„Dunărea de Jos” University of Galati
Doctoral School of Fundamental Science and Enginering

PhD THESIS

PhD thesis summary

Researches on obtaining a functional product for people with food intolerances

PhD student,
Carmen-Alina BOLEA

Scientific coordinator,
Prof.univ.dr.eng Camelia VIZIREANU

Seria I.1: BIOTECHNOLOGIES Nr. 8

GALATI
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The series of doctoral thesis publicly defended in UDJG starting with the 1st October 2013 are:

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- Seria I 2**: Computers and information technology**
- Seria I 3**. Electric engineering**
- Seria I 4**: Industrial engineering**
- Seria I 5: **Materials engineering**
- Seria I 6**: Mechanical engineering**
- Seria I 7**: Food Engineering**
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- Seria U 1: **Philology- Englisch**
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- Seria U 3**: History**

CONTENT

Introduction

"A food may be accounted as functional if it is considered to have effects or if it may beneficially affect one or more targeted functions in the body beyond the appropriate nutritional effects, in a way that is relevant either to the well-being or if it is capable to reduce the risk of a disease " (Roberfroid Marcel B., 2000).

Moreover, a functional food may be a component, a macronutrient if it has specific physiological effects (for example starch or resistant fatty acids) or an essential micronutrient if its intake is more than the daily recommendations. It can also be a food component that, even if it has a certain nutritional value, is not essential (some oligosaccharides) or has no nutritional value (living microorganisms or herbal chemicals). Indeed, beyond the nutritional value (metabolic requirements), a diet provides consumers with components capable of both modifying body functions and reducing the risk of important nutritional diseases.

A food may be considered functional if it respects any of these 5 approaches:

1) Elimination of a known component that causes or is identified to have a harmful effect when the product is consumed (e.g. an allergenic protein).

2) Increasing the concentration of a component naturally present in foods to a point where it will induce anticipated beneficial effects (a micronutrient fortification to reach a daily intake greater than the recommended daily dose but consistent with the dietary guidelines to reduce the risk of a disease), or to increase the concentration of a non-nutritive component to a known level in order for it to produce a beneficial effect.

3) Addition of a component that is not normally present in the food product and is neither a macronutrient nor a micronutrient for which its beneficial effects have been proven (antioxidants or prebiotics).

4) Replacement of a component, (usually a macronutrient like fats which its intake is usually in excess, and therefore can cause harmful effects) with another component for which its beneficial effects have been shown.

5) Increasing the bioavailability or stability of a component that was added in the product and that it is known to produce a functional effect or to reduce the risk of disease.

Several studies have suggested an association between black rice and the beneficial health effects that are attributed to the high amounts of fiber and phytochemicals such as tocopherols, tocotrienols, the vitamin B complex, the vitamin E complex, γ-oryzanol and phenolic compounds.

There are also reports on black rice bran extracts (seed husks) that suggest several beneficial health effects. The main secondary metabolites of the black rice are anthocyanins, cyanidin-3-O-glucoside and peonidin-3-O-glucoside, which are located in the pericarp and the aleuronic layers of the seeds. The anthocyanin intake of black rice has been shown to reduce hyperactivity, hyperglycemia and also facilitates the maintenance of an optimal cardio-vascular function. Black rice is rich in fibers, minerals and important amino acids, having a protein content much higher than white rice.

The Ph.D. thesis entitled "**Researches on obtaining a functional product for people with food intolerances**" focused on the study of the biologically active compounds from black rice, the structural behavior of these compounds at different temperature and pH values, and also the development of an innovative product.

The main aim of the thesis was to obtain a black rice flour product, namely biscuits, which may be regarded as an appetizer for people with gluten intolerance but they can also be consumed by all the categories of consumers. This category of biscuits can address a wide range of consumers but is especially destined for people who are intolerant to gluten (celiac disease).

In this context, the research carried out during the doctoral studies had the following objectives:

• black rice grinding in order to obtain the fractions of black rice flour;

• extraction of biologically active compounds from black rice flour;

• physico-chemical characterization of the extracts from the black rice flour fractions;

• evaluation of the biologically active compounds structural changes at different pH and temperature values;

• development of a functional product intended for people with food intolerances (aglutenic aperitif biscuits) and its physico-chemical, microbiological and sensorial characterization.

The PhD thesis follows two main parts such as:

I. DOCUMENTARY STUDY has 4 chapters presenting the most recent data from the literature regarding the functional product concept, gluten intolerance or celiac disease, the nutritional and physico-chemical characterization of black rice, the profile of bioactive compounds from black rice (anthocyanins, phenols, flavons) and the methods for identifying these compounds.

II. THE EXPERIMENTAL PART includes the original investigations that were carried out during the doctoral research, being structured into 3 chapters, as follows:

Chapter 5, entitled **Characterization of black rice flour**, exhibits the chemical composition and the physical properties of extracts from black rice flour fractions, the identification of the major anthocyanin compounds present in the black rice flour, the extraction and identification of the main proteins fractions by gel electrophoresis and the study of the structural changes of these protein fractions using fluorescence spectroscopy. Furthermore, the undertaken study also assayed the inactivation mechanisms of the protein fractions through heat treatment that were identified based on the emission spectra by determining the fluorescence intensity, phase diagram and variance of parameter A, fluorescence anisotropy, quenching experiments with acrylamide and KI.

Chapter 6, entitled **Characteristics of the main biologically active compounds from black rice**, displays the investigations results of the extraction of biologically active compounds from the black rice flour fractions and the assessment of the highest activity and stability under different pH and temperature conditions. The inactivation kinetics was also studied as to assess the thermal treatment effect, the inactivation mechanisms being described by first order kinetic models.

Chapter 7, entitled **Obtainment of a functional product for people with food intolerances**, presents the physico-chemical, microbiological and sensorial characterization of the aglutenic aperitif biscuits.

The chapters of the experimental part are structured based on a logical sequence, as follows: Introduction, which presents the research opportunity and the specific objectives of the undertaken study; Materials and methods, that describes the materials, the used reagents and the methods of investigation, analysis, processing and interpretation of the experimental data; Results and discussions, which highlights the obtained results as well as their transposition within the research literature; Partial Conclusions and References.

Chapter 8, **General Conclusions**, shows the main conclusions that were drawn based on the experimental results that followed the obtainment of a functional product for people with food intolerances, the physico-chemical characterization of black rice flour, the structural changes of the biologically active compounds as well as the main protein fractions identification and the determination of their structural changes.

The thesis includes *195 pages*, which includes *46 tables* and *84 figures*. The documentary study represents *20 %* and the experimental part represent *80 %.*

The research activities of the PhD thesis were carried out within the research laboratories of the Integrated Center for Research, Expertise and Technology Transfer in Food Industry BioAliment-Tehnia (www.bioliment.ro), from the Faculty of Food Science and Engineering, "Dunărea de Jos", University, Galati.

Throughout the PhD stage, the PhD student was involved in two projects research teams: PN-II-RU-TE-2014-4-0618 entitled "A bottom-up approach on the effects of food processing on the allergenic potential of food proteins" - project manager Prof.dr.eng. Iuliana Aprodu and PN-III-P2-2.1-BG-2016-0143 entitled "Solutions for Multicerealier Grinding", project manager Prof.dr.eng. Iuliana Banu.

The thesis was conducted under the scientific coordination of the scientific committee that has the following members:

- Prof.dr.eng. Camelia Vizireanu - PhD supervisor;

- Prof.dr.eng. Gabriela-Elena Bahrim;

- Prof.dr.eng. Iuliana Banu;

- Prof.dr.eng. Iuliana Aprodu.

5. Characterization of black rice flour

5.1. General information

Rice (*Oryza sativa* L.) is a cereal grown in developing countries and it is used as a basic food for more than three billion Asian people. Rice is a rich source of carbohydrates, which contains a moderate amount of protein and fat and is also a source of B vitamins such as thiamine, riboflavin and niacin. Rice carbohydrates are represented mainly by starch which is composed of amylose and amylopectin. The physico-chemical properties and cooking characteristics of rice depend on the amylose content. There are several types of rice around the world that are classified according to their color such as white rice, brown rice, red rice, black rice, etc., and also as aromatic and non-aromatic rice. In recent years, there has been a global trend towards the use of phytochemicals such as antioxidants and functional ingredients from natural resources such as vegetables, fruits, oleaginous seeds and aromatic plants.

5.2. Materials and methods

Black rice (*Oryza sativa* L) was purchased from a local supermarket (Galaţi) in the June-July 2014 period. Black rice was milled with a laboratory mill (Mlynek Laboratory JNY Tip WZ/2) in order to obtain the black rice flour. The product that resulted after the milling process was sifted through several sieves with 630, 550, 315, 180, 125 and 90 μm, thus obtaining seven streams (from F1 to F7).

The study investigations followed:

- Determination of black rice flour fineness and uniformity
- Physico-chemical characterization of black rice flour fractions
- Determination of monomeric anthocyanins from black rice flour extract
- Spectrum emission
- Synchronous fluorescence spectra
- Phase diagram
- Three-dimensional fluorescence spectroscopy
- Quenching experiments

5.4. Results and discussions

5.4.1. Physico-chemical characterization of fractions of black rice flour

The moisture content was found to be ~ 11-11.5 % for all the fractions. The ash values were significantly different amongst the seven fractions. An increasing trend was observed with the decrease of the particle size. The first fraction had the lowest ash content of 1.04±0.02 g/100 g whereas the highest content was found for F7 (4.66±0.20 g/100 g) (Table 5.1). These results could be explained by the high concentration of the conjugated compounds in the bran layers of the caryopsis.

The fat concentration increased amongst the fractions with the highest value of 6.27±0.16 g/100 g for F5, as it can be observed in table 5.4.

The increase of the protein's content was observed while decreasing the particle size from 9.80±0.12 g/100 g for F1 to 12.25±0.15 g/100 g for F7.

The increasing tendency of the protein content from the first fraction to the seventh is possible due to the fact that the proteins are concentrated in the endosperm, the highest concentration being determined in the last fraction.

The highest content of fibers was obtained for F4 and F5 of 3.33±0.69 % and 3.56±1.17%, respectively, which may be considered as an indicative of the fibers location in the aleuronic layer of the grain.

A decreasing trend for the carbohydrate content was observed from 73.07±0.06 g/100 g for F1 to 62.16±0.08 g/100 g found in F7 (Table 5.1). However, it can be considered that all the flour fractions are good sources of carbohydrates.

Thus, by decreasing the diameter of the flour particles, it has been found that the nutritional properties and fraction quality increased. Therefore, all these results also indicate that proteins, lipids and minerals are located in the outer layer of the endosperm. The rice milling process followed by flour sifting is important from a nutritionally point of view as it can provide functional foods when using this type of flour.

Table 5.1. Proximate compositions of the black rice flour fractions

Sample	***Moisture*** ***%***	***Ash*** ***g/100g***	***Lipid g/100g***	***Protein*** ***g/100g***	***Fiber*** ***%***	***Carbohydrate g/100g***
F1	*11.32±0.04*	*1.04±0.02*	*1.49±0.05*	*9.80±0.12*	*1.37±0.38*	*73.07±0.14*
F2	*11.47±0.03*	*1.91±0.02*	*1.58±0.03*	*9.86±0.14*	*1.97±0.43*	*71.42±0.11*
F3	*11.27±0.03*	*2.36±0.06*	*4.42±0.09*	*10.37±0.09*	*2.68±0.40*	*67.66±0.10*
F4	*11.35±0.06*	*3.41±0.06*	*5.76±0.12*	*10.77±0.05*	*3.33±0.69*	*63.52±0.14*
F5	*11.30±0.01*	*3.80±0.13*	*6.27±0.16*	*10.79±0.20*	*3.56±1.17*	*62.89±0.09*
F6	*11.22±0.07*	*4.33±0.16*	*4.80±0.08*	*11.85±0.06*	*1.66±0.44*	*62.65±0.11*
F7	*11.20±0.03*	*4.66±0.20*	*4.41±0.11*	*12.25±0.15*	*1.10±0.48*	*62.16±0.08*

5.4.2. Determination of compounds with antioxidant action in the fractions of black rice flour

5.4.2.1. Determination of the total content of monomeric anthocyanin

In figure 5.4, the results of the total anthocyanin content are presented based on a differential pH method that was used to determine this parameter. Absorbance was measured at two different wavelengths, 520 and 700 nm, while the anthocyanin content was expressed in g equivalents of cyanidin 3-glucosid (C_3G) / 100g of flour.

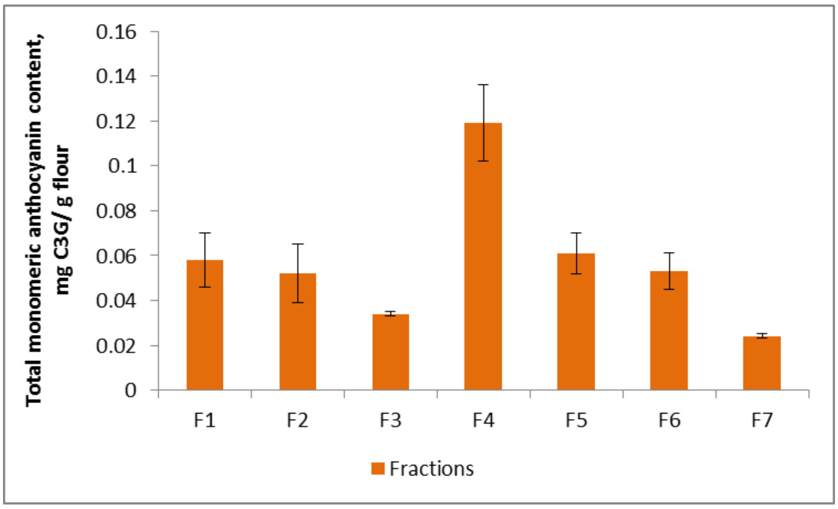
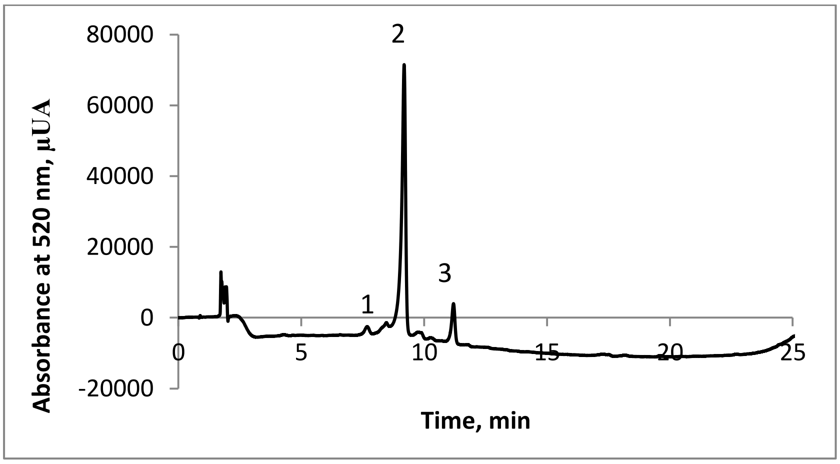


Figure 5.4. Total monomeric anthocyanin content of black rice fractions

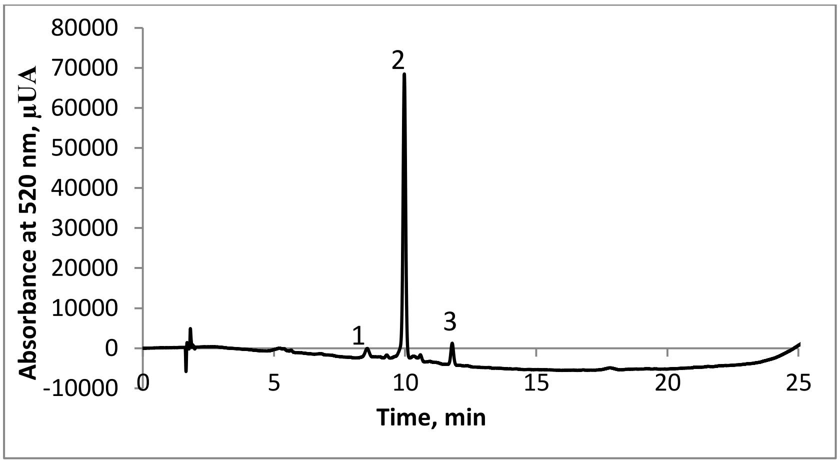
The anthocyanins are responsible for the purple, red or blue pigmentation of various plants. The most important differences between the anthocyanins are closely correlated to the nature and number of sugars that are attached to the molecule, the main position of the attachment, the number of hydroxyl groups, and the aromatic or aliphatic acids number. Among the seven fractions of black rice flour, F4 presented the highest amount of anthocyanin 0.0119±0.0017 g C_3G/100 g flour. All the other fractions presented close values such as 0.0052±0.0013 g C_3G/100 g flour in the case of F2, 0.0053±0.0008 g C_3G/100 g flour for F6, 0.0058±0.0012 g C_3G/100 g flour for F1 and 0.0061±0.0009 g C_3G/100 g flour for F5, whereas the lowest values were obtained in the case of F3 and F7 (0.0034±0.0001 and 0.0024±0.0001 g C_3G/100 g flour), respectively.

