the percentage of purple corn flour on the sensory, phytochemical, antioxidant and nutritional activity. In addition, taking into account the purple corn flour effect of stimulating the metabolic activity of the baking yeast, the addition of purple corn flour on the fermentation time (an important aspect in the industry) was also followed. The study allowed the development of technological schemes to obtain buns and to highlight the advantages derived from the use of purple corn flour.

5.3. Methods

5.3.1 Technological variants to obtain the buns

Two types of flour were used to make the buns: purple corn flour and white wheat flour, type 650. Two experiments were performed and the derived samples were coded as follows:

A. **The first experiment aimed at the influence of fermentation time on the sensory and nutritional characteristics and on the phytochemical profile of the buns**. For this purpose, three types of buns were made, in which the fermentation time varied (1 h or 2 h), coded as follows:

a. buns with 100% white wheat flour, type 650, used as a control sample – with a fermentation time of 1 h code M1, with a fermentation time of 2 h - code M2;

b. buns with 25% purple corn flour and 75% white wheat flour with a fermentation time of 1 h (P1) and a fermentation time of 2 h (F1);

c. buns with 75% purple corn flour and 25% white wheat flour, fermented for 1 h (P2) and fermented for 2 h (F2).

B. **The second experiment followed the influence of polyphenolic compounds from the purple corn flour on the activity of the yeast, implicitly on the fermentation time**. Three types of buns have been designed for this purpose, for which the amount of yeast has been varied:

a. buns with 100% white wheat flour, type 650, used as a control sample (M - standard amount of yeast - 2.5%);

b. buns with 25% purple corn flour and 75% white wheat flour (A- amount of yeast reduced by half - 1.25%); these buns were analysed based on the weight of the dough at the end of the fermentation period (1h).

c. buns (B) obtained from 25% purple corn flour and 75% white wheat flour, standard quantity of yeast, the required fermentation time was followed, the piece of dough reaching the weight obtained in the case of sample A.

In these circumstances, it was assessed whether the results obtained in the previous chapters are of practical importance in obtaining bakery products.

5.3.2 Sensory analysis of the buns

A panel of 10 panelists evaluated the sensory characteristics of the buns according to a seven-point hedonic scale (1- dislikes extremely to 7- likes extremely much). The evaluated parameters were, exterior appearance, section appearance, consistency, smell, taste, aroma, elasticity, mouthfeel, aftertaste, and overall impression. The samples were coded and served to the panelists on a white paper. Water was used to rinse the mouth before and between samples.

5.3.3 Extraction and phytochemical profile analysis of the buns

As extraction methods, a conventional and an ultrasounds-assisted extraction were used. The biologically active compounds extraction was performed using 70% ethanol and 1N HCl as solvents, this extraction being assisted by ultrasounds for 30 min, at 30°C. The determination of the monomeric anthocyanins content of the buns was performed similarly to the determination of the anthocyanins of the optimized extract. The protocols described by Ursu et al (2020) were used to determine the content of polyphenols and flavonoids.

The antioxidant activity was measured using the ABTS radical discoloration test [2.20 azinobis (3-ethylbenzothiazole-6-sulfonic acid)]. The determination of the antioxidant activity was performed using the ABTS • + cation radical method.

5.3.4 Physicochemical analysis of the buns

The nutritional characteristics determination was performed according to the methods and standards of analysis presented in Table 5.1.

Table 5.1. Physicochemical analysis of the buns

Physico-chemical characteristics	Color uniformity
Salt*, % of which sodium*, %	SR 91: 2007 Calculation (R 1169/2011)
Ash*, %	SR 91: 2007, Calcinare
Humidity *%	SR EN ISO 712: 2010
Acidity, degrees	SR 91: 2007
Fats*, %,	Hetero-hydrochloride method
Protein*, %	Kjeldahl method
Carbohydrates*, % of which,	By difference, the Iodometric Method

Sugar*, %	
Volume	Method with Fornet SR 91: 2007 type device
Porosity	SR 91: 2007
Elasticity	SR 91: 2007
Energy value *, %: kcal, kj	By calculation, Regulation 1169/2011

5.3.5. Storage stability of the phytochemical compounds in the buns

The M, A, B coded buns were kept for 48 hours at room temperature, in the dark, packed in polyethylene bags. The buns were analyzed for phytochemical compounds stability.

5.3.6. Statistical analysis

The results of this study represent the mean values ± standard deviation of the mean, representing the arithmetic mean of the analyzes performed in triplicate. The statistical analysis of the data was performed with the Minitab software.

