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The optimization of antioxidants extraction from winery waste

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Abstract
Winery waste serves as a valuable source of bioactive compounds with notable antioxidant properties and significant antiradical activity. The concentration of ethyl alcohol in the extraction medium plays a crucial role in influencing both the extraction rate and the yield of bioactive compounds, as well as affecting key parameters such as: the antioxidant activity of water-soluble and fat-soluble substances, the total anthocyanin content, the total polyphenol index, the tannin content, DPPH antiradical activity, and the ability to inhibit hydrogen peroxide in both acidic and basic environments. Based on the experimental data and applying appropriate calculation methods, first-order statistical characteristics were determined. The analysis revealed non-linear dependencies, leading to the use of spline functions for developing mathematical models. This evaluation of the experimental data provides insights into the influence of various measured parameters during the trials.

Keywords: winery waste, extracts, antioxidants, statistical characteristics, mathematical models.

1. Introduction

The continuous progress of human society has led to the ubiquity of technology in all areas. Nowadays, food is produced today using technologies and modern procedures, and the growing demand leads to overproduction and a wide offer provided by food producers. Thus, everything is made for food to be successful, so as to be preserved for longer periods, to have more attractive colors, to contain more vitamins even if they are artificially produced [1]. Human nutrition in modern society is increasingly rich in macronutrients and lacks micronutrients and biologically active substances. Plant material is vital for the survival of the entire population, serving as a source of aromatic, pharmaceutical, and industrial compounds, as well as biologically active substances [2, 3, 4]. These natural and biologically active substances can not be synthesized by the human body, therefore they must be consumed daily. Otherwise, any attack on the human body (infectious, toxic, mechanic etc.) initiates the beginning of free radical reactions [5]. Free radicals (oxidants and active forms of oxygen) are incomplete structures that do not have enough electrons and this deficit is trying to get compensated with the electrons of neighboring molecules, i.e. a reaction called oxidation starts. As a result, the destruction of cell membranes, cells or entirely separated tissues occurs [6]. Incorporating natural antioxidants into the diet, such as phenolic compounds, carotenoids, vitamins, minerals, and other pigments, helps neutralize the effects of reactive oxygen species. Numerous clinical and epidemiological studies suggest an inverse relationship between the risk of developing cardiovascular diseases and cancer and the consumption of foods rich in bioactive compounds with antioxidant properties. The diversity

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of natural polyphenols and their health benefits continue to be a subject of extensive research [10, 11]. In the Republic of Moldova and Romania viticulture and wine industry is well developed. Several ancient Greek philosophers praised the healing power of grapes, usually in the form of wine [12].

It has been demonstrated that grape phenolic compounds, such as hydroxy acids (caffeic, coumaric, ferulic, caftaric, cutaric), flavanols (catechin, epicatechin) and flavonols (quercetin) [10,13], possess health-enhancing properties [14,15]. These compounds play a key role in inhibiting carcino-genesis, mutagenesis, and cardiovascular diseases, and have been shown to help prevent peptic ulcers[16], various forms of diabetes [17], and even aid in lipid absorption [18]. Their antioxidant properties have been linked to these benefits in both in vivo and in vitro studies [19, 20]. In vivo research indicates that proanthocyanidins from grape seeds offer stronger protection against lipid peroxidation and DNA fragmentation than vitamins E, C, and β-carotene in mice [20]. During grape processing, approximately 20-25% of the material is converted into valuable wine products, including secondary by-products like pomace and grape seeds, which are rich in polyphenols [21, 22, 23].

At present, in the Republic of Moldova and Romania there are no factories which would apply a complex technology to exploit the secondary wine products. That is why the implementation of efficient technologies for recycling and treatment of secondary wine products, with low consumption of energy and materials is important [24]. The experimental research developed to study the process of extraction of antioxidants from winery waste cannot be in an arbitrarily large number of different reasons, including economic ones. For this reason, the theoretical study of this process has been established by using a mathematical model, an algorithm which describes the evolution in time or after certain mutual dependency between parameters [25].

The aim of this work is to optimize the parameters for the extraction process of some compounds with antioxidant capacity from winery waste. The analysis is based on analytical methods used for the characterization of extracts by determining important indices as: total content of polyphenols (anthocyanins, tannins), determination of the antioxidant activity and the antiradical activity.