5.4.2.2. Quantitative determination of anthocyanins using HPLC

The whole flour and the four fraction HPLC chromatograms obtained at 520 nm indicate three peaks (Figure 5.5) that were associated with two anthocyanins: unidentified compound peak 1, peak 2 cyanidin 3-glucoside (0.211 g / 100 g for F and 0.338 g / 100 g for F4) and peak 3 peonidin-3-glucoside (0.001 g / 100 g for F and 0.0051 g / 100 g for F4).



a)



b)

Figure 5.5. Anthocyanins profile in fraction four extract (a), and black rice flour (b) using HPLC. Peak 1 - unidentified compound; Peak 2 - cyanidin-3-glucoside; Peak 3 - peonidin-3-glucoside.

5.4.2.3. Determination of total polyphenol content

The phenolic compounds are found in different food varieties, including fruits, vegetables and cereals. The types of compounds and their concentration may vary among different food products, which may be due to genetic and environmental factors and the processing conditions. Rice is one of the main ingredients of the population's diet and has very important antioxidant properties. Phenols are compounds that contribute to the total antioxidant activity of products. In our study, it can be seen that F4 had the highest polyphenolic content of 48.3±0.53 g GA/100 g flour, closely followed by F3 with a content of 43.23±0.73 g GA/100 g flour, whereas F5 and F6 had almost the same content (40.22±0.80 and 40.53±0.63 g GA/100 g flour, respectively).

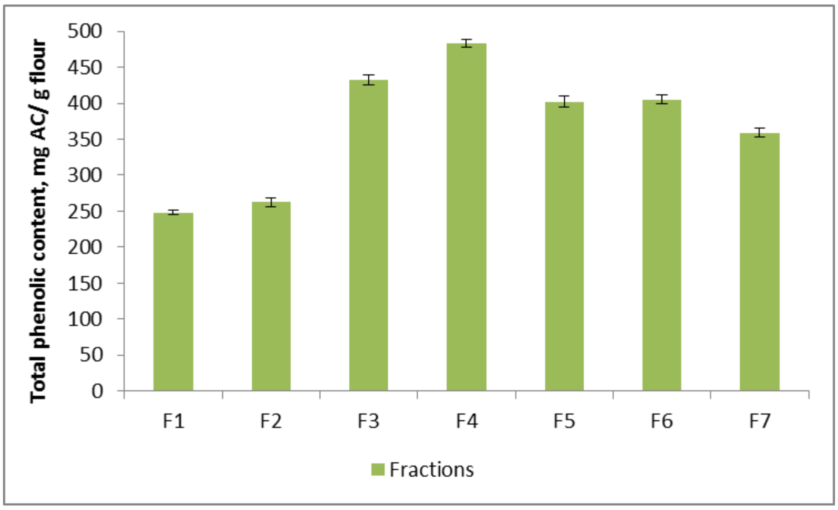


Figure 5.6. Total phenolic content of black rice fractions

5.4.2.4. Determination of the total flavonoid content of black rice flour

The total flavonoids content was determined by a spectrophotometric method using the wavelength of 765 nm and was expressed as catechin equivalents of EC / 100 g flour.

It can be seen in Figure 5.7, that the flavonoids content for F1, F2-6, had similar values that ranged between 8.15±0.24 g EC/100 g flour (F4) and 8.93±0.16 g EC/100 g flour (F6), while F2 and F7 presented smaller values of 7.01±0.29 and 7.68±0.29 g EC/100 g flour.

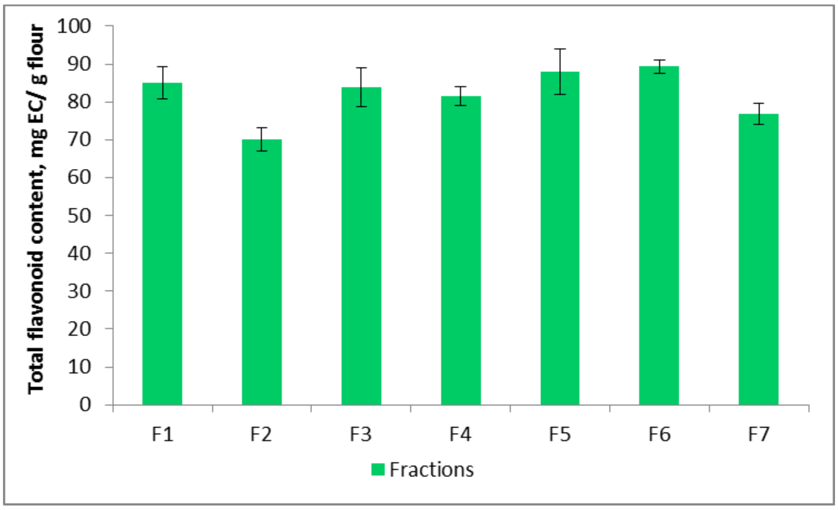


Figure 5.7. Total flavonoids content of black rice fractions

5.4.2.5. Determination of antioxidant activity of black rice flour

Antioxidant activity was expressed as % inhibition. Several researchers have reported that anthocyanins present a high antioxidant activity, together with flavonoids compounds and polyphenols. The antioxidant activity for each fraction varied between 60±2.3 (F1) - 68.23±4.01% (F5). It can be stated that after performing the experiments for the high DPPH activity the flavonoids content and to the total polyphenolic content are responsible.

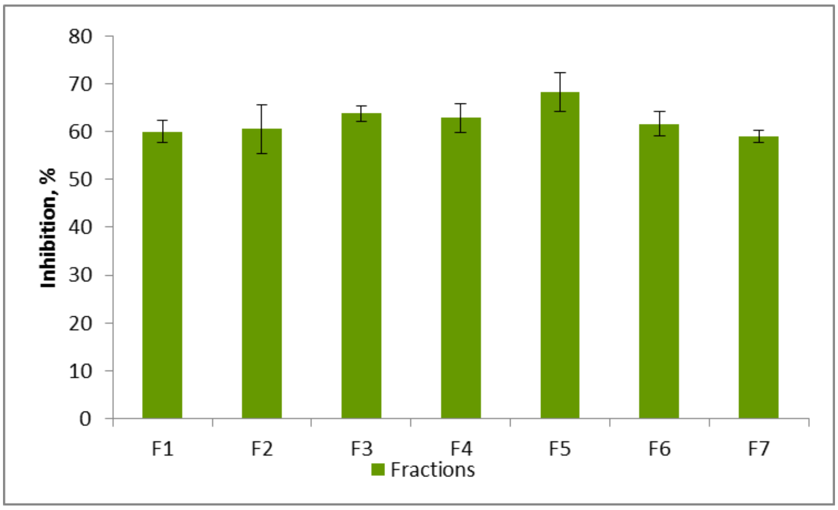


Figure 5.8. Antioxidant activity of black rice fractions

5.4.3. Separation and characterization of protein fractions in black rice flour

The rice flour with the highest protein concentration was further used to identify the main proteins fractions. It appeared that high amounts of polymers and monomers were concentrated into the fraction that had the particle size lower than 90 μm (F7) after the milling process of black rice. The SDS-PAGE pattern of the proteins from F7 is presented in Figure 5.9. By assessing the results of the SDS-PAGE analysis presented in Figure 5.9, it can be seen that the molecular weights of ALB fractions were distributed in the range of 13-16 kDa, 20-25 kDa and 35-50 kDa. GLU had bands around 13-25 kDa and 35 kDa regions, whereas the GLO bands were estimated to correspond to 12-17 kDa, 20-27 kDa and 50-70 kDa. Finally, the profile obtained for the prolamin extract was characterized by the existence of two intense bands around 10 and 15 kDa (Figure 5.9).

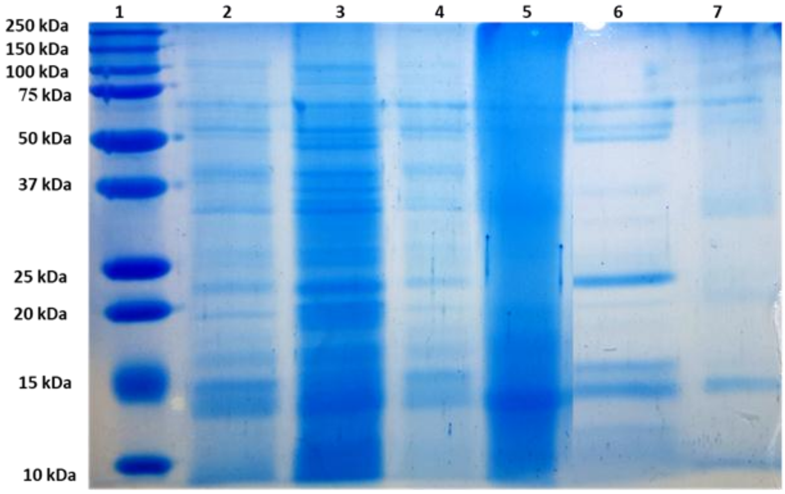


Figure 5.9. SDS-PAGE (4.5 % - 15 %) profile of the black rice flour proteins (lane 2), of F7 proteins and of the albumin, glutelin, globulin and prolamin extracts (lanes 3-7). Lane 1- Precision Plus Protein Dual Extra molecular weight marker

5.4.4. Effect of temperature on proteins in black rice flour

5.4.4.1. Spectrum emission

The fluorescence spectroscopy measurements were performed to observe the folding, unfolding events or the conformational changes that affect the microenvironment of tryptophan (Trp) and tyrosine (Tyr) residues found in the ALB, GLO and GLU fractions extracted from the black rice flour with particles smaller than 90 μm. The excitation wavelength of 292 nm was used to monitor the changes in the vicinity of Trp residues, 280 nm for both Trp and Tyr, and 274 nm for Tyr.

Emission spectra obtained after the selective excitation of Trp (Figure 5.10 c) indicated that the maximum fluorescence intensity was registered at 358 nm for ALB fraction, 384 nm for GLO and 360 nm for GLU, respectively. When excited at 280 nm, the λ_{max} corresponding to the maximum fluorescence intensity for the investigated protein fractions were 355 nm for ALB, 357 nm for GLO and 359 nm for GLU. These values indicated a higher exposure of Trp and Tyr residues to the solvent in the GLU fractions.

a)

b)

c)

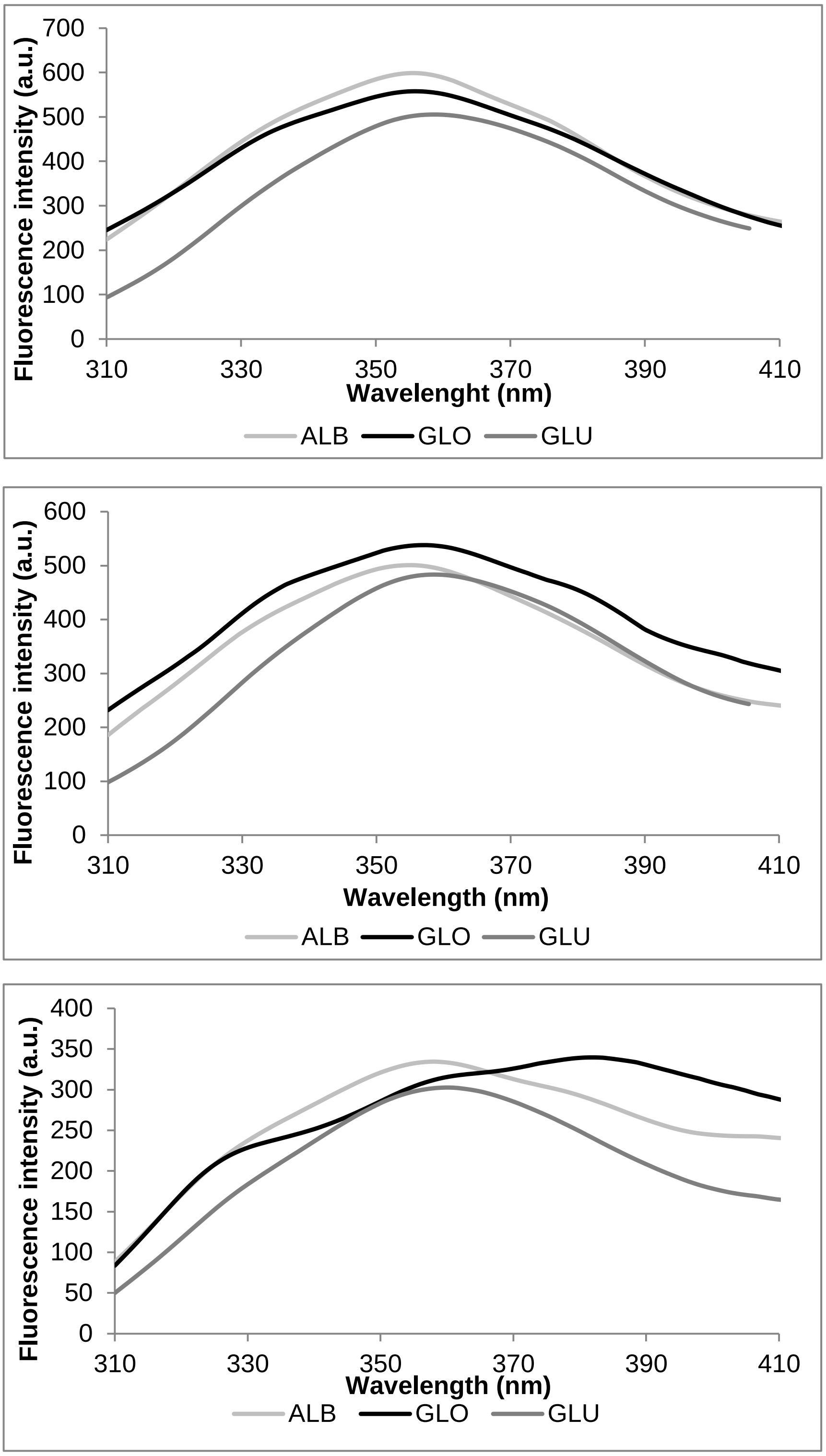


Figure 5.10. The emission's spectra of the rice proteins at 25°C. The excitation wavelength was 274 nm (a), 280 nm (b) and 292 nm (c) and the spectra were collected between 310 and 420 nm. Three independent tests were carried out in each case and the SD was lower than 10%.

5.4.4.2. Synchronous fluorescence spectra

In the tested temperature range, the synchronous spectra at *Δλ* of 15 nm indicated the presence of a red shift of 3 nm for the ALB fraction (from 286 nm at 25°C to 289 nm at 100°C), and small blue shifts of 1 nm and 2 nm for the GLO and GLU fractions (from 282 at 25°C to 281 nm at 100°C and from 295 nm to 293 nm, respectively). The red-shifts for the ALB fractions suggested the exposure of the Tyr residues to a more polar microenvironment, whereas the blue shifts in *Δλ* for GLO and GLU are an indicative of the heat-induced burial of Tyr residues to a more non-polar microenvironment.