5.4. RESULTS AND DISCUSSIONS

5.4.1. Physico-chemical and phytochemical profile of the buns

In Table 5.2. the phytochemical profile of the buns is presented, in which the influence of the fermentation time on the polyphenolic compounds and the antioxidant activity was analyzed. From table 5.2., it can be observed that in the case of the samples obtained from 25% PCF and 75% wheat flour with a fermentation duration of 1 h, the content of polyphenolic compounds increased linearly with the content of the added PCF. Thus, the TAC content was about 3 times higher for the P1 coded samples and 5 times higher in the case of the wheat flour replacement by 75%. The flavonoids content was higher in the control sample, while the TPC content presented higher values in the P2 sample, which also showed the highest antioxidant activity (137.02 ± 0.06 mM Trolox/100 g DW). It can stated, in this case, that the anthocyanins from the P2 samples are the main responsibles for the antioxidant activity increase.

In the case of the products obtained by prolonging the fermentation time, the values corresponding to the polyphenols content are clearly higher than those of the control samples, with an increase in the TAC content of 2.6 times (F1) and 4.22 (F2), respectively. The TFC and TPC content increased with the increasing PCF content, which resulted in significantly higher values for the antioxidant activity of F2 samples (155.07 ± 0.03 mM Trolox/100 g DW).

Table 5.2. Polyphenolic profile of the buns

Phytochemical profile (mg/100 g DW) and antioxidant activity	TECHNOLOGICAL VARIANTS					
	M1	**P2**	**P3**	**M2**	**F2**	**F3**
Total anthocyanins content, mg C3G	1.39 ± 0.002^{f}	4.08 ± 0.001^{d}	7.18 ± 0.00^{b}	1.98 ± 0.00^{e}	5.25 ± 0.00^{c}	8.36 ± 0.00^{a}
Total flavonoids content, mg EC	54.15 ± 0.03^{ab}	46.13 ± 0.05^{b}	52.51 ± 0.05^{ab}	52.63 ± 0.03^{ab}	55.96 ± 0.07^{ab}	65.00 ± 0.08^{a}
Total polyphenols content, mg GAE	144.6 ± 0.34^{bc}	117.1 ± 0.05^{bc}	155.3 ± 0.19^{ab}	105.2 ± 0.06^{c}	155.2 ± 0.09^{ab}	198.8 ± 0.03^{a}
Antioxidant activity, mM Trolox/g DW	70.64 ± 0.02^{e}	100.07 ± 0.01^{d}	137.02 ± 0.06^{b}	75.34 ± 0.01^{e}	119.83 ± 0.01^{c}	155.07 ± 0.03^{a}

*values in the same row that do not share a letter (a, b) are statistically different ($p < 0.05$) according to the Anova method, Tukey test (95% confidence level)

The analysis of the influence of the fermentation time (Table 5.2.) showed a much higher phytochemical content for the samples obtained after 2 h. In the case of the experimental variants, an increase of the TAC was observed from 4.08 ± 0.001 mg C3G/100 g to 5.25 ± 0.00 mg C3G/100 g, which coincides with an increase of approximately 30%, while for the samples in which the PCF presented a higher content, the increase was only of 16%. The TFC increased by about 20% in the 25% PCF replacement variants and 23% in the 75% PCF variants. There is also a significant increase of the TPC, by 30% and 28%, respectively. Following these results, it can

be concluded that the increase of the fermentation time positively influenced both the phytochemical profile and the antioxidant activity of the buns.

In order to analyze the influence of the polyphenolic compounds on the metabolic activity of yeasts, after the kneading operation, the dough pieces were weighed before and after the fermentation period. Both pieces of dough showed the same difference in weight, thus demonstrating the stimulating effect upon the metabolic activity of the baking yeast. Therefore, in the case of the B coded experimental variants, the fermentation time was reduced by 35 min, which was determined by weighing the piece of dough until a difference in weight similar to the A coded buns was obtained.

Table 5.3. showed that with the reduction of the fermentation time, the anthocyanins content decreased from 9.34 ± 0.001 C3G/100 g DW for A samples to 7.68 ± 0.001 C3G/100 g DW for B samples, but also for the flavonoids' contents from 63.58 ± 0.01 mg EC/100 g DW for A to 56.76 ± 0.01 mg EC/100 g DW for B. The content of polyphenols increased, even if the fermentation time decreased, reaching the values of 140.8 ± 0.04 mg GAE/100 g DW for A and 143.8 ± 0.19 mg GAE/100 g DW for B, which resulted in an increase of the antioxidant activity from 88.97 ± 0.04 mM Trolox/100 g DW for A and 95.12 ± 0.03 mM Trolox/100 g DW for B.