2. Materials and method

To obtain the extracts, locally sourced dried red grape winery waste was ground into powder and sifted. This powder was then subjected to extraction using ethanol in hydro-alcoholic solutions of varying concentrations: 20%, 40%, 50%, 60%, 80%, and 96%. The extraction process was carried out with a solid-to-liquid ratio of 1:8 for one hour in a dark environment at room temperature. The resulting extracts were filtered and stored in dark-colored containers. The winery waste extract samples were kept in a dark place at a temperature of 4±1°C. Various indices were measured in the winery waste extracts, and these indices, along with their symbols and notations, are listed in Table 1.

Table 1. Following indices were determined in the extracts of winery waste.

Symbol	Description
P1 (AASH)	The antioxidant activity of water soluble substances, C_{AA},
P2 (AASL)	The antioxidant activity of fat-soluble substances, TROLOX
P3 (CTA)	The total content of anthocyanins
P4 (IPT)	The index of total polyphenols
P5 (AAA)	The antiradical activity, DPPH, in the acidic medium
P6 (AAB)	The antiradical activity, DPPH, in the basic medium
P7 (CIPHA)	The ability to inhibit hydrogen peroxide in an acid medium
P8 (CIPHB)	The ability to inhibit hydrogen peroxide in basic medium
P9 (CTE)	The amount of extracted tannins

Based on the experimental data and corresponding calculations, first-order statistical characteristics were determined, including means, variance, standard deviation (r-squared), minimum, maximum, and median values [26, 27]. The analysis revealed non-linear dependencies, which led to the use of spline functions for developing mathematical models. This examination of the experimental data enables the assessment of the impact of various measured parameters during the tests [28, 29].

Determination of the amount of tannins means that all phenolic components are oxidized by Folin-Ciocalteu reagent.

The colour intensity is proportional to the content of phenolic compounds. The colour intensity is determined spectrophotometrically at λ=750 nm, and the content of phenolic substances is determined from the calibration curve. [30].

Index of total polyphenols (IPT) or **D_{280}** index is a parameter describing the contents of total phenolic compounds (phenolic acids, tannins, anthocyanins, flavones, etc.) in the extract. The principle of the method for determining the D_{280} index is based on the strong absorption of ultraviolet light by the benzene nucleous, characteristic for phenolic compounds, attaining a maximum absorption at the wavelength of λ= 275-280 nm [30].

Determination of the total amount of anthocyanins - pH variation method for the extracts is applied and examined by correlating the difference in intensity of coloring with the variation of pH. The method is based on the following principle: in the acidic environment there is a balance between colored and colorless forms of anthocyanins; this balance depends on the pH (0.6 and 3.5) and the variation of coloring intensity between two pH values is proportional to the content of anthocyanins. The absorbance (optical density) at λ=520 nm is measured for both pH values. The calculating equation is inferred from a calibration curve [30].

Photochemluminescence PCL method. Photochem method of quantifying the antioxidant capacity of the extracts includes the determination of antioxidant capacity of water-soluble compounds (ACW) and the determination of antioxidant capacity of lipid-soluble compounds (ACL) [30].

Antioxidant action of soluble compounds in water (ACW)-principle of the method: free radicals (superoxide anion radicals) are produced by optical excitation (irradiation) of a photosensitive substance. These radicals are eliminated by the reaction with the antioxidants present in the sample - wine in our case. The remaining radicals are determined due to the luminescence which they produce in the cell of the measuring apparatus. The antioxidant capacity of the sample is quantified by comparing to a standard (using a calibration curve with ascorbic acid) and is given in equivalent units of the standard.

Antioxidant action of lipids soluble compounds (ACL). is assessed by evaluating their ability to neutralize free radicals, such as superoxide anion radicals, which are generated through the optical excitation (irradiation) of a photosensitive substance. In this process, these radicals partially react with the antioxidants present in the wine sample. The remaining radicals are detected based on the luminescence they emit in the measuring apparatus. The antioxidant capacity of the sample is quantified by comparing it to a standard (using TROLOX scale calibration) and is expressed in equivalent units of this standard.