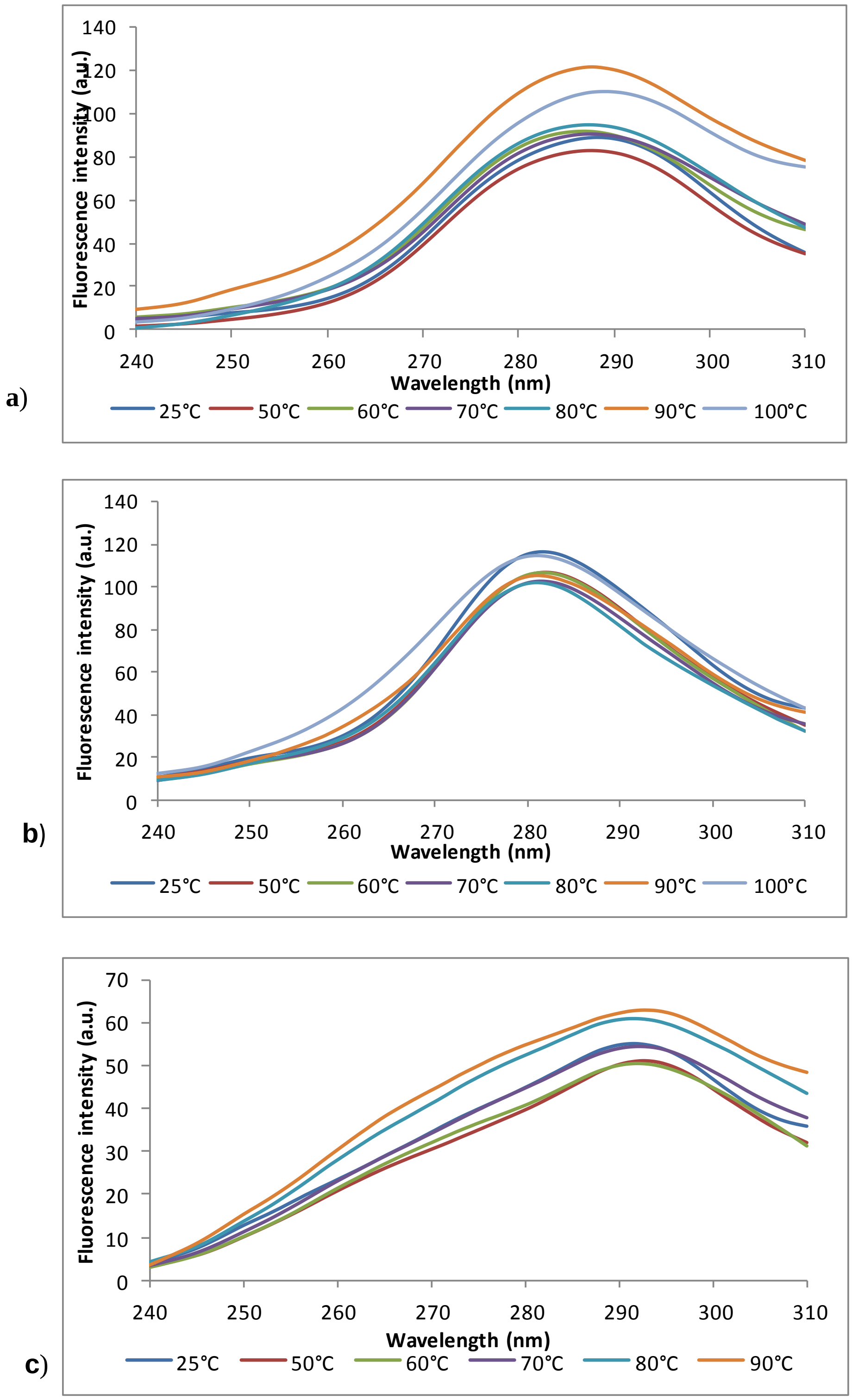


Figure 5.11. Synchronous fluorescence spectra of the rice proteins at *Δλ* = 15 nm at different temperatures values: (**a**) albumins, (**b**) globulins and (**c**) glutelins. Three independent tests were carried out in each case and the SD was lower than 3.5%

The synchronous spectra at $\Delta\lambda$ of 60 nm showed small red shifts for all the tested fractions when the temperature increased from 25°C to 100°C (from 282 nm to 283 nm, from 278 nm to 280 nm and from 282 nm to 284 nm for ALB, GLO and GLU, respectively), therefore indicating the exposure of the Trp residues to a more polar microenvironment.

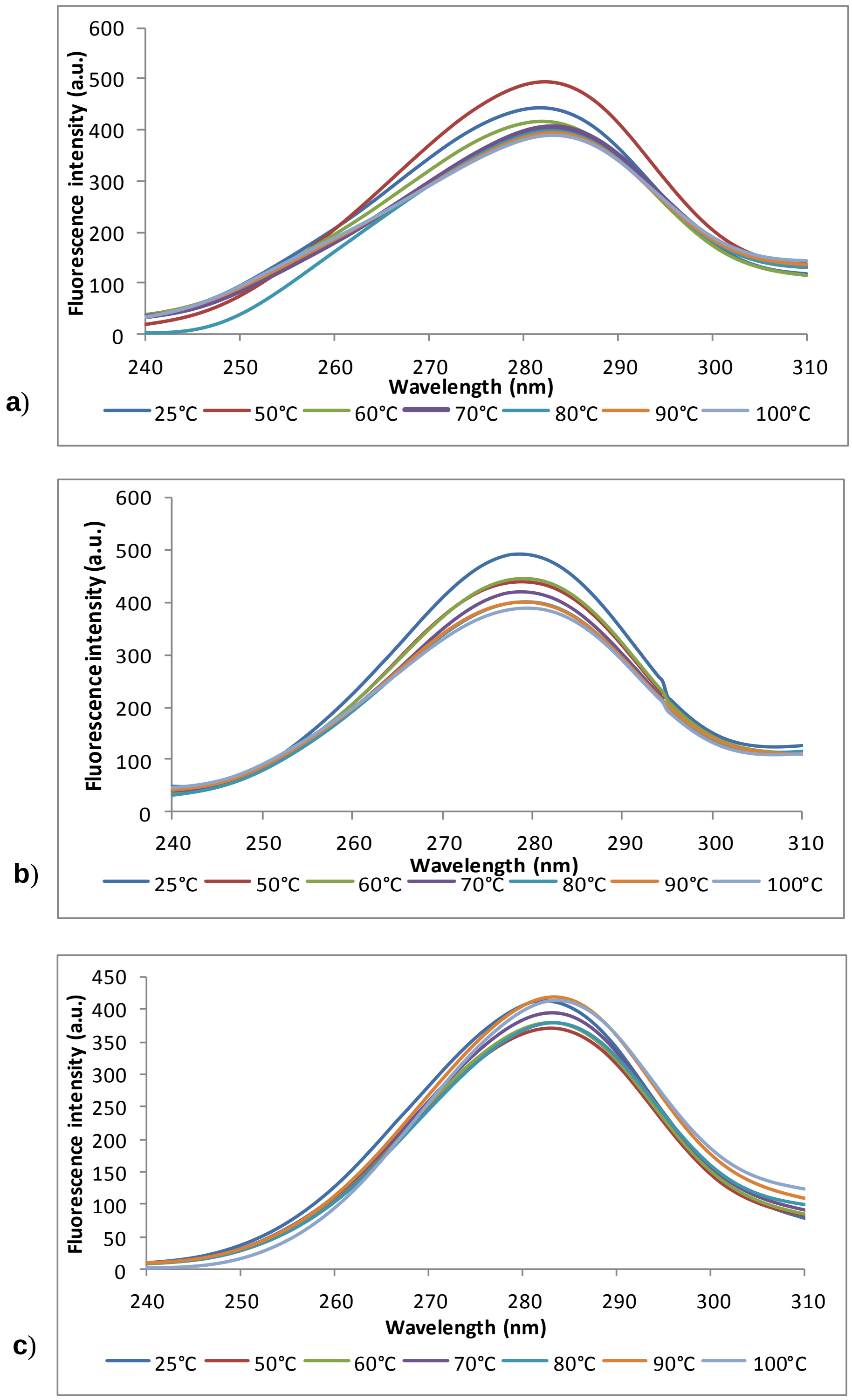


Figure 5.12. Synchronous fluorescence spectra of the rice proteins at $\Delta\lambda$ = 60 nm at different temperatures values: (**a**) albumins, (**b**) globulins and (**c**) glutelins. Three independent tests were carried out in each case and the SD was lower than 3.5%.

5.4.4.3. Phase diagram

The phase diagram showed a linear correlation for GLO, GLU fractions, which indicates the presence of two distinct molecular species both folded and unfolded ones. Furthermore, a non-linear correlation was assessed for the ALB fractions, which corresponds to the presence of two or more distinct molecular species. Based on the results presented in Figure 5.11, it can be noted that the increase of temperature determined a sequential decrease of the fluorescence intensity so that after the heat treatment some conformational changes occurred, these changes being associated with the destruction of some bonds that stabilize the conformation of the proteins.

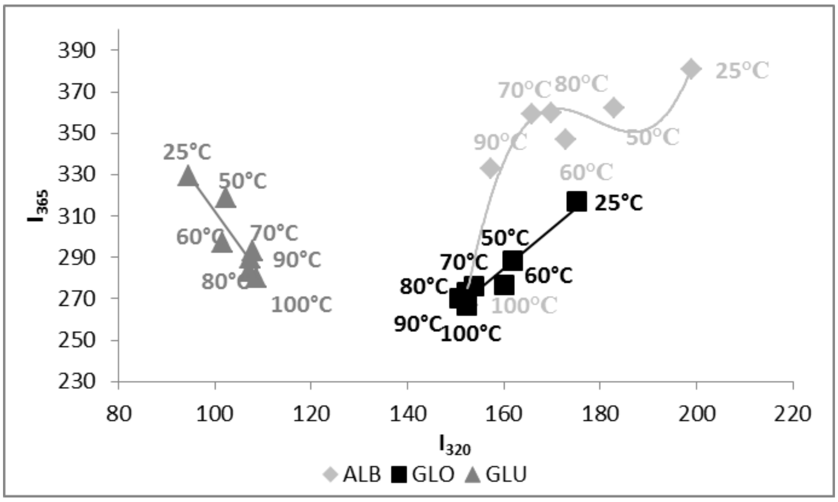


Figure 5.11. Phase diagram analysis of heat-induced conformational changes of rice proteins based on intrinsic fluorescence intensity values measured at the wavelengths of 320 and 365 nm. The temperature values are indicated in the vicinity of the corresponding symbol. Three independent tests were carried out in each case and the SD was lower than 3.5%.

5.4.4.4. Three-dimensional fluorescence spectroscopy

In order to obtain detailed information about the proteins conformational changes, three-dimensional fluorescence spectra were used. The wavelength changes evaluation at excitation or emission followed the presence or disappearance of peaks. The heat treatment conformational changes of all the protein fractions (ALB, GLO and GLU) are shown in Figure 5.14. The main parameters that characterized the spectra are presented in Table 5.2.

Table 5.2. Three-dimensional fluorescent spectra parameters of albumin (**a**), globulin (**b**) and glutelin (**c**) at different temperatures

a)

Temperature °C	***Peak 2***			***Peak 1***		
	Peak position (λ_{ex}/nm. λ_{em}/nm)	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*	*Peak position (λ_{ex}/nm. λ_{em}/nm)*	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*
25	230/354	124	449.93	280/356	76	505.36
50	230/354	124	429.04	280/356	76	470.82
60	230/354	124	426.91	280/356	76	465.78
70	230/358	128	340.79	280/358	78	424.75
80	230/356	126	397.69	280/357	77	461.14
90	230/358	128	399.36	280/357	77	448.35
100	230/360	13	387.35	280/358	78	439.64

b)

Temperature °C	***Peak 2***			***Peak 1***		
	Peak position (λex/nm. λem/nm)	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*	*Peak position (λex/nm. λem/nm)*	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*
25	230/353	123	495.62	280/355	75	461.00
50	230/354	124	440.23	280/355	75	403.59
60	230/350	120	530.55	280/355	75	452.98
70	230/355	125	467.80	280/356	76	423.28
80	230/355	125	410.93	280/356	76	395.23
90	230/358	128	477.02	280/357	77	414.79
100	230/357	127	449.70	280/356	76	394.11

c)

Temperature °C	***Peak 2***			***Peak 1***		
	Peak position (λex/nm. λem/nm)	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*	*Peak position (λex/nm. λem/nm)*	*Stokes shift Δλ (nm)*	*Fluorescence intensity (UA)*
25	*230/361*	*131*	*442.19*	*280/359*	*79*	*636.25*
50	*230/361*	*131*	*390.05*	*280/361*	*81*	*588.58*
60	*230/361*	*131*	*344.66*	*280/360*	*80*	*539.09*
70	*230/361*	*131*	*345.38*	*280/361*	*81*	*537.93*
80	*230/369*	*139*	*382.66*	*280/363*	*83*	*567.70*
90	*230/366*	*136*	*455.68*	*280/362*	*82*	*624.22*
100	*230/376*	*146*	*494.66*	*280/362*	*82*	*358.91*

In Figure 5.14 a and 5.14 b, the presence of 4 peaks can be observed. Peaks A and B correspond to the Rayleigh diffusion ($\lambda_{ex} = \lambda_{em}$), peak 1 corresponds to the spectral characteristics of tryptophan residues while Peak 2 reveals the spectral behavior of the polypeptide chain structure (backbone structure). The fluorescence intensity of peak 1 and 2 displayed in Fig. 5.14 as well as the two peaks shown in Fig. 5.14 b had a maximum value at 25 ^{o}C, afterwards a significant decrease at 70ºC was observed which indicates the fact that the reorientation associated with the folding of the albumin fraction polypeptide chain was induced by the thermal treatment.

The glutelin fraction showed significant conformational changes in the protein structure, which were associated to the heat treatment. The presence of three peaks can be observed in figures 5.14 a and 5.14 b, the first two peaks revealing the same spectral characteristics both for albumin and globulin, while the third peak may be associated with the presence of an intermediate compound.