Table 5.3. Polyphenolic profile of the buns and storage stability

Phytochemical profile and antioxidant activity	TECHNOLOGICAL VARIANTS					
	M		A		B	
	T0	T48	T0	T48	T0	T48
Total anthocyanin content, C3G, mg / 100 g DW	5.11 ± 0.003 ^{bC}	5.78 ± 0.001 ^{BC}	9.34 ± 0.001 ^{aA}	8.83 ± 0.00 ^{bA}	7.68 ± 0.001 ^{BC}	7.42 ± 0.002 ^{aB}
Total flavonoid content, mg CE/100 g DW	44.35 ± 0.005 ^{bC}	54.94 ± 0.02 ^{aB}	63.58 ± 0.01 ^{aA}	68.42 ± 0.03 ^{aA}	56.76 ± 0.01 ^{aB}	54.41 ± 0.05 ^{aB}
Total polyphenol content, mg GAE / 100 g DW	99.5 ± 0.06 ^{aB}	107.7 ± 0.03 ^{aB}	140.8 ± 0.04 ^{aA}	136.2 ± 0.08 ^{aA}	143.8 ± 0.08 ^{aA}	128.4 ± 0.01 ^{bA}
Antioxidant activity, mM Trolox / g DW	42.02 ± 0.05 ^{aB}	46.94 ± 0.01 ^{aB}	88.97 ± 0.04 ^{bA}	94.99 ± 0.01 ^{aA}	95.12 ± 0.03 ^{aA}	94.45 ± 0.01 ^{aA}

*values in the same row that do not share a letter (a, b - time variation, A, B - sample variation) are statistically different (p <0.05) according to the Anova method, Tukey test (confidence level 95 %)

Pasqualone et al. (2019) investigated the effect of the fermentation time on the antioxidant activity and the polyphenolic profile of a bread enriched with grapes and pomegranate seeds. They noticed that the total content of polyphenolic compounds and antioxidant activity were significantly improved over a prolonged fermentation time. The polyphenolic profile of the control dough ranged from 280 to 376 mg gallic acid equivalent (GAE)/kg; that of the dough containing pomegranate seeds ranged from 402 to 466 mg GAE/kg; and the content of polyphenolic compounds in the grape seed dough varied between 551 and 591 mg GAE/kg.

5.4.2. Sensory analysis of buns

In Table 5.4., the average values of the characteristics analyzed by the panelists for the buns are presented. The sensory characteristics are one of the most important properties of food, because it determines the acceptability of the consumer. Of all the characteristics evaluated by the panelists, the appearance in the section, the taste, the aroma and the mouthfeel were the most appreciated attributes evaluated, because the long fermentation time had a positive impact on the smell, taste and the aroma of the products.

Table 5.4. Average scores of the attributes evaluated in the buns' acceptance test

Samples	Characteristics									
	Outer appearance	Appearance in section	Smell	Taste	Flavor	Consistency	Elasticity	Mouthfeel	After taste	Overall impression
M1	6.7 ± 0.48^{a}	6.3 ± 0.67^{a}	6.4 ± 0.69^{a}	6 ± 0.66^{a}	6.2 ± 0.78^{ab}	6.6 ± 0.62^{a}	6.5 ± 0.70^{a}	6.7 ± 0.48^{a}	6.7 ± 0.48^{ab}	5.9 ± 0.56^{b}
P1	5.6 ± 0.51^{b}	6.8 ± 0.42^{a}	6.7 ± 0.48^{a}	6.4 ± 0.96^{a}	6.4 ± 0.69^{ab}	6.6 ± 0.69^{b}	6.6 ± 0.51^{a}	6.6 ± 0.51^{a}	6.6 ± 0.69^{ab}	6.5 ± 0.7^{ab}
P2	5.5 ± 0.52^{b}	6.4 ± 0.51^{a}	6.9 ± 0.31^{a}	6.1 ± 0.56^{a}	6 ± 0.47^{b}	6.7 ± 0.48^{a}	6.4 ± 0.51^{ab}	5.9 ± 0.56^{b}	5.9 ± 0.31^{c}	6 ± 0.0^{b}
M2	6.6 ± 0.51^{a}	6.7 ± 0.48^{a}	6.9 ± 0.31^{a}	5.8 ± 0.42^{a}	6.9 ± 0.31^{a}	6 ± 0.47^{b}	6.8 ± 0.42^{a}	6.7 ± 0.48^{a}	6.8 ± 0.42^{ab}	6.1 ± 0.31^{b}
F1	6.6 ± 0.51^{a}	6.7 ± 0.48^{a}	6.6 ± 0.51^{a}	6.5 ± 0.52^{a}	6.7 ± 0.48^{ab}	7 ± 0.0^{a}	6.5 ± 0.52^{a}	7 ± 0.0^{a}	7 ± 0.0^{a}	6.9 ± 0.31^{a}
F2	5.6 ± 0.69^{b}	6.5 ± 0.70^{a}	6.4 ± 0.51^{a}	6.5 ± 0.52^{a}	6.7 ± 0.48^{ab}	6.8 ± 0.42^{a}	5.8 ± 0.42 ^{b}	6.8 ± 0.42^{a}	6.3 ± 0.48 ^{bc}	6.4 ± 0.51^{ab}

*values in the same row that do not share a letter (a, b) are statistically different (p <0.05) according to the Anova method, Tukey test (95% confidence level)

The panelists gave a higher score to the general impression for the P1 buns, respectively F1. This may be due to the optimal percentage of composite flour that led to the obtainment of good products in terms of appearance, elasticity and flavor, especially the F1 buns.