Antiradical activity using free radicals DPPH•. The spectrophotometric method with free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl) is based on the decrease in absorbance of the radical in the presence of antioxidants. DPPH • is characterized as a stable radical, due to the delocalization of the unpaired electron over the entire molecule. The delocalization of the unpaired electron results in the appearance of the violet color which forms an absorption band with a maximum located at about 520 nm. In this work, the investigations were carried out at a wavelength λ=515 nm [31].

Antioxidant activity (HPSA). The method for determining the capacity of inhibiting the hydrogen peroxide peroxide is estimated by titration through the method of substitution (the analytical solution does not react directly, so it is transformed into a chemical combination which can then be titrated with the solution of known concentration) [32].

For the statistical processing the **Matlab** program was used.

3. Results and discussion

The results obtained from the extracts of winery waste depending on the concentration of ethyl alcohol are presented in the table 2.

The total content of tannins extracted from the winery waste ranges from 7.81 mg/3g and 11.36 mg/3g. The highest value was recorded in the extract obtained with 50% alcohol solution. Another value close to the maximum concentration is observed at 60%. The amount of tannins extracted with ethyl alcohol solution of 50% is 1.45 times higher than the one extracted with the solution of ethyl alcohol 96% and 1.26 times higher than the one extracted with 20% ethyl alcohol solution. Different studies suggest that the alcoholic concentration of 50% favors the extraction process; this hydro-alcoholic solution has the maximum of extractive power and higher selective capacity [12].

The index of total polyphenols from wine waste samples varies between 6.47 and 19.00, the highest value being recorded at the alcoholic content of 50%. The amount of polyphenols extracted differs in function of the concentration of the alcohol used. Thus, the polyphenols in winery waste extract is extracted better at the concentration in the range of 40-60%

Table 2. The scientific results obtained from the extracts of winery waste depending on the concentration of ethyl alcohol

Concentration of ethyl alcohol, %	Amount of tannins, mg/3g	Index of total polyphenols	Total quantity of anthocyanins mg/3g	Antioxidant activity of lipid-soluble substances, TROLOX, mg/3g	Antioxidant activity of water soluble substances, mg/3g
20	9.00±0.05	6.47±0.23	0.11±0.01	4.46±0.12	0.08±0.01
40	10.33±0.05	11.83±0.33	0.75±0.02	5.71±0.06	0.23±0.01
50	11.36±0.06	19.00±0.30	0.86±0.02	3.45±0.02	0.19±0.01
60	11.09±0.06	13.50±0.50	1.29±0.02	5.59±0.07	0.39±0.02
80	10.35±0.03	8.30±0.20	0.82±0.01	4.09±0.08	0.21±0.01
96	7.81±0.02	8.7±0.20	0.09±0.01	1.83±0.04	0.10±0.01

The amount of polyphenols extracted with ethyl alcohol solution of 50% is 2.94 times higher than the one extracted with ethyl alcohol 20% and 2.29 times higher than the one extracted with ethyl alcohol of 80%.

By analyzing the data, the anthocyanin content in extracts from the six different ethyl alcohol concentrations was determined, with values ranging from 0.09 mg/3g to 1.29 mg/3g. The highest anthocyanin content was found in the extract with 60% ethyl alcohol, followed by the 50% concentration. The lowest anthocyanin content, 0.09 mg/3g, was observed with 96% ethyl alcohol. The quantity of anthocyanins extracted with 60% ethyl alcohol is 14.33 times greater than that extracted with 96% ethyl alcohol and 11.73 times greater than that extracted with 20% ethyl alcohol.

The study of the influence of ethyl alcohol concentration on the antioxidant activity of water soluble substances extracted from winery waste emphasized the fact that ethanol concentrations in the range of 40% - 80% values ranged between 0.21 mg/3g and 0.39 mg/3g. The highest antioxidant activity of water soluble substances extracted from winery waste constitutes 0.39 mg/3g with 60% ethyl alcohol. The lowest antioxidant activity of water-soluble substances extracted from winery waste constitutes 0.08 mg/3g, in extracts obtained with distilled water followed by those extracts obtained with ethyl alcohol 96%-0.10 mg/3g.