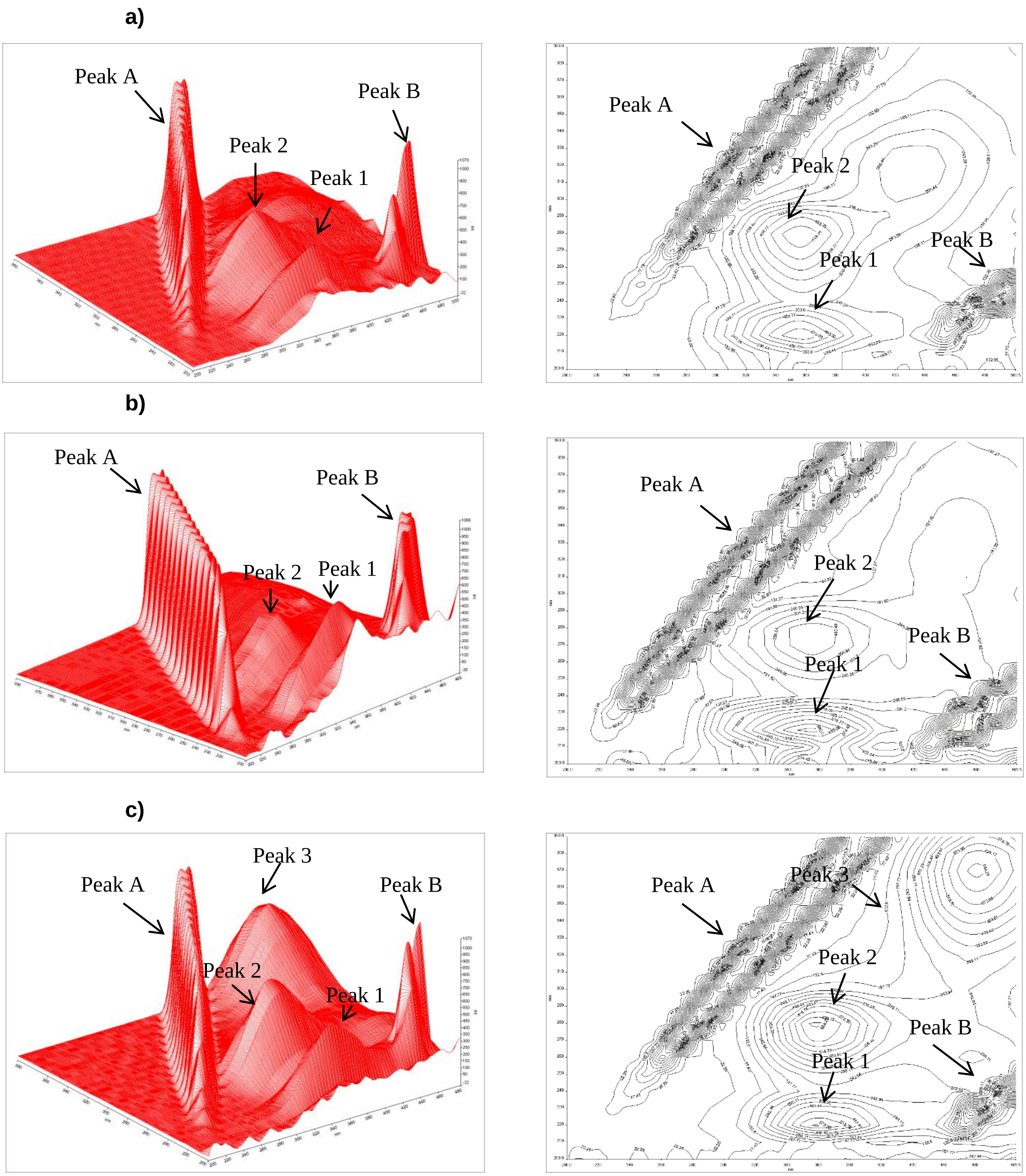


Figure 5.14. Three-dimensional fluorescence spectra and contour diagrams of black rice proteins at 25 °C: (a) albumin, (b) globulin and (c) glutelin

5.4.4.5. *Quenching experiments*

In order to check the accessibility of fluorescent residues of black rice proteins fractions to different quenchers, experiments with acrylamide and KI were performed. Acrylamide and KI are external quenchers (charged and non-charged) used to analyze the solvent accessibility and the polarity of the microenvironment close to the Trp residues. The selection of the two quenchers was based on the different accessibility of the quenchers, as acrylamide quenches the exposed and partially exposed Trp residues, while KI quenches only the fluorescence of the exposed Trp located at or near to the surface of the molecules.

The highest values for the acrylamide quenching constants were measured after a thermal treatment at 60°C for ALB (15.11 ± 3.32 mol^{-1} L), at 100°C for GLO (19.63 ± 2.70 mol^{-1} L) while for the GLU fraction the highest value was recorded at 100°C (14.15±1.67mol^{-1} L).

Table 5.3. The Stern Volmer quenching constant (K_{SV}) with acrylamide at different temperatures

Temperature. °C	K_{SV} ***(10^{-3}L·mol^{-1})***		
	Albumins	*Globulins*	*Glutelins*
25	*9.40±0.55*	*18.07±1.79*	*13.60±0.94*
50	11.72±0.47	*15.57±0.54*	*8.55±0.68*
60	15.11±3.32	*19.41±0.06*	*12.47±0.04*
70	13.79±1.11	*12.51±0.19*	*13.25±0.46*
80	14.30±1.84	*12.22±1.96*	*10.96±0.67*
90	13.75±2.84	*14.93±0.86*	*13.79±1.77*
100	13.52±0.80	*19.63±2.70*	*14.15±1.67*

For all the rice protein fractions, the K_{SV} values variations indicated the sequential character of the structural and conformational changes that were rmally induced.

For the KI quenching experiments, the maximum K_{SV} value for ALB was calculated at 100°C (6.47 ± 0.53 mol^{-1} L) and the minimum value was recorded at 80°C (4.70 ± 0.57 mol^{-1} L). For the GLO fraction, the maximum K_{SV} was found at 70°C (6.65 ± 1.80 mol^{-1} L) and the minimum at 80°C (4.53 ± 1.50 mol^{-1} L), while the highest value for the GLU fraction was recorded at 90°C (4.38 ± 1.15 mol^{-1} L) and the lowest one at 70°C (1.29±0.65 mol^{-1} L).

Table 5.4. The Stern Volmer quenching constant (K_{SV}) with KI at different temperatures

Temperature. °C	K_{SV} ***(10^{-3}L·mol^{-1})***		
	Albumins	*Globulins*	*Glutelins*
25	*4.92±0.87*	*7.03±1.17*	*1.37±0.42*
50	*5.04±0.79*	*6.56±0.21*	*2.37±0.11*
60	*5.65±1.01*	*5.10±0.95*	*1.99±0.51*
70	*5.79±1.41*	*6.65±1.80*	*1.29±0.65*
80	*4.70±0.57*	*4.53±1.50*	*1.81±0.32*
90	*6.41±1.00*	*5.43±2.26*	*4.38±1.15*
100	*6.47±0.53*	*6.21±1.10*	*3.12±0.91*

5.5. Partial conclusions

- As a result of the physico-chemical characterization of the black rice flour fractions it was found that the seventh fraction had a high protein content of 12.25 ± 0.15 g/100 g, while the fifth had a high fiber content of 3.56 ± 1.17%;

- Also, the physico-chemical characterization revealed that the proteins are mostly found in the endosperm of the rice grain whereas the fibers are in the aleuronic layer;
- The results highlighted a wide range of phytochemicals that are present in the black rice. Therefore, significant differences between the black rice fractions regarding the anthocyanins content, the phenolic acids content and the antioxidant properties were observed, which led to the conclusion that black rice is a good source of phytochemicals;
- Among the seven fractions of black rice flour, F4 showed the highest amount of anthocyanins 0.0119 ± 0.0017 g C_3G/100 g flour and polyphenols 48.30 ± 0.53 g AG/100 g flour, whereas the flavonoid content and the antioxidant activity had close values.
- The antioxidant activity was mainly given by the total flavonoids content, but also by the total content of polyphenols and anthocyanins;
- Based on the HPLC analysis, two anthocyanins were identified in the black rice flour, namely cyanidin-3-glucoside (the largest amount) and peonidin-3-glucoside;
- The molecular masses of the albumin protein fraction were distributed in the range of 13-25 kDa and 35-50 kDa. Glutelin had bands around 13 and 35 kDa while the globulin bands were estimated to be between 12-17 kDa, 20-27 kDa and 50-60 kDa. The profile obtained for the prolamin extract showed two prominent bands around 10 and 13 kDa;
- The fluorescence spectroscopy experiments were performed to observe the conformational changes or the folding or unfolding processes of proteins, that produced the local reorientation of the tryptophan and tyrosine residues;
- The intrinsic fluorescence measurements revealed that the thermal treatment caused the protein molecules partial folding at moderate temperatures and the unfolding at higher temperatures, with changes in the solvent exposure of tryptophan and tyrosine residues;
- The phase diagram showed a linear correlation for the globulin and glutelin fractions, while for the albumin fraction a non-linear correlation was determined, suggesting that the heating led to the formation of two or more distinct molecular species;
- The quenching studies revealed a higher availability of the acrylamide to the amino acid residues with fluorescent properties in the globulin fractions compared to KI. For all the studied fractions, the quenching constants varied with the temperature at which the heat treatment was performed, indicating the sequential character of the structural and conformational changes induced in the proteins structure;
- The increase of the quenching constants values with the increasing temperature indicates that the conformational transition of proteins decreases the distance between the tryptophan residues and the quenching agent. The high K_{SV} values at higher temperatures are an indicator of the structural rearrangements that cause the partial exposure of the tryptophan residues within the protein molecules;
- The temperature increase caused a sequential decrease of the fluorescence intensity, indicating that some thermal conformational modifications occurred following the heat treatment, these changes being associated with the destruction of some bonds that stabilize the protein's conformation;
- The present study provides the fundamental knowledge in regards to the physiological functionality and processing behavior of different components from black rice, therefore promoting a more efficient consumption of black rice products by increasing the consumer's awareness of the health benefits of grains.

6. Characteristics of the main biologically active compounds in black rice flour

6.1. General information

In recent years, to identify new vegetable sources of pigments that could replace synthetic dyes and can also be used in the food industry became a challenge. The scientific interest in improving the separation and identification techniques of anthocyanins from plant sources has increased significantly due to their potential use as natural dyes. Anthocyanins exhibit significant biological activity with remarkable antioxidant properties that play an important role in the prevention of neuronal, cardiovascular disease, certain types of neoplasms, diabetes, etc. (Kong et al., 2003, Konczak and Zhang, 2004, Lule and Xia, 2005).

6.2. Materials and methods

The experiments involved the use of the following reagents: 1N HCl solution, phosphate buffer pH = 4.0, 70% ethanol solution, HPLC purity methanol, 5% $NaNO_2$ solution (w/v), 10% $AlCl_3$ solution (w/v), 1M NaOH solution, Folin- Ciocâlteu reagent, 20% Na_2CO_3 solution (w/v), 0.025 M KCl solution, pH = 1.0, 0.4M $CH_3COONa \cdot 3H_2O$ solution, pH =4.0, 3 - 5% formic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, TROLOX solution, gallic acid solution, 4% boric acid.

The laboratory equipment used to carry out the experiments was: Raypa TRADE BBA-4 water thermostat, (Spain), METTLER TOLEDO XS 403 SM high precision analytical balance (Switzerland), HETTICH Universal 320 R cooling ultracentrifuge (Germany), METTLER TOLEDO S 20 K pH-meter (Switzerland), LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT, USA), LAB COMPANION COMECTA SA orbital shaker with analog control of shaking frequency and thermostat, JENWAY UV spectrophotometer - Double-VIS VIS with data analysis software.

The investigations from this chapter followed:

- Studies on the thermal degradation of the biologically active compounds from black rice:
  - The effect of heat treatment on the anthocyanins content of the black rice flour fractions
  - The effect of heat treatment on the total polyphenols content of the black rice flour fractions
  - The effect of thermal treatment on the flavonoids content of the black rice flour fractions
  - The effect of heat treatment on the antioxidant activity of the black rice flour fractions
- Studies on the influence of temperature and pH on the biologically active compounds from black rice using fluorescence spectroscopy techniques
  - Influence of the pH on the anthocyanins from black rice flour
  - Influence of the thermal treatment on the biologically active compounds from black rice flour

6.3. Results and discussions

6.3.1. Studies on thermal degradation of biologically active compounds in black rice

6.3.1.1. Effect of thermal treatment on the anthocyanin content in the fractions of black rice flour

The degradation degree of the anthocyanin content of all the black rice flour fractions was analyzed in the temperature range of 60-100°C, in a time Interval between 5 and 20 minutes.

Figure 6.1, presents the temperature dependence of the heat degradation rate constants of the anthocyanins from the seven black rice fractions in the 25-100°C temperature range. Independently of the particle size of the black rice seven fractions, the degradation kinetics of the applied thermal treatment displayed a first order degree model (Figure 6.1).

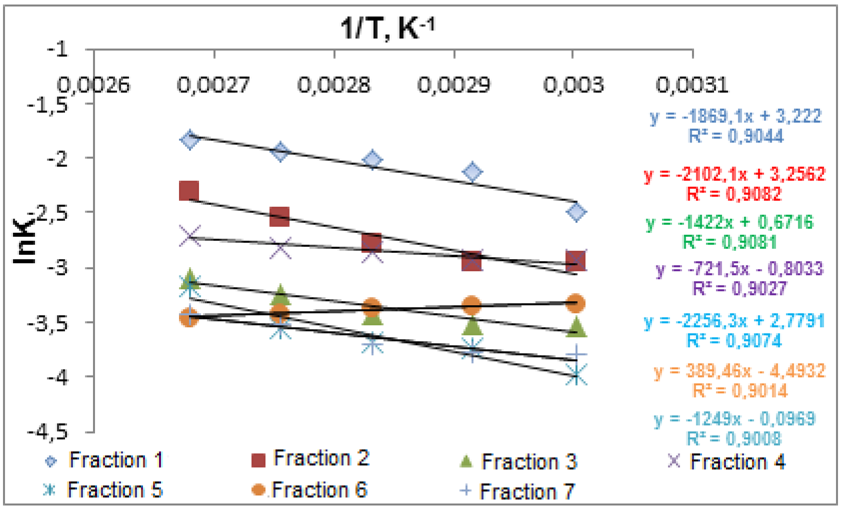


Figure 6.1. Temperature dependence of the heat degradation rate constants of the anthocyanins from the seven black rice flour fractions

Figure 6.2, shows the thermal degradation of the extract from the first fraction of black rice flour in the temperature range of 60-100 ºC, noting that a first order degradation kinetics was assessed.

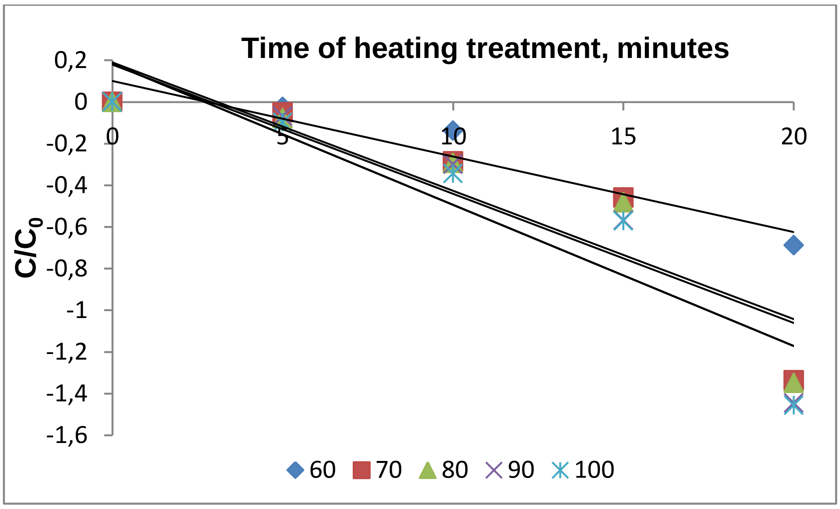


Figure 6.2. Thermal kinetics degradation of the extract from fraction 1 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

Analyzing the results from Table 6.2, it can be observed that the value of the degradation constant, k, increased as the temperature rose to 80 ºC, after which the value of k decreased, whereas the value of the decimal reduction time (D) decreased as the temperature rose to 80 ºC. The degradation of the anthocyanins in fraction 1 was characterized by an activation energy of 0.25 ± 2.60 kJ/mol, which means that a much higher temperature is required in order to achieve the degradation of the total anthocyanins content.

Table 6.2. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the first fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	13.21±2.71*	0.174±0.010
70	11.27±1.42	0.204±0.042
80	11.29±1.21	0.203±0.012
90	12.43±1.00	0.185±0.031
100	12.50±1.01	0.184±0.021
***Ea*, kJ/mol**	0.25±2.60	

*standard deviation

Figure 6.3 shows that the thermal degradation of the extract from the second fraction of black rice flour in the temperature range of 60°C-100°C. It can also be observed that the degradation of anthocyanins from this fraction showed a first order degradation kinetics, with the total anthocyanin content values decreasing from 0.052±0.013 C_3G mg/g value to 0.001±0.001 C_3G mg/g.