5.4.3. Storage stability of the buns

The results of the storage stability performed on the M, A, B coded bun samples kept for 48 hours at room temperature are presented in Table 5.3. For the A coded samples, no significant variation in the anthocyanins and polyphenols content was found, while the flavonoids content increased from 63.58 ± 0.01 mg EC/100 g DW to 68.42 ± 0.01 mg CE/100 g DW, which led to an increase of the antioxidant activity from 88.97 ± 0.04 mM Trolox/100 g DW to 94.99 ± 0.01 mM Trolox/100 g DW. On the other hand, for the B coded samples, a slight decrease of the polyphenolic compounds content was observed, therefore also of the antioxidant activity.

5.4.4. Nutritional profile of the buns

Table 5.5 shows the nutritional profile of the buns.

Table 5.5. Nutritional profile of the buns

Physico-chemical characteristics	M1	P1	P2	M1	F1	F2
Salt*, % of which Sodium*, %	1.2 0.48	1.1 0.44	1.12 0.44	1.24 0.49	1.18 0.47	1.16 0.46
Ash*, %	1.54	1.42	1.44	1.55	1.44	1.46
Humidity*%	44.1	44.5	43.10	44.6	42.3	42.4
Acidity, degrees	1.84	2.61	3.39	1.17	2.92	3.51
Fats*, %,	0.11	0.22	0.31	0.11	0.21	0.32
Protein*, %	9.3	9.5	9.7	9.3	9.4	9.6
Carbohydrates*, %	44.95	44.76	45.45	44.44	46.75	46.22
Volume	316.48	253.51	220.43	288.04	277.04	198.83
Porosity	76.09	70.71	-	75.50	72.26	-
Elasticity	93.22	78.94	-	89.09	78.33	-
Energy value*, %: kcal K	223.44	224.5	228.99	221.35	232.16	231.83

From Table 5.5., it can be observed that there were no significant variations in the salt and sodium content, ranging from 1.1% (P1) to 1.2% (M1). The fermentation time did not have a significant influence on the salt and ash content. The increase of the ash content led to the increase of the mineral substances content, this representing a key factor in assessing the percentage of mineral substances existing in the substrates (Ntuli et al. 2013). This finding suggests the effectiveness of adding the purple corn flour to improve the mineral composition of the substrates (Ogodo et al. 2017).

When the humidity of the samples was compared, a decrease was observed with the increase of the purple corn flour percentage. The fermentation time significantly influenced the

humidity of the samples, so that a decrease was observed from 44.5% (P1) to 42.3% (F1), respectively from 43.10% (P2) to 42.4% (F2). The acidity increased with the addition of PCF in the composition of the buns (from 1.84 degrees in M1 to 3.39 degrees in P2; from 1.17 degrees in M2 to 3.51 degrees in F2) but did not significantly depend on the fermentation time (2.61 degrees in P1, 2.92 degrees in F1; 3.39 degrees in P2 and 3.51 degrees in F2).

The fat content increased with the replacement of a part of the wheat flour with the purple corn flour and with the increase of the percentage of corn flour between 0.11% (m1) - 0.31% (P2) and 0.33%, respectively in F2. The fermentation time did not significantly influence the fat content percentage of any of the studied buns. The content of proteins increased with the addition of corn flour. Thus, in the case of the samples with the addition of PCF, the percentage of the protein content remained around the average of 9,5%, regardless of the fermentation time. The fat content increased in proportion to the amount of PCF added, but the fermentation time did not significantly influence the lipid content. The increase of the lipid content was due to the higher fat content of the PCF compared to the wheat flour. The most obvious increase was the carbohydrates content, with an impact on the energy value. Thus, it seems that a higher added PCF content and the prolongation of the fermentation time causes an increase in the carbohydrates content in the analyzed samples, which can be explained by the different bioavailability degree of the compounds in the two matrices. In the flours from different cereals, the carbohydrate matrix is simpler than the matrix found in the buns, the compounds having a much higher availability for the enzymatic and microbiological processes.