The influence of alcoholic concentration on the antioxidant activity of fat-soluble substances is more highlighted. In the range of ethanol concentrations 20%-96% the values ranged between 1.83 mg/3 g and 5.59 mg/3 g. The highest antioxidant activity of fat-soluble substances extracted from winery waste constitutes 5.59 mg/3g with 60% ethyl alcohol. The lowest antioxidant activity of fat-soluble substances extracted from winery waste constitutes 1.83 mg/3g with ethyl alcohol 96%. The antioxidant activity of soluble substances extracted from the winery waste with ethyl alcohol solution of 60% is 3.05 times higher than those extracted with 96% ethanol and 1.25 times higher than those extracted with distilled water.

The antiradical and the antioxidant activity of the hydro-alcoholic extracts of winery wastes were determined at different pH: acid - 2.00 ± 0.03, and basic - 8 ± 0.1, because the human body pH varies between 1.2-3.0 for gastric juice and 7.6-8.6 for biliary secretion.[33] Thus, the influence of pH and concentration of ethanol on the antiradical and antioxidant activity of hydro-alcoholic extracts have been determined.

As shown in Table 3, the antiradical activity determined by DPPH• for hydro-alcoholic extracts from winery waste reveals the highest values of 92.65 - 90.80% (basic medium) and 89.77 - 82.45% (acid environment) of DPPH• radical inhibition in the extracts obtained with a concentration of 96 to 50% ethyl alcohol in the basic environment and 96 to 20% concentration of alcohol in the acidic environment. In the case of antioxidant activity, it can be mentioned that the percentage of hydrogen peroxide inhibited in acidic medium differs little from the one in a basic medium. The highest percentage of hydrogen peroxide inhibition is observed in winery wastes extract solvent concentration 60% alc. (76.89%) and the lowest in the extract concentration of 20% (47.49%), in the case of acidic medium. On the other hand, in the basic environment, the highest percentage of inhibited hydrogen peroxide was obtained in the extract from the 80% solvent concentration (72.51%), and the lowest one in the extract with 50% alcohol concentration (37.47%).

Table 3. The antiradical and antioxidant activity of extracts from winery waste with various concentrations of solvent - ethanol in different pH environments

Concentration of ethyl alcohol, %	Antiradical activity, %		Antioxidant activity, %	
	Acidic medium, pH=2.00±0.03	Basic medium, pH=8.00±0.10	Acidic medium, pH=2.00±0.03	Basic medium, pH=8.00±0.10
20	82.45±0.45	85.84±0.43	47.49±1.03	49.57±0.40
40	80.00±0.01	90.10±0.02	53.87±0.63	52.81±0.22
50	78.65±0.53	90.80±0.52	52.64±0.39	37.47±0.40
60	75.03±0.65	89.66±0.60	76.89±0.38	70.74±0.47
80	82.25±0.11	86.22±0.10	69.73±0.28	72.51±0.21
96	89.77±0.30	92.65±0.27	46.97±0.76	46.97±0.26

The experimental data indicated in tables 2 and 3 represent discrete finite series which were obtained by 3 values of the 9 parameters at each concentration of ethyl alcohol (20%, 40%, 50%, 60%, 80%, and 96%). Figure 1 shows the values, the average and the dispersion of parameter P1 (antioxidant activity of soluble substances, C_{AA}, AASH).

The graph in figure 1 reveals that the lowest values of P1 are at low concentrations (20%) and at high levels (96%) of alcohol.