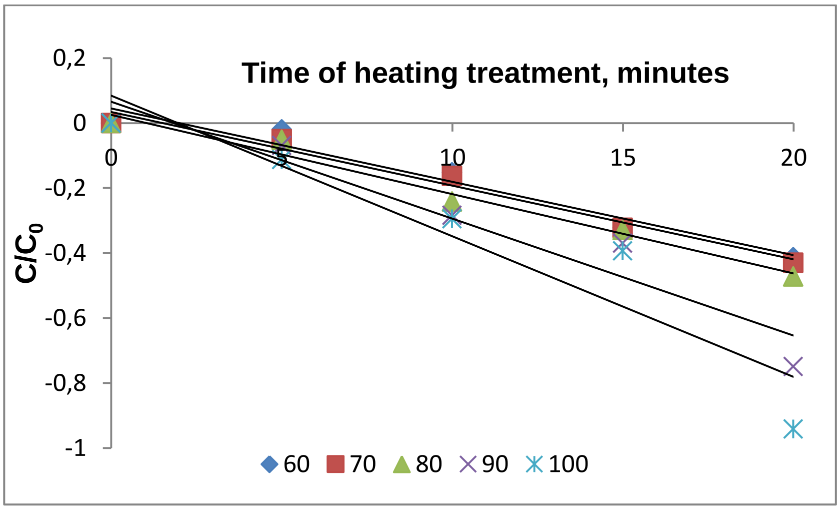


Figure 6.3. Thermal kinetics degradation of the extract from fraction 2 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

As table 6.3 shows, the kinetics parameters k and D for the total monomeric anthocyanin content from the second fraction depended on the applied thermal treatment. In the case of the degradation constant k it can be seen that its value increased in the temperature range of 90-100ºC. At 100°C, the degradation constant k recorded the highest value, 0.171±0.022 min^{-1}. Regarding the degradation constant D, its value increased between the applied temperatures of 60-80ºC. Thus, at 60°C the D constant is 21.14±1.02 min, and it increases up to 24.21±3.00 at 70ºC. At 80°C, the D degradation constant is 23.58±1.09 min whereas at 100ºC it decreases up to 13.45±1.01 min.

The second fraction of the black rice flour recorded an activation energy of 4.69±5.93 kJ/mol.

Table 6.3. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the second fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	21.14±1.02 *	0.108±0.037
70	24.21±3.00	0.095±0.017
80	23.58±1.09	0.097±0.028
90	18.48±2.23	0.124±0.017
100	13.45±1.01	0.171±0.022
***Ea,* kJ/mol**	4.69±5.93	

*standard deviation

As observed for the first two fractions that were investigated, the study of the activity of anthocyanins in fraction 3 of black rice flour in the temperature range of 60ºC - 100ºC indicated that the degradation of these compounds follows a first order kinetics model.

Following the thermal treatment, the anthocyanin content of the third fraction decreased from 0.034 ± 0.011 C_3G mg/g to 0.010 ± 0.002 C_3G mg/g.

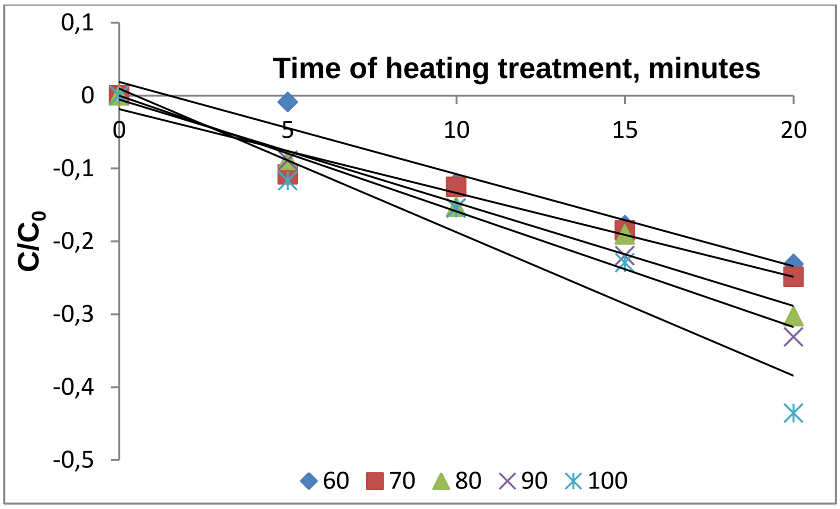


Figure 6.4. Thermal kinetics degradation of the extract from fraction 3 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

Table 6.4 shows the kinetic degradation constants k and D whose values vary within the temperature range.

The value of the kinetic degradation constant k increased between the temperature range from 60ºC to 100ºC. At the temperature of 60ºC the value of the constant was 0.037±0.011 min^{-1}. In the 80-90°C temperature range, k value increased from 0.047±0.012 min^{-1} to 0.055±0.024 min^{-1}.

The decimal reduction time (D) decreased in the applied temperature range for the third fraction. Thus, around the temperatures of 60°C D's value was 61.34±7.05 min, while at the temperature of 70°C this value decreased up to 54.05 ± 7.00 min. At 80°C, the decimal time was 48.78±7.02 min, value that decreased at 90°C to 41.15±4.01 min.

The activation energy for the third fraction recorded a value of 10.77±1.53 kJ/mol.

Table 6.4. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the third fraction of black rice flour

Temperature, °C	***D*, min**	***k*, min^{-1}**
60	61.34±7.05*	0.037±0.011
70	54.05±7.00	0.042±0.021
80	48.78±7.02	0.047±0.012
90	41.15±4.01	0.055±0.021
100	41.84±4.00	0.055±0.024
***Ea*, kJ/mol**	10.77±1.53	

*standard deviation

The thermal degradation kinetics of the extract from black rice fraction 4 follows a first order kinetic model at all the studied temperatures. The value of anthocyanins as a result of the heat treatment decreased from 0.119±0.017 C_3G mg/g to 0.017±0.011 C_3G mg/g.

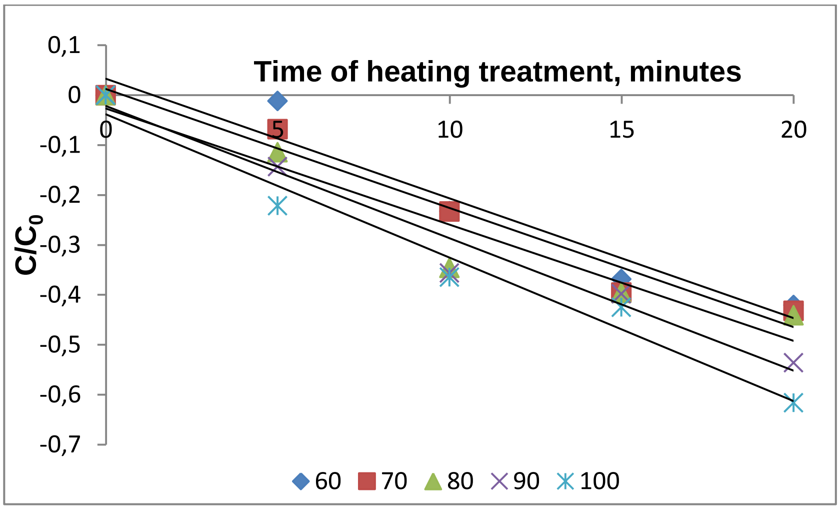


Figure 6.5. Thermal kinetics degradation of the extract from fraction 4 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

According to table 6.5., the thermal degradation constants (k) and the decimal reduction time (D) have different values at the degradation temperatures. At 80°C the value of the degradation constant increased from 0.074±0.011 min^{-1} to 0.081±0.023 min^{-1} at 90°C.

In the temperature range of 60-70ºC, the decimal reduction time D increased from 27.10±7.00 min to 30.39±2.00 min. In the temperature range between 80 and 90°C, the decimal reduction time decreased from 30.95±7.01 min to 28.16±2.31 min.

After performing the heat treatment at different temperatures, for the extract obtained from fraction 4 an activation energy of 11.49 ± 1.47 kJ/mol was calculated.

Table 6.5. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the fourth fraction of black rice flour

Temperature, ºC	***D*, min**	***k*, min^{-1}**
60	27.10±7.00*	0.084±0.019
70	30.39±2.00	0.075±0.014
80	30.95±7.01	0.074±0.011
90	28.16±2.31	0.081±0.023
100	26.52±2.11	0.086±0.033
***Ea,* kJ/mol**	11.49±1.47	

*standard deviation

It can be seen from figure 6.6. that for the fifth fraction of black rice the degradation was calculated to be a first order degradation kinetics while the anthocyanin content decreased from a value of 0.061±0.021 C_3G mg/g to a value of 0.016 ± 0.011 C_3G mg/g.

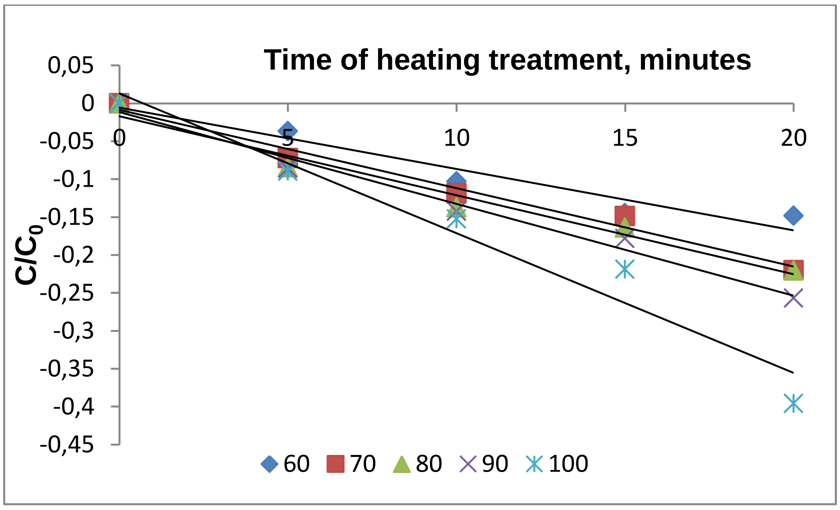


Figure 6.6. Thermal kinetics degradation of the extract from fraction 5 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

The results presented in table 6.6. show that in the case of the total monomeric anthocyanin content of the fifth fraction, there was an increasing trend of the degradation constant (k) in the tested temperature range, while the degradation constant (D) decreased. At the temperature of 60ºC, k presented a value of 0.014±0.009 min^{-1} and 0.133±0.045 min^{-1} at 70ºC. The value of k at 80°C was 0.132±0.026 min^{-1}, whereas at 90°C the value increased up to 0.136 ± 0.028 min^{-1}. At 100°C, the constant's value increased up to 0.150±0.012 min^{-1}.

Between 60 and 70°C, D value decreased from 16.51±7.09 min to 17.24±7.32 min. In the 80-90°C temperature range, the value of D also decreased from 17.33±7.32 min to 16.83±4.02 min. The value of the kinetic degradation parameter at 100°C decreased up to 15.31±2.43 min.

In the case of the extract obtained from fraction 5, the activation energy value was 10.18±5.89 kJ/mol.

Table 6.6. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the fifth fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	16.51±7.09*	0.014±0.009
70	17.24±7.32	0.133±0.045
80	17.33±7.32	0.132±0.026
90	16.83±4.02	0.136±0.028
100	15.31±2.43	0.150±0.012
***Ea*, kJ/mol**	10.18±5.89	

*standard deviation

As shown in figure 6.7. the thermal degradation kinetics of anthocyanins from the sixth fraction of black rice followed a first order degradation model. The total anthocyanin content decreased from the value of 0.053±0.018 C_3G mg/g to 0.015±0.006 C_3G mg/g in the 25-100ºC temperature range for a time period between 5 and 20 minutes.

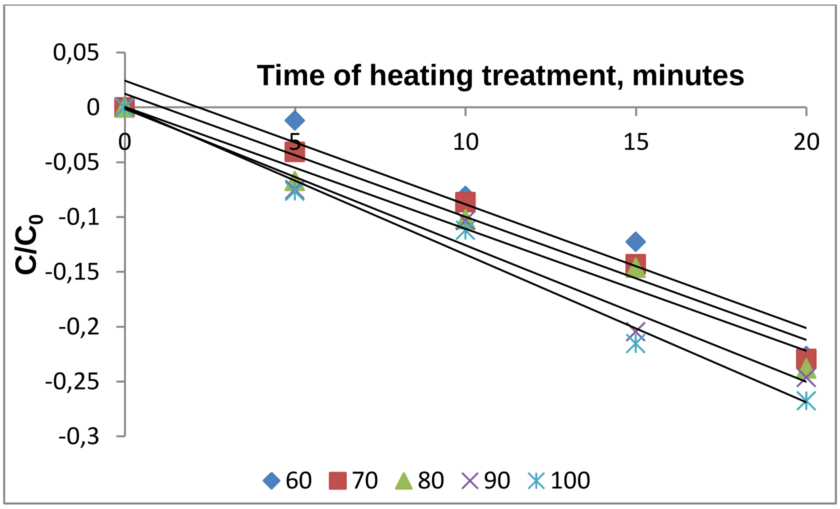


Figure 6.7. Thermal kinetics degradation of the extract from fraction 6 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

Unlike the fractions previously characterized, for the sixth fraction the degradation constant (k) values ranged between 0.050 and 0.056 min^{-1} in the studied temperature range. The same behavior was also observed for the decimal reduction parameter (D). In the 60-70°C temperature range, k decreased from 0.056±0.012 min^{-1} to 0.051±0.022. Between the temperatures of 80-90°C, the value of k increased from 0.050±0.014 min^{-1} to 0.053±0.021 min^{-1}. At 100ºC, k value increased up to 0.056±0.011 min^{-1}.

The second thermal degradation constant, the decimal reduction time (D), had a similar behavior as the degradation parameter (k). At temperatures between 60-70°C the value of D increased from 40.98±2.16 min to 45.04±4.04 min. Between 80 and 90°C, the value of D decreased from 45.24±1.03 min to 43.10±7.09 minutes whereas at 100°C the degradation constant value decreased up to 40.98±4.05 min.

The activation energy for the extract from fraction 6 was 11.99±1.81 kJ/mol.

Table 6.7. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the sixth fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	40.98±2.16*	0.056±0.012
70	45.04±4.04	0.051±0.022
80	45.24±1.03	0.050±0.014
90	43.10±7.09	0.053±0.021
100	40.98±4.05	0.056±0.011
***Ea*, kJ/mol**	11.99±1.81	

*standard deviation

At all the studied temperatures, the kinetics of the thermal degradation of the extract from the seventh fraction of black rice followed a first order degradation model (Figure 6.8). The anthocyanins content gradually decreased from 0.024±0.001 mg C_3G/g to 0.008±0.001 C_3G mg/g.

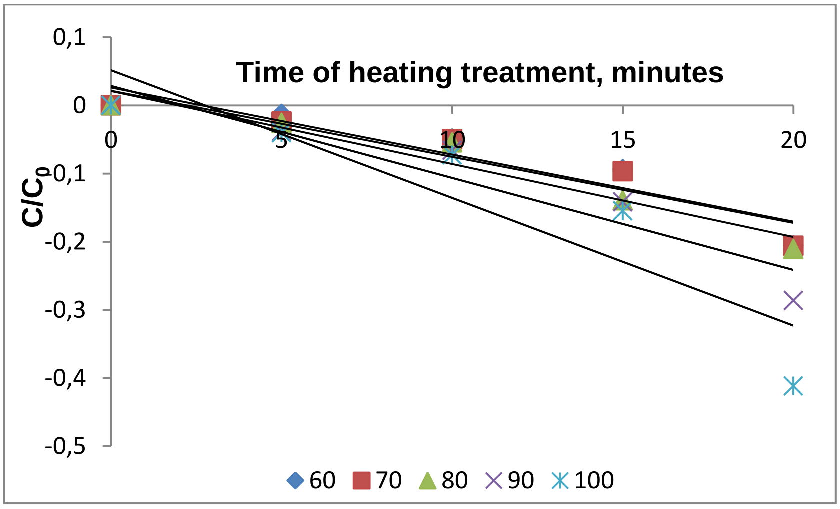


Figure 6.8. Thermal kinetics degradation of the extract from fraction 7 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

According to table 6.8, where the values of the thermal degradation constants k and D are represented, one can see that the kinetic thermal degradation values of k increased in the studied temperature range, whereas the values of the decimal reduction time (D) decreased with the increase of the temperature.