The volume, porosity and elasticity of the buns obtained from the composite flours (wheat flour and purple corn flour in different percentages) showed a significant reduction. The volume of P1 samples showed a reduction of 20% compared to the control sample, while P2 samples displayed a volume reduction of 31% compared to the control sample. The same aspect was observed also in the case of F1 products, the volume decreasing by 14%, respectively by 32% in the case of F2 samples, compared to the M2 control. On the other hand, the fermentation time influenced differently the volume of the buns: for F1 an increase of 9% was observed, and for F2 a decrease of 10% compared to the M2 samples. This can be explained by the action of polyphenolic compounds on the rate of yeast multiplication; the higher the amount of polyphenolic compounds (respectively the amount of purple corn flour) the higher the multiplication rate, and a longer fermentation time leads also to a significant change in the structural properties of the doughs. Due to the absence of a natural network, such as wheat gliadin and glutenin, needed to retain the carbon dioxide released during the fermentation process, the buns with purple corn flour cannot reach the spongy texture of the wheat bread. From Table 5.5, it can be seen that both the porosity and the elasticity of the samples encoded P1 and F1, which had a percentage of 25% purple corn flour, presented lower values compared to the control samples (M1 and M2); in the case of buns with a 75% percentage of PCF, the porosity and elasticity had lower values than the standard. The fermentation time had a positive influence on the porosity of the F1 samples (72.26) compared to the P1 samples (70.71). There are no statistically significant differences in the energy value of the samples obtained by fermentation for 1 hour. In the case of the buns that had a fermentation time of 2 hours, the highest energy value was represented by the F2 coded samples (232,16 kcal/100 g product). In the case of the products with an 75% addition of PCF, the resulting products showed a lower value (231,83 kcal/100 g and 232.16 kcal/100 g). Comparatively, regardless of the percentage of PCF added, the prolongation of the fermentation time led to an increase of the energy value.

5.4.5. Development of general technological schemes to obtain buns with the addition of purple corn flour

For the development of the experimental variants of buns, the raw and the auxiliary materials and also the technological process are presented in Figure 5.2.

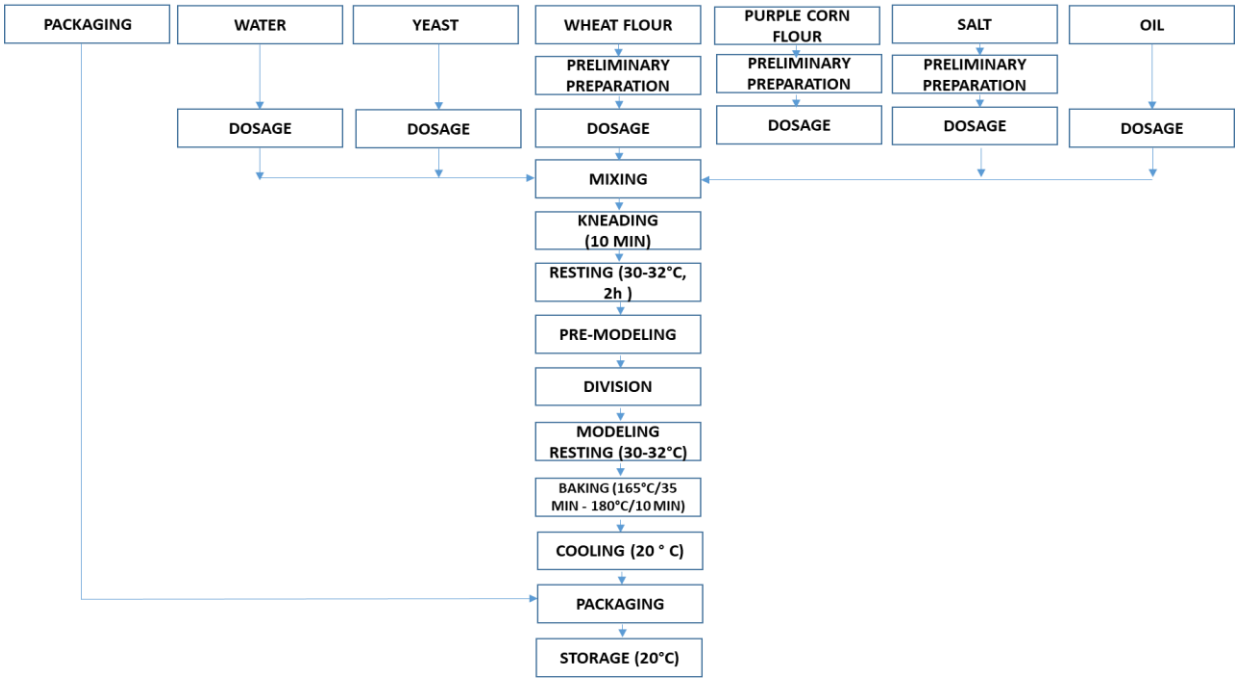


Figure 5.2. Technological scheme to obtain the buns (purple corn flour 25% and white wheat flour 75%)