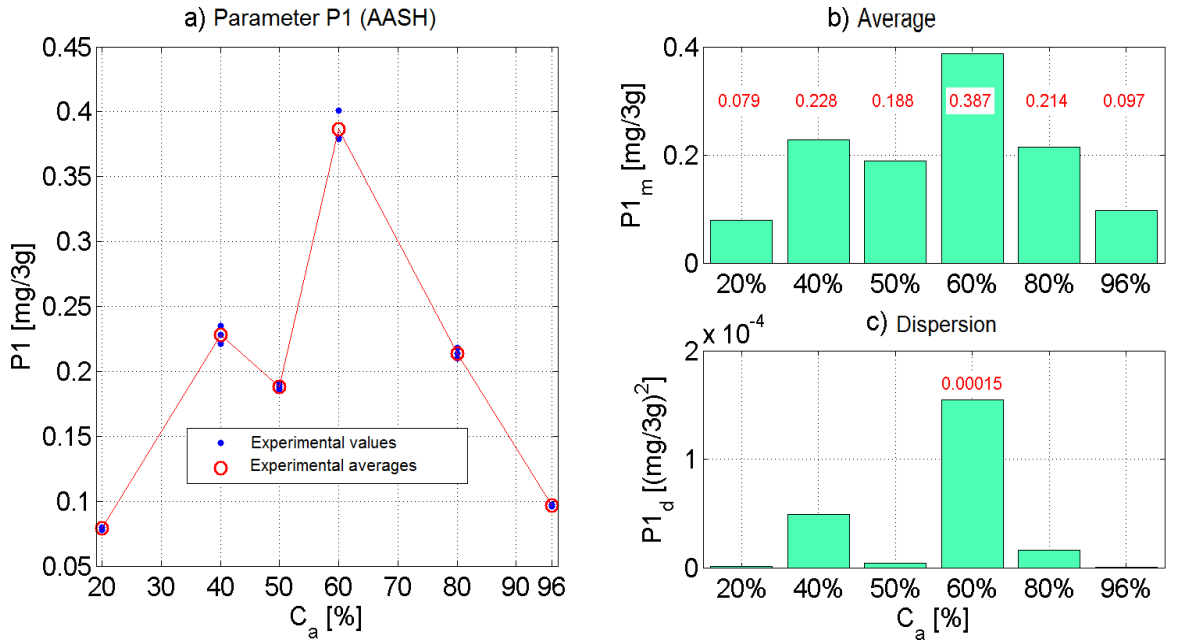

Figure 1. The values, the average and dispersion of P1 (antioxidant activity of water-soluble substances, AASH) winery waste, depending on ethanol concentration

Similarly, fig. 2 shows the values, the average and dispersion of parameter P2 (antioxidant activity of lipid-soluble substances, TROLOX, AASL) for winery waste, all for the 6 values of ethylic alcohol concentration. Fig. 3 also shows the values, the average and the dispersion parameter P3 (total content of anthocyanins, CTA) and, in fig. 4 of parameter P4 (index of total polyphenols, IPT).

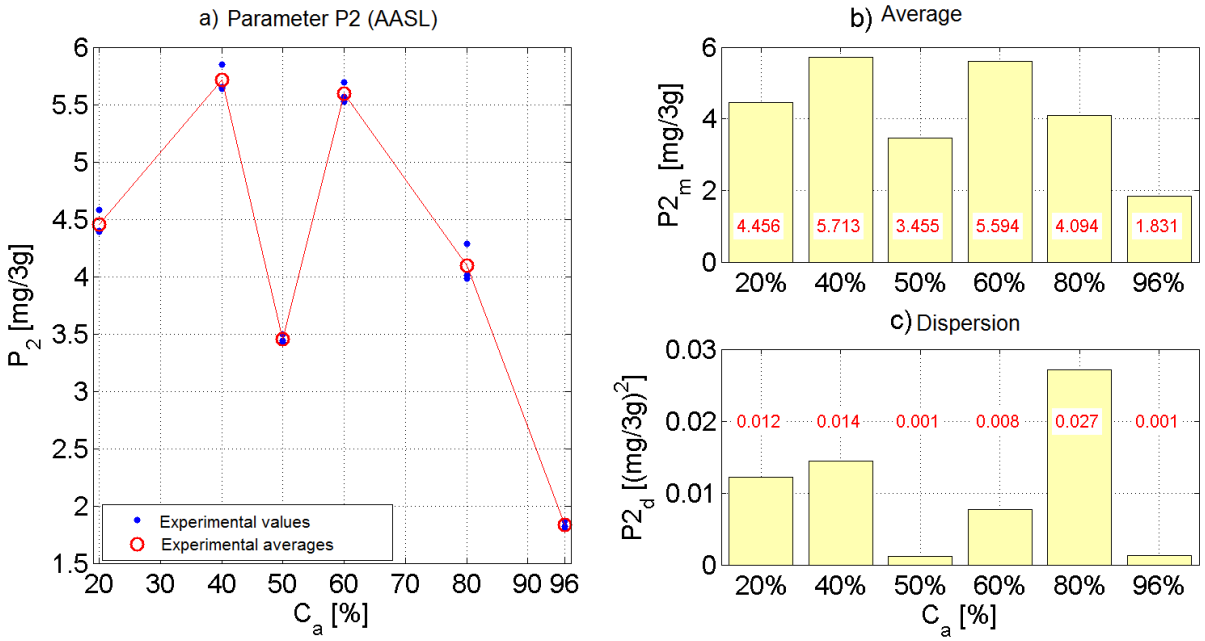

Figure 2. The values, the average and the dispersion of P2 (antioxidant activity of lipid-soluble substances, AASL) winery wastes, depending on the ethanol concentration.