At 60°C and 70°C, the k value decreased from 0.030±0.012 min^{-1} to 0.029±0.008 min^{-1}. At 80 and 90°C, k values increased from 0.033±0.022 min^{-1} to 0.039±0.023 min^{-1}. The thermal degradation constant recorded an increasing value of 0.051±0.022 kJ/mol at 100ºC.

With respect to the decimal reduction time, it can be seen that between 60 and 70°C the values increased from 76.33±17.00 min to 76.92±17.09 min. The D value between 80 and 90°C decreased from 68.02±10.20 min to 58.13±4.33 min. At the temperature of 100ºC, the value of the decimal reduction time decreased up to 44.64±2.11 min.

For the seventh fraction the activation energy was calculated as being 13.79±3.20 kJ/mol following the thermal treatment.

Table 6.8. Kinetic parameters estimated for the total monomeric anthocyanin content (degradation constants k, D and activation energies-*Ea*) for the seventh fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	76.33±17.01*	0.030±0.012
70	76.92±17.01	0.029±0.008
80	68.02±10.20	0.033±0.022
90	58.13±4.33	0.039±0.023
100	44.64±2.11	0.051±0.022
***Ea*, kJ/mol**	13.79±3.20	

*standard deviation

6.3.1.4. Effect of thermal treatment on antioxidant activity in the fractions of black rice flour

The results of the thermal degradation in the tested temperature range between 25 and 100°C showed that the anthocyanins in terms of antioxidant activity followed a first order degradation kinetics. The antioxidant activity after the heat treatment dropped from 60.004±2.3% to 47.183±1.45%.

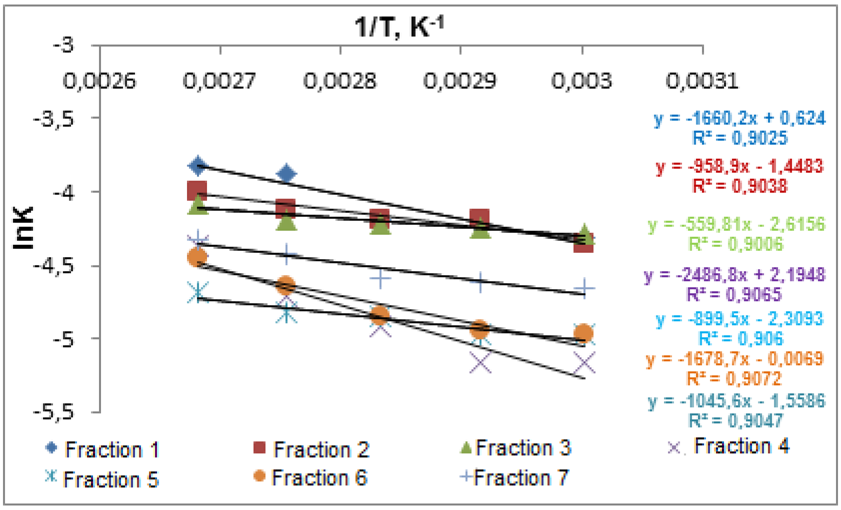


Figure 6.25. Temperature dependence of the heat degradation rate constants in terms of antioxidant activity of the black rice flour fractions

In figure 6.26, the degradation kinetics of the extract's antioxidant activity from fraction 1 followed a first order kinetic model.

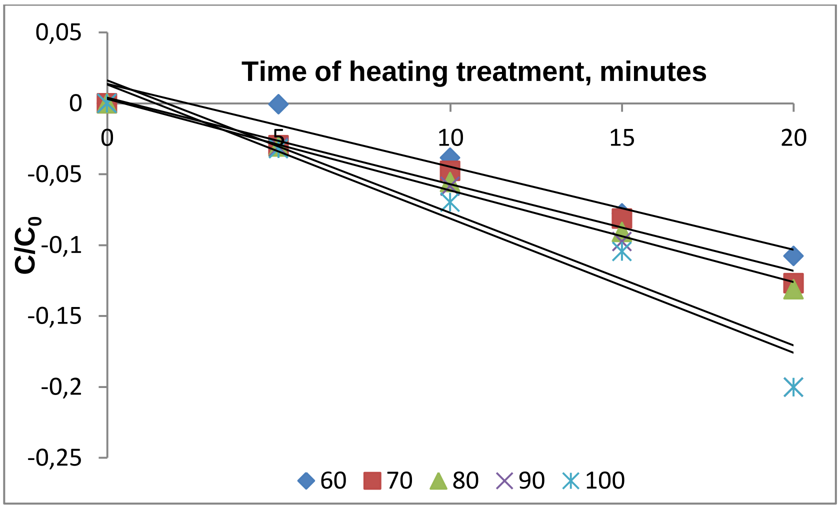


Figure 6.26. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 1 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

The value of the degradation constant (k) increased with the increasing temperatures while the value of the decimal reduction time (D) decreased as the temperature increased, which means that the antioxidant activity of fraction 1 was reduced.

The thermal degradation of the antioxidant activity of the first fraction was characterized by an activation energy of 16.61±3.98 kJ/mol.

Table 6.26. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the first fraction of black rice flour

Temperature, ºC	***D*, min**	***k*, min^{-1}**
60	172.41±17.04*	0.013±0.011
70	158.73±14.01	0.014±0.011
80	151.51±10.00	0.014±0.009
90	111.11±7.01	0.021±0.012
100	105.26±7.00	0.021±0.005
***Ea*, kJ/mol**	16.61±3.98	

*standard deviation

According to figure 6.27, the thermal degradation of the extract from fraction 2 in the temperature range between 60ºC and 100ºC showed a degradation kinetics of the first order.

The antioxidant capacity of the second fraction at different temperatures decreased from 60.521±5.03% to 41.041±1.75%.

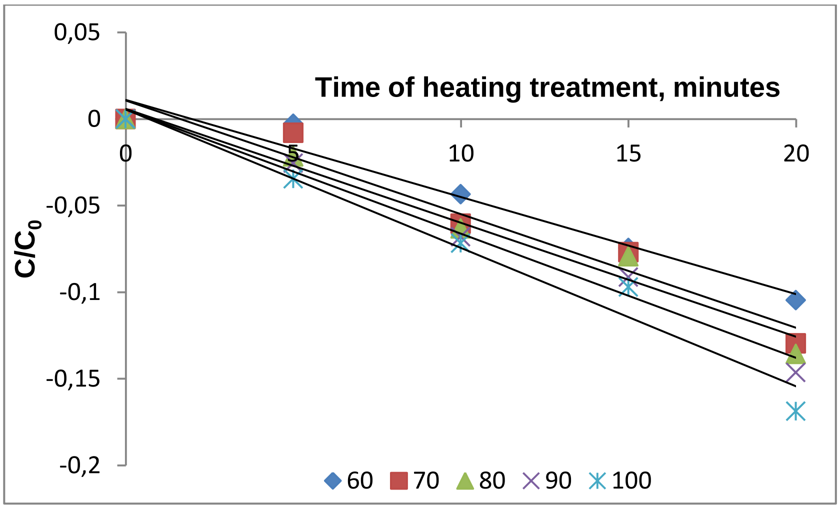


Figure 6.27. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 2 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

According to table 6.27, the kinetic parameters for the antioxidant activity of the second fraction, k and D, varied within the applied temperature range as the parameters obtained for the first fraction. In the case of the degradation constant, an increase of its value was assessed in the 60-100ºC temperature. Regarding the degradation constant (D), a decrease of the values was observed between the applied temperatures.

The second fraction of black rice flour recorded an activation energy of 8.27±1.30 kJ/mol.

Table 6.27. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the second fraction of black rice flour

Temperature, ºC	***D*, min**	***k*, min^{-1}**
60	178.57±17.56*	0.012±0.009
70	151.51±10.02	0.015±0.011
80	151.51±10.02	0.015±0.007
90	142.85±10.00	0.016±0.012
100	125.00±8.01	0.018±0.011
***Ea*, kJ/mol**	8.27±1.30	

*standard deviation

In terms of the antioxidant activity of the black rice third fraction, the thermal degradation results in the 60-100°C temperature range showed that it follows a first-order degradation kinetics.

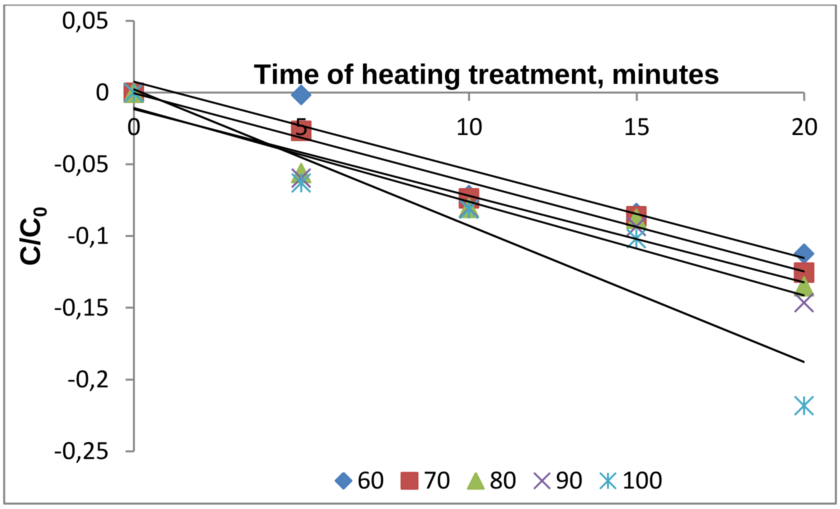


Figure 6.28. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 3 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

As a result of the heat treatment of the third fraction of black rice flour, the antioxidant activity decreased from 63.758±1.61% to 38.580±2.32%.

Table 6.28. highlights the kinetic degradation constants, k and D, whose values vary within the studied temperature range.

The increase of the degradation constant values conducted to the decrease of the decimal reduction time, which resulting in the reduction of the antioxidant activity.

The activation energy calculated for the third fraction extract recorded a value of 9.39±4.87 kJ/mol.

Table 6.28. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the third fraction of black rice flour

Temperature, ºC	*D,* min	*k,* min^{-1}
60	166.66±14.04*	0.013±0.011
70	161.29±14.02	0.014±0.005
80	156.25±12.00	0.014±0.006
90	151.51±10.03	0.014±0.011
100	136.98±10.00	0.021±0.013
***Ea,* kJ/mol**	9.39±4.87	

*standard deviation

The thermal degradation kinetics of the extract from fraction 4 displayed a first order kinetic order at different temperatures.

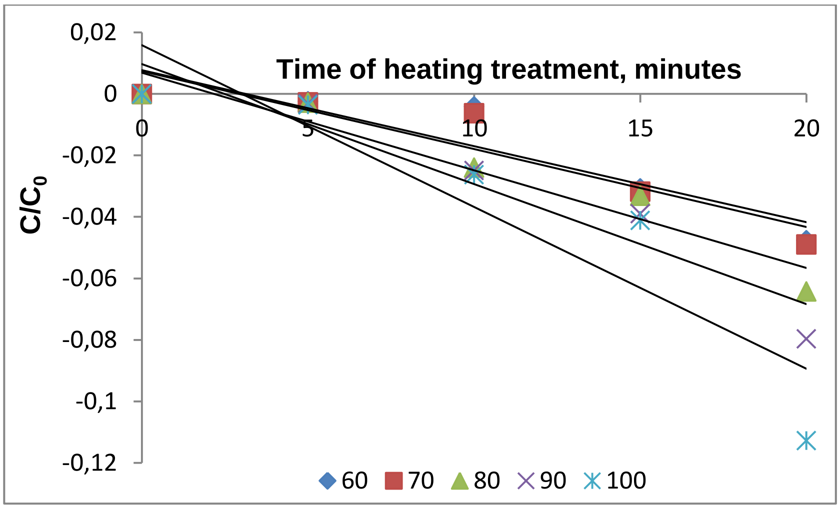


Figure 6.29. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 4 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

In the studied temperature range, 25-100ºC, in the time period of 5-20 minutes, the antioxidant activity of the fourth fraction gradually decreased from 62.901±3.02% to 48.516± 2.11%.

Like for all the other fractions, the thermal degradation constants, k and the decimal reduction time D, for the fourth fraction different values at certain degradation temperatures were assessed. Regarding the antioxidant activity, the degradation constant values increased within the temperature range of 60-100°C.

The extract from the fourth black rice fraction after undergoing the heat treatment at different temperatures presented an activation energy of 19.93 ± 3.49 kJ/mol.

Table 6.29. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the fourth fraction of black rice flour

Temperature, ºC	*D*, min	*k*, min^{-1}
60	400.00±21.02*	0.005±0.002
70	400.00±21.02	0.005±0.003
80	312.50±19.03	0.007±0.004
90	256.41±17.05	0.008±0.002
100	181.81±14.20	0.012±0.001
***Ea,* kJ/mol**	19.93±3.49	

*standard deviation

It can be seen from figure 6.30, that for the fifth fraction of black rice the degradation followed a first order kinetic model.

The antioxidant activity for fraction 5 decreased from 68.231±4.10% to 51.764±2.8%.

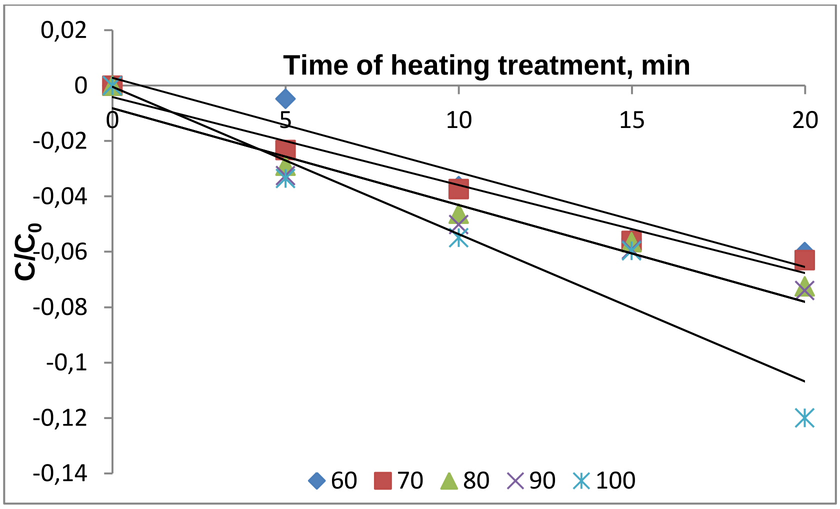


Figure 6.30. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 5 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

Table 6.30. shows that in the case of the antioxidant activity of the fifth fraction the degradation constant values increased in the temperature range. The values of D decreased, which indicated the decrease of the antioxidant activity.

In the case of the extract obtained from fraction 5, the activation energy value was 9.82±5.23 kJ/mol.

Table 6.30. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the fifth fraction of black rice flour

Temperature, ºC	***D* (min)**	***k* (min^{-1})**
60	333.33±20.06*	0.007±0.002
70	333.33±20.06	0.007±0.002
80	294.11±20.06	0.007±0.003
90	285.71±20.00	0.008±0.002
100	250.00±17.02	0.012±0.005
***Ea*, kJ/mol**	9.82±5.23	

*standard deviation

As shown in figure 6.31, the thermal degradation kinetics of the extract from the sixth black rice flour fraction at different temperatures followed a first order degradation model.

For the fraction 6 extract, the antioxidant activity decreased from 61.627±2.54% to 46.029±1.18%.