The raw and auxiliary materials used to obtain the buns were: wheat flour type 650 (in the following proportions: M1 and M2 - 100%, P1 and F1 - 75%, P2 and F2 - 25%), PCF (P1 and F1 - 25 %, P2 and F2 - 75%), yeast 2.5%, water 55-66%, water temperature between 25-35°C, salt 1.3-1.6%, sunflower oil 4.3%. The raw materials, after the preliminary preparation, were subjected to kneading operations for 10-15 minutes and fermentation for 1 hour for the M1, P1, P2 and A buns, respectively 2 hours for M2, F1, F2 buns and 25 minutes for B. The dough was sliced into 180 g pieces, shaped and left to rest for 15 minutes, at a temperature of 35°C, followed by baking for 35 minutes at 160°C and 10 minutes at 180°C. After cooling, the buns were packed in containers that did not allow the loss of moisture or the buns to dry out during storage.

5.5. Partial conclusions

The purpose of this study was to study the influence of purple corn flour additive on a series of technological parameters in order to optimize the technology of obtaining bakery products, which would exploit at the same time the polyphenolic profile by developing functional products. The results obtained in this study allowed the formulation of the following partial conclusions:

❖ In order to study the influence of the fermentation time on the sensory, nutritional characteristics and the phytochemical profile, three types of buns were obtained in which the fermentation time varied (1 h or 2 h): white wheat flour buns with a leavening period of 1 hour (M1) and 2 h (M2) respectively, buns with 25% purple corn flour and 75% white wheat flour (P1 and F1) and 75% purple corn flour and 25% white wheat flour (P2 and F2) buns.

❖ The phytochemical profile analysis of the buns revealed a significant increase in the polyphenolic profile proportional to the increase of fermentation time and the percentage of purple

corn flour. Thus, the experimental variants of 75% purple corn flour and 25% white wheat flour, with a fermentation of 2 h, presented the highest content of polyphenolic compounds.

❖ It has been also observed that the purple corn flour significantly reduced the fermentation time by increasing the yeast multiplication rate. This fact was observed in the case of the samples with half of the amount of yeast, which showed the same weight after one hour of fermentation. In the case of bun B (standard yeast quantity) the fermentation time was reduced by 35 min compared to control bun M.

❖ The experimental M, A, B variants showed a good stability of the polyphenolic compounds during storage. Variant A showed an increase of its antioxidant activity (94.99 ± 0.01 mM Trolox/100 g DW compared to 88.97 ± 0.04 mM Trolox/100 g DW) due to an increase of the flavonoids content to 68.42 ± 0,03 mg CE/100 g DW after 48 hours of storage compared to 63.58 ± 0.01 mg CE/100 g DW, value registered initially. In the case of the products coded with B, there was a slight decrease in the content of polyphenolic compounds, and therefore in the antioxidant activity from 95.12 ± 0.03 mM Trolox/100 g DW to 94.45 ± 0.01 mM Trolox/100 g DW after 48 hours of storage.

❖ When evaluating the sensory characteristics of the buns, the panelists especially appreciated their taste and aroma that were obtained due to the long fermentation time. The buns that obtained the highest score were the fermented variants for 2 h, with a content of 25% purple corn flour, an optimum content highlighted by the rheological analysis of purple corn flour in the previous chapter.

❖ The analysis of the nutritional profile showed that both the amount of purple corn flour and the fermentation time had a significant influence on the physico-chemical characteristics. Thus, it was noted that the acidity decreased in proportion to the percentage of purple corn flour and the fermentation time, while the purple corn flour brought extra fat to the dough. Also, the fermentation time did not have a significant influence on the fat content, but it influenced the carbohydrates content.

❖ The volume, porosity and elasticity of the buns decreased with the replacement of the wheat flour with purple corn flour, due to the lack of gluten proteins in the purple corn flour.Thus, the buns with 75% purple corn flour presented sub-standard values.

❖ The energy value of the buns increased with the addition of purple corn flour and the fermentation time, so that the variants with a more prolonged fermentation time showed a higher energy value (231.83 kcal/100 g product), mainly due to the acceleration of the metabolic processes.

❖ A technological scheme has been proposed to obtain buns that contain 25% purple corn flour, which can contribute to the development of functional foods.