The figure 3 and figure 4 show that the greatest values of P3 and P4 parameters are attested at the average concentrations of ethyl alcohol of 60% for P3, and 50% for P4, respectively.

In addition, the latter graphs show the existence of more or less non-linear dependencies between the concentration of ethyl alcohol and the parameters measured.

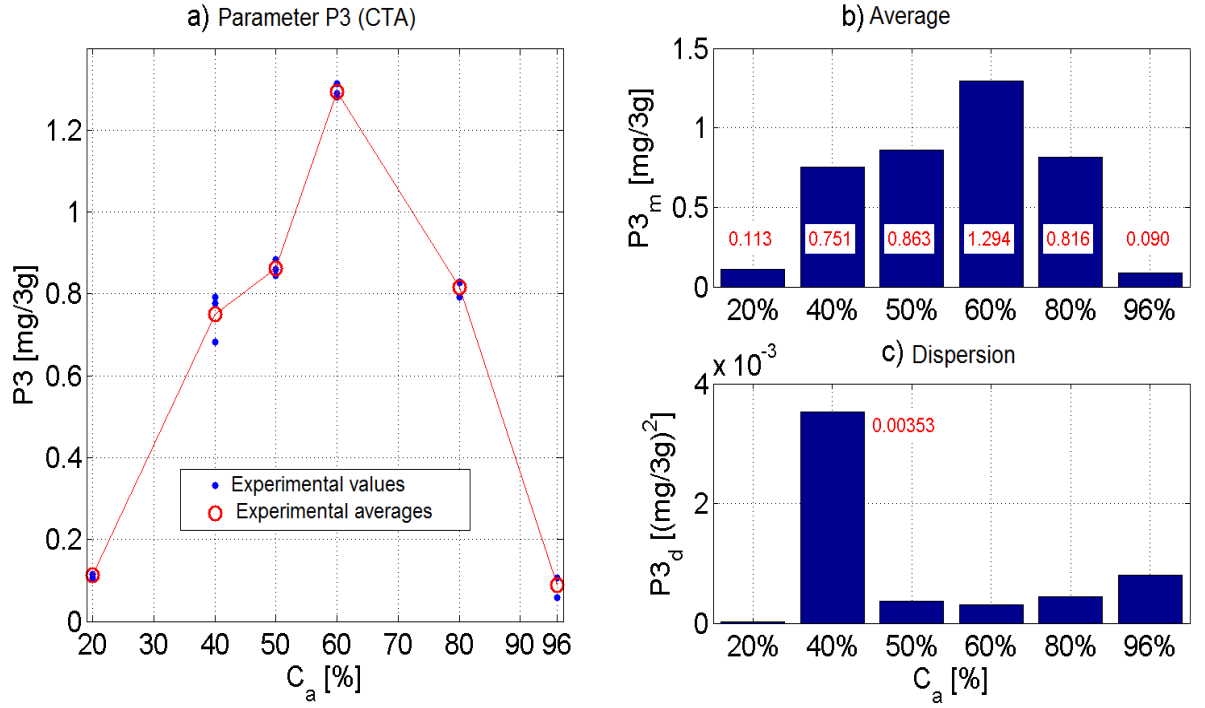

Figure 3. The values, the average and dispersion of P3 (total quantity of anthocyanins, CTA) winery waste, depending on ethanol concentration.