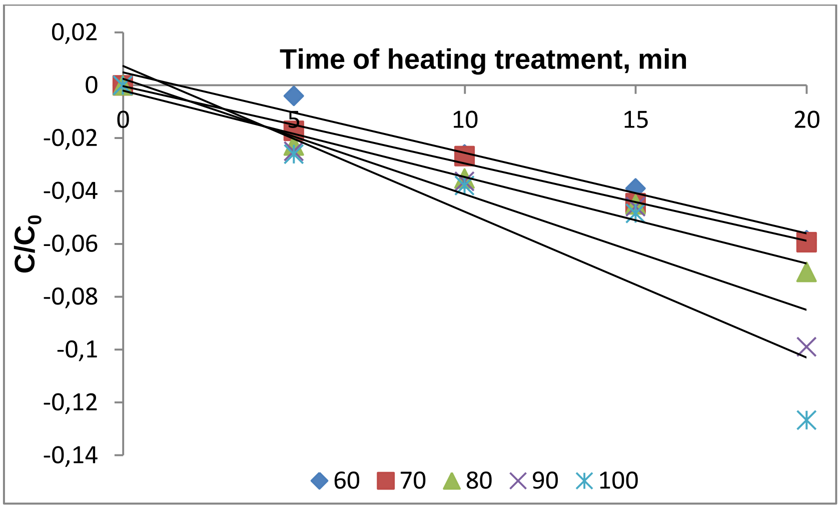


Figure 6.31. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 6 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

For fraction 6, the degradation constant, k, increased in the studied temperature range whereas the decimal reduction time parameter decreased. In the 60-100°C range the value of k increased from 0.006±0.004 min^{-1} to 0.012±0.005 min^{-1}.

The activation energy for the extract from fraction 6 was 16.61±3.98 kJ/mol.

Table 6.31. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the sixth fraction of black rice flour

Temperature, ºC	*D*, (min)	*k* (min^{-1})
60	333.33±9.00*	0.006±0.004
70	322.58±8.06	0.007±0.003
80	294.11±8.00	0.007±0.003
90	238.09±8.00	0.010±0.006
100	196.07±7.09	0.012±0.005
***Ea*, kJ/mol**	16.61±3.98	

*standard deviation

The thermal degradation kinetics of the seventh fraction extract from black rice at different temperature values followed a first order degradation model and the antioxidant activity of fraction 7 decreased from the value of 59.001±1.31% to 41.066±2.62%.

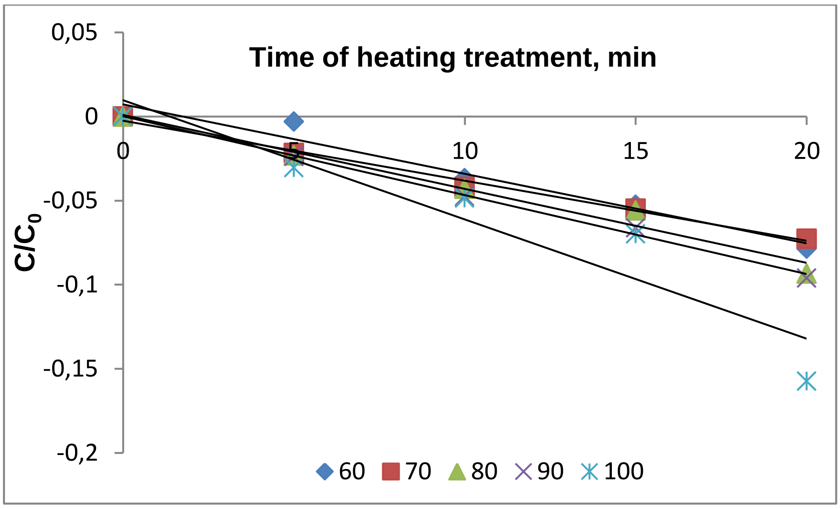


Figure 6.32. Thermal degradation kinetics of the antioxidant activity of the extract obtained from fraction 7 at different temperatures (C is the concentration of the extract at t time, C_0 is the initial concentration of the extract)

According to Table 6.32., one can see that the k values increased in the studied temperature range while the decimal time values decreased as the temperature rose.

For the seventh fraction, an activation energy of 13.80±5.49 kJ/mol was calculated following the thermal treatment. To significantly reduce the antioxidant activity of the black rice flour fractions, a much higher temperature is required.

Table 6.32. Kinetic parameters estimated for the antioxidant activity (degradation constants k, D and activation energy *Ea*) for the seventh fraction of black rice flour

Temperature, °C	***D*, min**	***k*, min^{-1}**
60	243.90±10.02*	0.009±0.012
70	232.55±10.01	0.009±0.006
80	227.27±10.00	0.010±0.011
90	192.30±8.02	0.011±0.010
100	175.43±4.04	0.016±0.011
***Ea*, kJ/mol**	13.80±5.49	

*standard deviation

6.4. Partial conclusions

- The effect of temperature on the degradation process of phenolic compounds from black rice flour extract was examined based on a kinetic study;
- The content of anthocyanins of the first fraction decreased as a result of the heat treatment in the 25-100°C range, from 0.058±0.012 C_3G mg/g to 0.001±0.0001 C_3G mg/g, a decrease which was also observed in the case of the other black rice flour fractions;
- In terms of thermal degradation, the total content of phenols, flavonoids and monomeric anthocyanins followed a first order kinetic model;
- The degradation rate constants showed that with the temperature increase, the degradation process intensified also, this fact being expressed by the means of the activation energy values (*Ea*);

- Following the heat treatment, the thermal degradation constants and the decimal reduction time values, k and D, were calculated for each fraction. For the first fraction, the thermal degradation of anthocyanins took place between 60-80°C. As a result the value of the degradation constant, k, increased with the temperature increase to 80°C. Afterwards, the value of k decreased, while the value of the decimal reduction time (D) decreased as the temperature rose to 80°C;
- The fraction with the highest activation energy value was the seventh fraction with a value of 13.7±3.20 kJ/mol whereas the lowest activation energy value was calculated for for the first fraction with a value of 0.25±2.60 kJ/mol, specifying also that a small value was also obtained for the second fraction;
- In the temperature range of 60-100ºC, the antioxidant activity of the studied fractions displayed a significant decrease;
- In order to degrade the existing compounds from all the fractions, a longer period of treatment and a higher temperature are required;
- The degradation rates constants have shown that with the increase of temperature the degradation process was intensified, this fact being expressed by the small activation energies (*Ea*), which were lower than those identified in the literature, thus demonstrating the high thermostability of anthocyanins present in black rice flour.

7. Obtaining a functional product for people with food intolerance

7.1. General information

Biscuits are the most popular bakery items consumed at almost all levels of society. This is mainly due to their nature, nutritional quality and the availability of different assortments at affordable costs. Most of the bakery products are used as a source for incorporating various nutritionally rich ingredients. Several types of health care products have now become available. Dietary fibers play a very important role in human diet. Food fibers, consisting of digestible cellulose, hemicellulose, lignin, gums and mucilages, offer a variety of health benefits.

7.2. Materials and methods

The *raw materials* used to make the biscuits were purchased from a local supermarket in Galaţi, these being: black rice, oat bran, chia seeds, almonds, black pepper, rosemary, thyme, salt and baking powder.

The black rice flour was obtained by fine grinding the rice grains in a laboratory mill (Mlynek Laboratory JNY Type WZ / 2). This type of flour stands as the main ingredient used to make the aperitif biscuits. The black rice flour has various biologically active compounds (anthocyanins, phenols, flavonoids) and does not contain gluten. Black rice flour provides minerals and vitamins, thus helping to maintain the health, to improve the metabolism, nerve function, to prevent heart attacks, to lower cholesterol, to improve the bone and muscle, memory and the blood circulation, to prevent heart disease, neoplasm, diabetes, hypertension, asthma and Alzheimer's disease, being a good antioxidant and a natural anti-inflammatory.

The used reagents were: methyl methylene blue red indicator, bromocresol methyl green indicator, sodium hydroxide, sodium thiosulphate, 70% ethanol solution, HPLC purity methanol, 5% (m/v) $NaNO_2$ solution, 10% (m/v) $AlCl_3$ solution, 1M NaOH solution, Folin-Ciocaltelu reagent, 20% (m/v) Na_2CO_3 solution, 0.025M KCl solution, pH = 1.0, 0.4M $CH_3COONa·3H_2O$ solution; pH 4.0, 3-5% formic acid solution, DPPH reagent (2,2-diphenyl-1-picrylhydrazyl), TROLOX solution, gallic acid solution, boric acid, 4%, 0.002 n clorhidric acid, potassium sulphate, mercury oxide, petroleum ether with a distillation range of 40-60°C, 0.23 mol/l sodium hydroxide or 0.313 mol/l sodium hydroxide, 0.13 mol/l H_2SO_4, dissociated water, rose-bengal culture media, 3M Enterobacteriaceae Count Plate petrified petroleum products, sterile distilled water, patented cocktail (R7006 / R7016, official AOAC method).

7.5. Results and discussion

7.5.1. Physico-chemical characterization of aperitif biscuits

The results of the physico-chemical analysis performed on the functional product for people with food intolerances (aperitif biscuits) are presented in Table 7.1.

Table 7.1. Physico-chemical composition of aperitif biscuits

	Parameters	
Biscuits	Ash g/100g	5.49±2.03
	Lipid g/100g	16.71±4.06
	Protein %	22.25 ± 1.14
	Moisture %	4.46±1.12
	Fiber %	8.99±2.50

According to the results presented in Table 7.1 it can be appreciated that the aperitif biscuits obtained at the laboratory level by using black rice flour as a basic ingredient have a high protein and fiber content (22.25±1.14 and 8.99±2.5%). In regards to the high fiber content of the aperitif biscuits, it is possible to label the product "rich in fiber" due to the raw materials used to make them.

7.5.2. Evolution of the content of biologically active compounds during storage

The initial content of biologically active compounds was evaluated for the dough used to make the biscuits before the baking operation. The dough obtained according to the operations displayed in Figure 7.1 showed the following content of biologically active compounds: TAC 0.0035±0.0002 g C_3G/100 g; TPC 1.32±0.01 g AC/100 g; CFT 0.87±0.01 g EC/100 g; 47.96±0.71% DPPH. Table 7.2 shows the evolution of the total anthocyanins content (TAC), total polyphenol content (TPC), flavonoids content (TFC), and the antioxidant activity over a 21-days storage period for the biscuits.

Table 7.2. Evolution of the content of biologically active compounds during storage at room temperature

Biologically active compounds	Storage period, day			
	0	7	14	21
TAC, g C_3G/100 g	0.0050±0.0009	0.0024±0,0001	0.0024±0.0003	0.0024±0.0001
TPC, g AC/100 g	1.62±0.05	1,66±0.08	1.77±0.15	1.88±0.07
TFC, g EC/100 g	1.51±0.77	2.28±0.24	2.28±0.06	2.3±0.57
DPPH, %	66±1.66	74.32±0.16	76.94±3.06	77.01±0.80

Analyzing the results presented in Table 7.2, it can be seen that the aperitif biscuits recorded a decrease in the total anthocyanin content in the first 7 days of storage, after which the value was maintained for up to 21 days (0.0024±0.0001 mg C_3G / 100 g), while the total content of polyphenols and flavonoids slightly increased throughout the studied period. The evolution of the antioxidant activity of the aperitif biscuits followed the same trend as observed for the polyphenols and flavonoid contents. Thus, it can be appreciatde that both the total polyphenols and flavonoids predominantly influence the antioxidant activity of the biscuits.

7.5.3. In vitro digestibility

In the present study, a release kinetics of anthocyanins compounds from the biscuits during the *in vitro* digestibility (Figure 7.2) was performed. In the simulated gastric juice, a decrease of the total monomeric anthocyanins content was observed, with about 34% in the first 30 minutes of digestion and about 44% after 120 minutes. In the simulated intestinal juice, an increase of the anthocyanins compounds release (Figure 7.2) was observed, about 3 times higher after 30 minutes of intestinal digestion and about 5 times higher after 120 minutes. It can be concluded that the complex biscuit matrix protected the anthocyanins in the gastric juice, allowing them to be released into the intestinal juice. The release of anthocyanins during the gastric digestion simulation suggests that the proteins protect the anthocyanins, thus allowing a controlled release of the bioactive compounds under the influence of gastric and intestinal enzymes in the simulated juices.

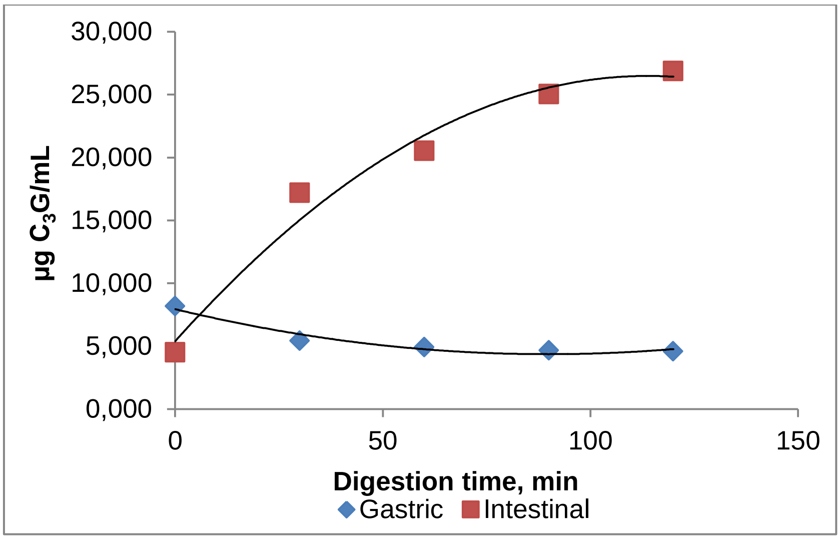


Figure 7.2. Content of anthocyans following the simulated gastric and intestinal digestion of aperitif biscuits

7.5.4. Testing the antigenic potential of aperitif biscuits

The ELISA test based on the monoclonal antibody R5, which was suggested by Codex Alimentarius (2005) for food analysis, was used to quantify the antigenic properties of gliadins possibly present in the black rice biscuits. After the ELISA analysis, it was found that the obtained value was below the acceptable limit of 20 mg/kg gluten, indicating the absence of the agglutination of the potential allergenic proteins in the aperitif biscuits. According to the ELISA analysis, the aperitif biscuits can be successfully categorized as a gluten-free product.

7.5.5. Colorimetric determination results

The values of the color index L * a * b * measured with the HuniscLab Miniscan XEPlus tool are shown in Table 7.3. In the CIE color space L * a * b * the rectangular color coordinates are: L - brightness coordinate: 0 (black) to 100 (white); a * - the red / green coordinate, with +a * indicating red and -a* indicating green; b * - yellow / blue coordinate, with + b * indicating yellow and -b * indicating blue. The values of the colorimetric indices a * and b * indicated that the aglutenic biscuits have the color of red-orange and yellow, whose intensity gradually decreased during storage. The brightness (L *) remained constant over the entire shelf life of the black rice biscuits.

Table 7.3. Colorimetric indicators of the aglutenic aperitif biscuits

Indices	Initial	After 7 day	After 14 day	After 21 day
L*	39.14±0.36	39.20±0.21	39.24±0.13	39.26±0.02
a*	7.63±0.11	7.41±0.04	7.14±0.11	7.05±0.05
b*	14.26±0.16	13.04±0.08	11.23±0.06	9.52±0.07

From Table 7.3, it appears that the values of the L *, a * and b * colorimetric indices did not vary greatly over the 21 days, which means that the aglutenic biscuits retained their color throughout their shelf-life.

7.5.6. Microbiological analysis

The microbiological analysis of the total number of yeasts and molds was done according to SR ISO 21527/1. The procedure showed that the aglutenic aperitif biscuits were microbiologically safe until the end of the 21-days storage period. Based on these results it can be appreciated that the parameters used for baking were adequate, causing the destruction of all microorganisms. Thus, the functional product intended for people with food intolerances (gluten-free biscuits) was microbilogically safe.

Table 7.4. Microbiological and hygiene criteria for the functional product for people with food intolerances (gluten-free aperitif biscuits)

Product	Microorganisms	Initial	After 7 day	After 14 day	After 21 day
Biscuits	Yeasts and molds	Absent	Absent	Absent	Absent

7.5.7. Sensorial analyses

In deciding whether to accept or reject food, sensorial qualities have a particularly important role, influencing the consumer's reaction to food. The characterization of the flavor and taste of a product is only possible through sensorial analysis.

The sensorial analysis results of the aperitif biscuits are presented in Figures 7.3 and 7.4.