CHAPTER 6. Final conclusions

Hippocrates' statement, "Let food be your medicine and medicine your food," is a concept promoted in this century and it is of growing interest both among researchers and in the medical, economical, industrial, and social spheres. This fact has led to an increasing interest in functional foods that can prevent certain diseases or alleviate certain side effects. The doctoral thesis study, entitled "**The development of value-added foods by exploiting the biologically active potential of purple corn"**, aimed to quantify and capitalize the biologically active compounds from purple corn in order to develop functional products for the consumers. All the objectives were met, this matter being highlighted by the partial conclusions at the end of each chapter of the experimental part as well as some general conclusions presented summarily in this chapter.

Comparatively, 5 extraction techniques were tested in terms of the biologically active compounds content and antioxidant activity. The extract obtained by ultrasound-assisted extraction at 30˚C/60 min was considered superior in terms of the biologically active compounds' concentration; two major compounds being identified through a chromatographic analysis as cyanidin-3-*O* -glucoside and its acylated form of cyanidin 3-*O*-(6"- malonylglucoside).

After analyzing three parameters: temperature, liquid/solid ratio and ethanol concentration, and adjusting the data using a quadratic polynomial model, a positive correlation has been revealed between anthocyanins' recovery and temperature, ethanol concentration, while over time the liquid/solid ratio exerted a negative effect. The optimum conditions were established for the highest anthocyanins extraction yield (5 hours, at 39°C, liquid/solid ratio of 30 mL/g and an ethanol concentration of 73%), while the chromatographic analysis of the optimized extract showed the highest concentration of myricetin, followed by quercetin 3-β-D-glucoside, kaempferol, cyanidin 3-O-glucoside and gallic acid.

The thermal degradation kinetics study showed that the anthocyanins presented a high thermostability in the temperature 80-110˚C range, noting that the thermal degradation parameters related to the antioxidant activity were significantly lower compared to those estimated for the thermal degradation of anthocyanins, which indicates a different thermostability of the biologically active compounds in the extract.

The inhibitory effect was higher than that of the reference drugs on the tyrosinase, α-amylase, α-glucosidase and lipase enzymes, an effect given mainly by the polyphenolic compounds. Due to this aspect, it can be concluded that the purple corn extract has a potential antidiabetic, hypocholesterolemic and preventive effect against Parkinson's disease, Alzheimer's and melanoma.

After testing the effect of the metabolic stimulation on yeasts (*Saccharomyces cerevisiae*), the highest multiplication rate was obtained after 48 hours of cultivation, being correlated to an added purple corn flour extract concentration of 1.2 mg/100 mL. Regarding the viability of the yeast cells, the autolysis process was observed after 72 hours, so it can be stated that the purple corn flour extract had a protective effect on the yeasts' cell. The results also showed a continuous fermentation process, due to the release of CO_2.

As a result of the thermo-mechanical profile analysis of the purple corn flour, rice flour and some composite flours, three biscuit variants were obtained, which showed a good acceptability in general, and following the analysis of the phytochemical profile, these products can be regarded as being functional. The ELISA test revealed the absence of antigenic prolamins in the biscuits,

so the biscuits can also be considered gluten-free products. They presented a good storage stability and a textured structure, in the form of successive layers of starch granules and anthocyanins aggregates.

Two types of buns were obtained in which 25% and 75% purple corn flour were used to test the influence of the fermentation time on the characteristics of the buns and the influence of polyphenolic compounds on the fermentation time. Generally, it was shown that the fermentation time positively influenced the sensory, phytochemical and nutritional characteristics of the buns, providing a good acceptability, especially the buns with 25% purple corn flour. The presence of polyphenolic compounds led to a reduction of the fermentation time by 35 min.

The results of the analysis of this study showed that the gluten-free biscuits with purple corn flour can be easily accepted by consumers and recommended for celiac patients. The buns with 25% purple corn flour, due to their phytochemical profile, can be considered biologically functional products. From a nutritional point of view they meet the standards and can be part of a healthy and balanced diet.

CHAPTER 7. Original contributions and future perspectives

The original contributions of the doctoral thesis derive from the following aspects:

- A positive correlation was established between the effect of the optimized extract on the metabolic activity of the *Saccharomyces cerevisiae* yeast and the action of polyphenolic compounds from the purple corn flour on the metabolic activity of the same yeast during the technological process of obtaining the buns, a correlation highlighted by the reducing fermentation time. So far, no similar study has been identified, hence the originality of the doctoral thesis.
- To characterize the advanced polyphenolic profile, high performance liquid comatography and spectrophotometry techniques were used, techniques that allowed a detailed analysis that together with the kinetic parameters study aimed to obtain products with important biochemical properties, all from the perspective of the structure-function-process-product relationship.
- Carrying out rheological studies on the basis of which the two functional products were developed.
- The structure-function-process-product relationship represents the key element for the originality of the thesis that opens new directions of exploitation of other cereals not used so far on the Romanian market or of some fruits (e.g. black mulberry) and disregarded by the vast majority of consumers but so rich in polyphenolic compounds with high antioxidant capacity.