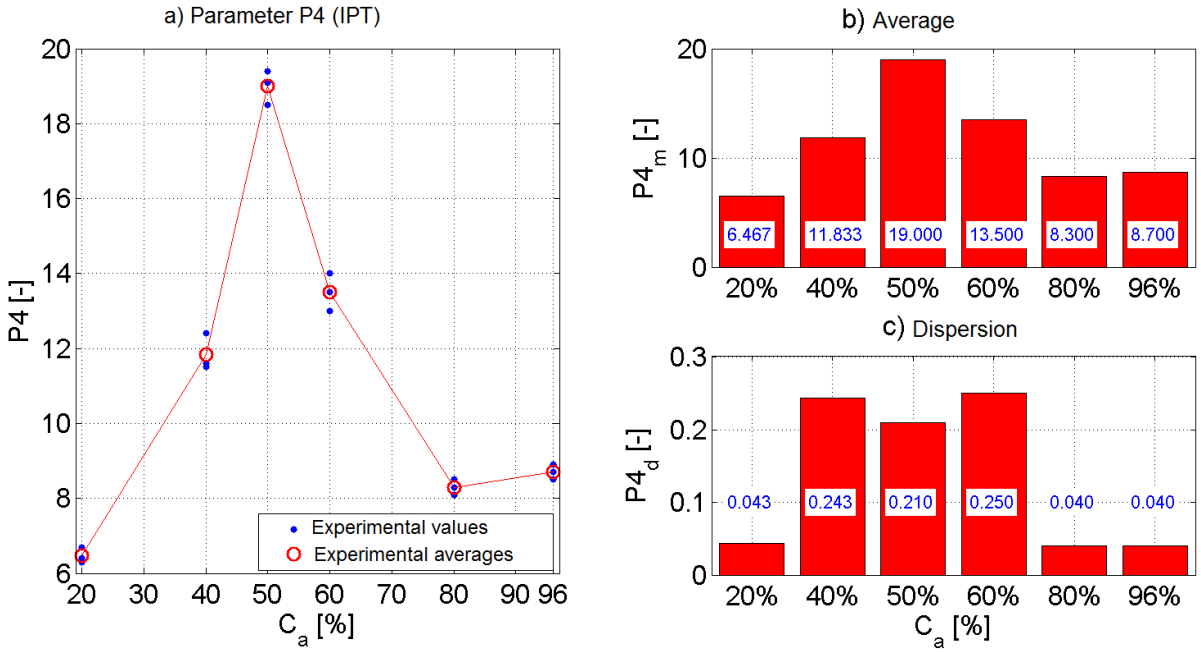

Figure 4. The values, the average and the dispersion of P4 (index of total polyphenols, IPT) winery waste, depending on ethanol concentration.

This is also confirmed by the graphs in figure 5 and figure 6 for the other four parameters from winery waste; as the next section will show later, such dependencies exist even between the parameters measured.

Figure 5 shows that the lowest values of the parameters P5 and P6 are at the concentration of 60% ethyl alcohol.