The quality of a product represents all the features capable of meeting the needs of consumers. Sensorial analysis is part of the concept of quality and it is the method in which a product is examined based on the basic human sensibilities such as hearing, sight, taste and smell. The methods of sensorial analysis may be objective and subjective. The subjective method is the hedonic analysis or the preference test that measures the appreciation of a product by "naive" consumers while the objective method is the sensorial analysis performed in the laboratory by a group of trained experts. Regarding the sensorial profile of gluten-free biscuits, it can be seen from Figure 7.3 that during tasting the smell, flavour, aftertaste and taste were perceived by the panhelists to a larger extent, giving them a high score . Instead, the mouthfeel perceived by the panelists scored lower than the other analyzed sensorial characteristics.

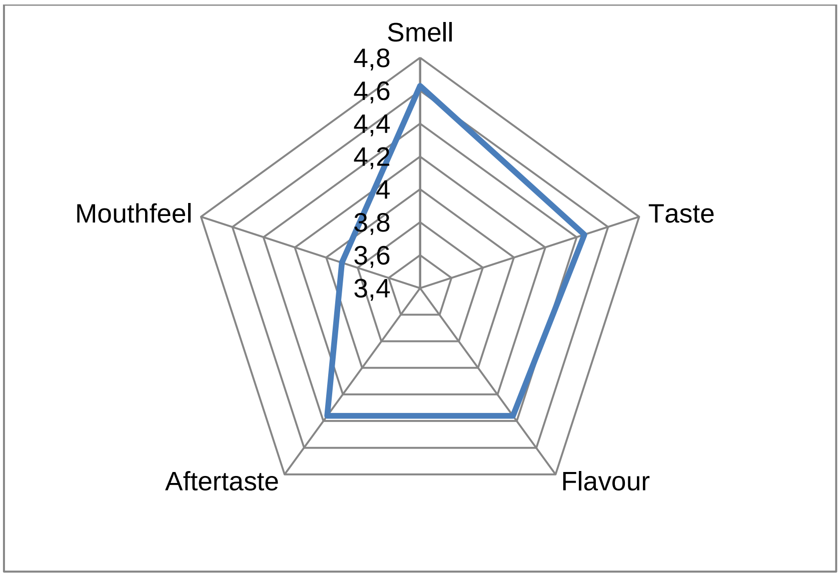


Figure 7.3. The sensorial profile of the functional product for people with food intolerances (aglutenic aperitif biscuits)

According to the results presented in Figure 7.4, it was observed that the cross-sectional appearance resulting from the cracking of the biscuits in two had a high score as well as their thickness. Instead, the other sensorial attributes had a lower score given by the panellists because the biscuits did not have a smooth surface, being very thin and crispy.

Based on the results of the sensorial analysis, it can be appreciated that the agulutenic aperitif biscuits were highly appreciated by the panellists, especially because of their taste and satiety degree.

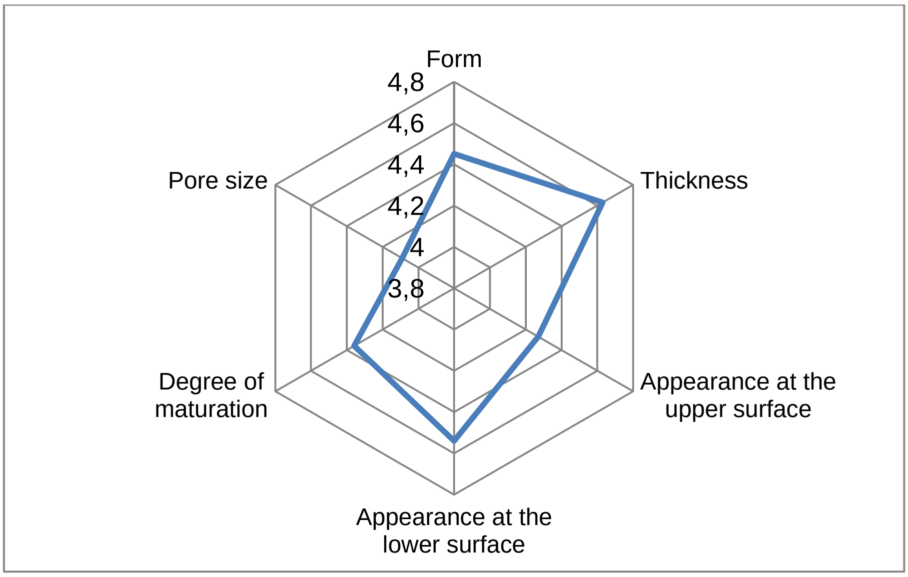


Figure 7.4. The sensorial image of the functional product for people with food intolerances (aglutenic aperitif biscuits) in terms of appearance and section appearance

7.6. Partial conclusions

Following the research on the preparation and characterization of the functional product intended for people with food intolerances (aglutenic aperitif biscuits), the following conclusions can be drawn from the physico-chemical, microbiological and sensorial characterization:

- It was possible to develop a recipe and a technological scheme for the preparation of gluten-free biscuits from black rice;
- All the ingredients used in the making of these biscuits come with a high intake of proteins, fibers, biologically active compounds that confer numerous beneficial effects to the human body;
- The obtained product was analyzed from a physico-chemical, microbiological and sensorial point of view;
- The results of the physico-chemical and microbiological analysis was in accordance to the Specific Standards;
- Aperitif biscuits based on black rice have a high protein and fiber content;
- The functionality of gluten-free biscuits is also given by the high content of biologically active compounds having an effect on sanogenesis;
- In *vitro digestibility* studies have highlighted that the complex matrix of biscuits allowed the protection of anthocyanins compounds in the gastric juice, allowing their gradual release into the intestinal juice;
- Appetizer biscuits based on black rice presented no antigenic potential and could be categorized as gluten-free products;
- This category of biscuits can address a wide range of consumers but especially those who are intolerant to gluten (celiac disease);
- The microbiological tests have shown the stability of the product during storage for 21 days; yeasts and molds were absent, and thus the biscuits were considered safe for consumption;
- The product was manufactured without the use of synthetic additives or genetically modified ingredients, being in harmony with the nature and the environment. As a result of the ecological principles, complementary additives, synthetic chemical products, artificial flavors, artificial colors and all genetically modified organisms were excluded in the manufacture of these innovative biscuits;
- The used package can be reused or recycled and it was proposed for the packaging of the functional product intended for people with food intolerances (aglutenic aperitif biscuits);
- The raw materials, especially black rice flour, the ingredient that has been emphasized in this paper, are considered to be functional ingredients;
- This type of biscuits can be sold in eco and bio stores and can be consumed by all categories of consumers at any time of the day.

8. General conclusions

The doctoral thesis study aimed at the advanced characterization of the milled black rice in order to obtain a functional product for people with food intolerances. Based on the obtained results of the experiments and the partial conclusions highlighted at the end of each chapter, a series of general conclusions are presented as follows:

Due to the high nutrient content, black rice (*Oryza sativa* L.) has become increasingly appreciated by consumers and researchers. The high content of biologically active compounds of black rice provides various sanogenic properties to this raw material.

The black rice has been shredded and fractionated by sifting through sives of different sizes. The resulting black rice flour was shown to be suitable for the use in various food formulas. The physico-chemical analysis highlighted the increase of the protein and lipid content with the reduction of the particle size of the sifted flour fractions. The carried out investigations provided valuable information on the predominant distribution of the black rice grain components in different fractions resulting from sifting.

The determination of the total anthocyanins content, total polyphenols content, total flavonoids and the determination of the antioxidant activity of black rice flour fractions was performed by a spectrophotometric method at different wavelengths (520-700 nm, 510 nm, 765 nm and 515 nm nm).

Black rice flour is a good source of biologically active compounds. By sifting the black rice flour seven fractions of flour resulted with different particle sizes, which were characterized in terms of the biologically active compounds distribution. Significant differences were observed between the various fractions of black rice flour in terms of the anthocyanins and phenolic acids content but also regarding the antioxidant properties. Of the seven fractions of black rice flour, the fourth fraction displayed the highest amount of anthocyanins and polyphenols, while the flavonoids content and the antioxidant activity did not vary significantly from one fraction to another. It can be therefore appreciated that the high DPPH antioxidant activity was due to the content of flavonoids, and also due to polyphenols and anthocyanins.

The HPLC method revealed the presence of two anthocyanins, namely cyanidin-3-glucoside (in the largest amount) and peonidine-3-glucoside in both whole flour and fraction F4.
The results on the content of the biologically active compounds and their distribution could help rice producers and food industry specialists to better promote the consumption of rice products by increasing the consumer awareness regarding the health benefits.

The effect of temperature on the process of the degradation of phenolic compounds in the black rice flour extract was examined based on a kinetic study. The degradation rate constants showed that as the temperature increased the degradation process also intensified and this was expressed by the activation energy values (*Ea*). The thermal degradation constants, constant k and the decimal reduction time D, of the biologically active compounds from the black rice flour fractions were calculated after applying a thermal treatment.

The results obtained after applying the thermal treatment suggested that anthocyanins are unstable at high temperatures, this being evidenced by the increase of the fluorescence intensity and the presence of red and blue shifts, wavelengths at which the maximum fluorescence intensity was recorded. The thermal treatment at high temperatures led to the degradation of phenolic compounds from the black rice flour fractions.

The studies have both fundamental and applicative research value. The results of the fundamental research carried out in the first part of the study were exploated to develop a new product based on black rice. In this respect, the recipe and the technology for obtaining the aglutenic aperitif biscuits were achieved and optimized. The final product was analyzed physiochemically, microbiologically and sensorially. The ingredients used to make the aperitif biscuits provided a high content of proteins, fibers and biologically active compounds of the product, which can be considered a functional product including for those suffering from allergy or intolerance to gluten. The aperitif biscuits are safe-to-eat products that retained their properties for a 21-days storage period at room temperature.

9. Contributions to field knowledge development and future perspectives

The research presented in the PhD thesis includes both a fundamental and an applicative part, that contain several original contributions, as follows:

The fundamental research study aimed at the black rice flour advanced characterization, especially in terms of its biologically active compounds content. The originality of the studies derives from the chosen approach to conduct the investigations which aimed to highligth the functionality of black rice. Thus, after grinding the black rice, based on the particle size different fractions of flour were used to separate the biologically active compounds and the protein fractions.

Modern techniques such as fluorescence spectroscopy were used to investigate the effect of temperature and pH on the conformational and structural modifications of the biologically active compounds molecules from black rice flour.

The HPLC technique was used to identify the major anthocyanins compounds present in the black rice flour.

The effect of heat treatment on the major protein fractions of black rice was also investigated.

The results on the thermal treatment stability of the biologically active compounds and proteins allow the processing of black rice flour in order to obtain food products that do not present significant biological potential reductions.

The results of the fundamental research were emphasized in order to develop an innovative functional product which was sent for patenting at OSIM. Furthermore, to produce the aperitif biscuits based on black rice, a technology process has been proposed. The obtained product does not contain gluten and is rich in biologically active compounds so that it can be considered a functional food.

The obtained data represents the reference for future research with significant scientific and applicative importance in understanding the conformational changes that resulted from the thermal treatments applied during processing. This type of analysis allows the obtainment of high-functionality food products.

In the future, the studies will be carried out to further exploit the biologically activity and/or the technological potential of some fractions or extracts from black rice flour by including them in different food matrices. Several products such as dairy products (yoghurt, cheese) and products obtained by alcoholic fermentation (beer) will be considered.

10. Dissemination of research results

The dissemination of the research results during the doctoral studies was materialized through the publication or communication of several scientific papers as follows:

Published articles in ISI journals

1. **Carmen Bolea**, Mihaela Turturică, Nicoleta Stănciuc, Camelia Vizireanu. 2016. Thermal degradation kinetics of bioactive compounds from black rice flour (*Oryza sativa* L.) extracts. Journal of Cereal Science. 71,160-166. http://www.sciencedirect.com/science/article/pii/S0733521016301655, (**2,223**).
2. Iuliana Aprodu, **Carmen Bolea**, Nicoleta Stănciuc, Livia Pătrașcu. 2017. Structural and antigenic properties of thermally treated gluten proteins. Food Chemistry. http://dx.doi.org/10.1016/j.foodchem.2017.03.018, (**4,529**).
3. **Carmen Bolea**, Leontina Gurgu (Grigore), Iuliana Aprodu, Camelia vizireanu, Nicoleta Stănciuc. 2018. Comparison of functional and structural heat induced properties of black rice flour milling fractions. Submited at Journal of Cereal Science, YJCRS_2018_322, (**2,223**).

Published articles in international database journals

1. **Carmen Bolea**, Camelia Vizireanu. 2017. Polyphenolic content and antioxidant properties of black rice flour. The Annals of the University Dunărea de Jos of Galați Fscicle VI-Food Technology, 41(2), 75-85.
2. **Carmen Bolea,** Iuliana Aprodu, Iuliana Banu. 2018. Impact of multigrain milling on the chemical profile of the mill streams. Scientific Study & Research – Chemistry & Chemical Engineering, Biotechnology, Food Industry (SCS-CICBIA), vol. 19, nr. 1, 2018.

Papers communicated at international scientific conferences

1. **Carmen Bolea**, Iuliana Aprodu, Iuliana Banu, Gabriela Bahrim, Camelia Vizireanu, Effect of Thermal Treatment on Phenolic Compounds from Black Rice. European Biotechnology Congress, May, 7th – 9th, 2015. Bucharest, România.
2. **Carmen Bolea**, Camelia Vizireanu, Iuliana Banu, Thermal Degradation of Some Bioactive Compounds from Black Rice Flour. The 7th International EuroAliment Symposium, September 24th – 26th, 2015, Galați, România.
3. **Carmen Bolea**, Iuliana Aprodu, Iuliana Banu, Comparative analysis of enzyme activity in wheat, rye, triticale, oat and barley. European Biotechnology Congress, May, 25th – 27th, 2017, Croația.
4. **Carmen Bolea**, Camelia Vizireanu, Iuliana Aprodu, Leontina Grurgu-Grigore, Nicoleta Stănciu, Camelia Vizireanu, Conformational changes and protein content of sifted black rice flour fractions. The 8th International EuroAliment Symposium, Octomber 7th – 8th, 2017, Galați, România

Papers communicated at national scientific conferences

1. **Carmen Bolea**, Camelia Vizireanu, Physico-chemical characterization of black rice used to obtain functional food. Scientific Conference of Doctoral Schools from UDJ – Galaţi CSSD-UDJG, May 15th-16th, 2014, Galați, România.

2. **Carmen Bolea**, Camelia Vizireanu, The Impact of Thermal Treatment on Phenolic Content of Black Rice Anthocyanins. Scientific Conference of Doctoral Schools from UDJ – Galaţi CSSD-UDJG, June 4th-5th, 2015, Galați, România.
3. **Carmen Bolea**, Nicoleta Stănciuc, Camelia Vizireanu, Thermal degradation effect on bioactive compounds from the black rice extracts (Oriza sativa L.). Scientific Conference of Doctoral Schools from UDJ – Galaţi CSSD-UDJG, June 2th-3th, 2016, Galați, România.
4. **Carmen Bolea**, Camelia Vizireanu, Functional Product for People with Food Intolerances. Scientific Conference of Doctoral Schools from UDJ – Galaţi CSSD-UDJG, June 8th-9th, 2017, Galați, România.
5. **Carmen Bolea**, Turturică Mihaela, Livia Pătrașcu, Iuliana Banu, Nicoleta Stănciuc, Iuliana Aprodu, Investigation on the Stability of Soy Proteins under Different Processing Condition. Scientific Conference of Doctoral Schools from UDJ – Galaţi CSSD-UDJG, June 8th-9th, 2017, Galați, România.

Patent

1. **Carmen Bolea**, Camelia Vizireanu, Romulus Burluc, Daniela Istrati. 2017. Dough composition for aglutenic aperitif biscuits. A/00158.

Other publications

1. Iuliana Aprodu, **Carmen Alina Bolea**, Irina Ştefănescu, Iuliana Banu. 2017. Influence of tempering moisture on the milling potential of some cereals. The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology, 41(2), 21-30.