CHAPTER 8. Dissemination of results

I. Published articles

Slavu (Ursu), MG, Milea, Ș.A., Aprodu, I., Râpeanu G., Stănciuc N. (2020).Thermal Degradation Kinetics of Anthocyanins Extracted from Purple Maize Flour Extract and the Effect of Heating on Selected Biological Functionality. *Foods*, 9 (11), 1593, https://doi.org/10.3390/foods9111593

Slavu (Ursu), M., Banu, I. Milea, Ș.A., Aprodu, I., Enachi, E., Cotârleț, M., Râpeanu, G, Stănciuc, N. (2021). Designing gluten-free, anthocyanins-enriched cookies on a scientific basis. *International Journal of Food Science and Technology*, https://doi.org/10.1111/ijfs.15457

II. Articles submitted for publication

Slavu (Ursu), MG, Milea Ș.A., Păcularu-Burada, B., Dumitrașcu, L., Râpeanu, G., Stanciu, S., Stănciuc, N. Optimizing the liquid-solid conventional extraction conditions for anthocyanin's from purple corn flour (*Zea mays* L) using Response Surface Methodology: evidences on selected properties of optimized extract. Submitted to *Food Chemistry: X.*

III. Participation at national conferences

Slavu (Ursu), MG, Aprodu, I., Enachi, E., Râpeanu, G., Banu, I., Stănciuc N. (2020). Fostering Purple Corn as a source of biologically active compounds. Poster, *8 th edition of Scientific Conference of Doctoral Schools,* "Dunarea de Jos" University of Galati.

Slavu (Ursu), MG, Aprodu, I., Enachi, E., Râpeanu, G., Banu, I., Stănciuc N. (2020). Extraction of antocyanins from purple corn (*Zea mays* L.) by conventional and ultrasound assisted method, Poster, *8 th edition of Scientific Conference of Doctoral Schools* , "Dunarea de Jos" University of Galati.

Slavu (Ursu), MG, Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2020). The nutraceutical properties of purple maize and its inhibitory effect on α-glucosidase and α-amylase, Poster, *Student Scientific Session XVIII th Edition*, November 26-27, Arad.

Slavu (Ursu), MG, Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2020). Kinetics of thermal degradation of anthocyanins in correlation with the antioxidant activity of biologically active compounds in the extract of purple corn (*Zea mays* l.), Poster, "*Young people and multidisciplinary research in applied life sciences"*, Section: Food Chemistry, Engineering & Technology, Faculty of Food Engineering, Timișoara.

Slavu (Ursu), MG, Milea, Ș.A., Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2021). Development of anthocyanins-rich buns based on purple corn flour (*Zea mays* L.), Poster, *Multidisciplinary Conference on Sustainable, Development, Section: Food Chemistry, Engineering & Technology*, Faculty of Food Engineering Timişoara.

Slavu (Ursu), MG, Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2021). Rheological characteristics of composite flours with brown rice and purple corn, Poster*, 9 th edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University of Galati.

Slavu (Ursu), MG, Milea, Ș.A., Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2021). Gluten-free, anthocyanins-enriched biscuits based on purple corn flour (*Zea mays* L.), Poster, *9 th edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University of Galati.

Slavu (Ursu), MG, Milea, Ș.A., Aprodu, I., Râpeanu, G., Banu, I., Stănciuc N. (2021). Gluten-free, anthocyanins-enriched biscuits based on purple corn flour (*Zea mays* L.), Poster, *EuroAliment*, Galati.

IV. Awards

Third Prize, Poster, The nutraceutical properties of purple maize and its inhibitory effect on α-glucosidase and α-amylase, *Student Scientific Session XVIII th Edition*, 26-27 November 2020, Arad.

Third prize, Poster, Kinetics of thermal degradation of anthocyanins in correlation with the antioxidant activity of biologically active compounds in the extract of purple corn (*Zea mays* l.)" *Young people and multidisciplinary research in applied life sciences"*, Section: Food Chemistry, Engineering & Technology, Faculty of Food Engineering, 27 November 2020, Timisoara.

Honorable Mention, Poster, Gluten-free, anthocyanins-enriched biscuits based on purple corn flour (*Zea mays* L.) *9th edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University, 10 -11 June 2021, Galați.

Honorable Mention, Poster, Anthocyanins-enriched buns based on purple corn flour (*Zea mays* L.) *10th edition of Scientific Conference of Doctoral Schools*, "Dunarea de Jos" University, 9 -10 June 2022, Galați.