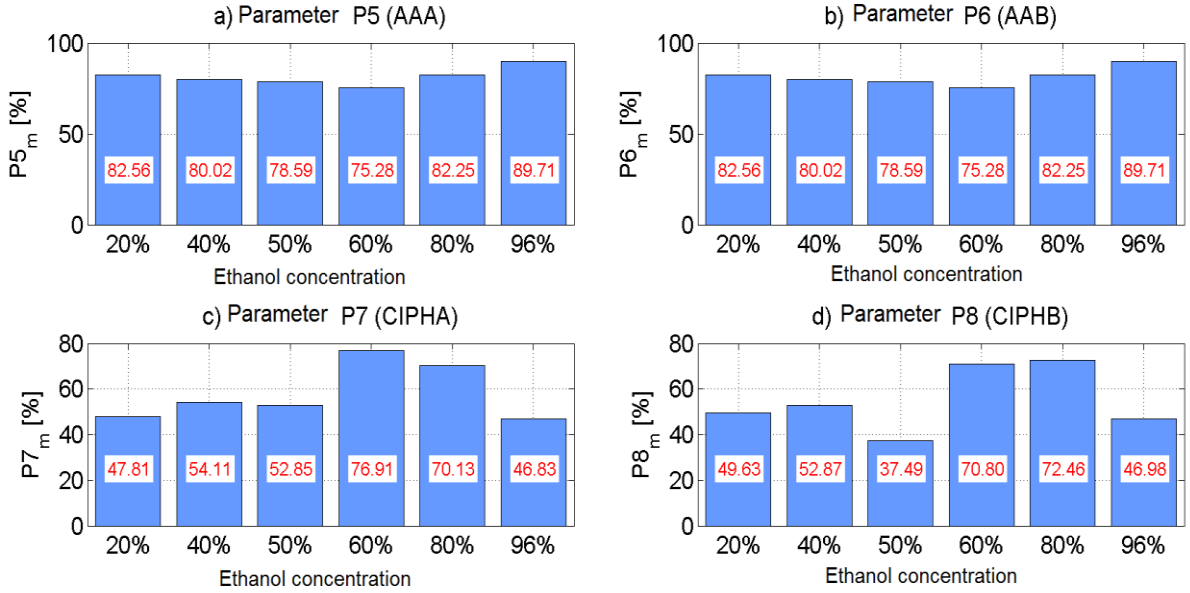

Figure 5. The averages of parameters P5-P8 winery waste, depending on ethanol concentration.

In fig. 6, the values of the standard deviation in the case of 9 parameters from winery waste are given (for 6 concentrations of ethyl alcohol). These graphs show the existence of different values of the standard deviation for various products, different parameters measured and different concentrations of ethyl alcohol, with implications on data analysis under the conditions of uncertainty and the implications of establishing mathematical models, as the next section will show.

The analysis of the information provided by experimental data in the tests allows the establishment of the influences of various indices measured during tests. The information analysis is based on two main concepts: entropy and information[34,35]. In the information theory, the unit of measurement of information and entropy is the bit, if it uses the base 2 logarithm of the previous expressions.

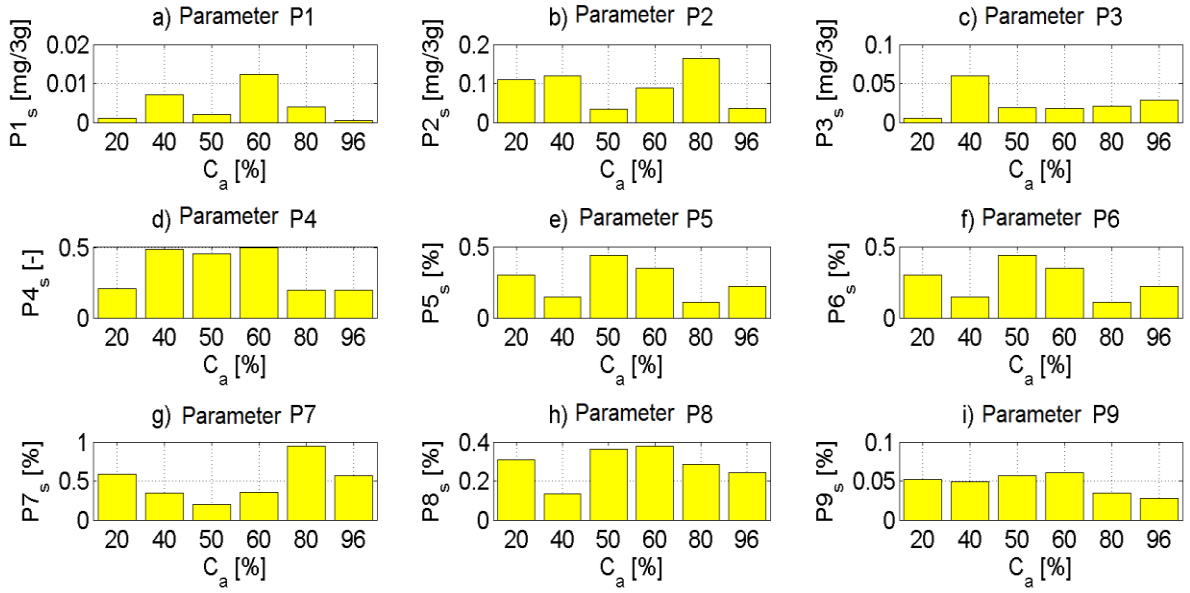

Figure 6. The values of the standard deviation for 9 parameters in winery waste.

The mutual information is a concept that provides a quantitative measure to reduce uncertainty, so to increase the level of prediction. The higher the values of the mutual information, the smaller the uncertainties are, therefore the higher is the degree of prediction. Consequently, the mutual information enables to establish the influence of ethyl alcohol concentration on the parameters measured. With regard to the information mentioned in the correlation analysis, it appears that mutual information ensures as well the establishment of the dependence between the factors measured.

Based on the experimental results, an information analysis[36] for winery waste was done and presented in Fig. 7.

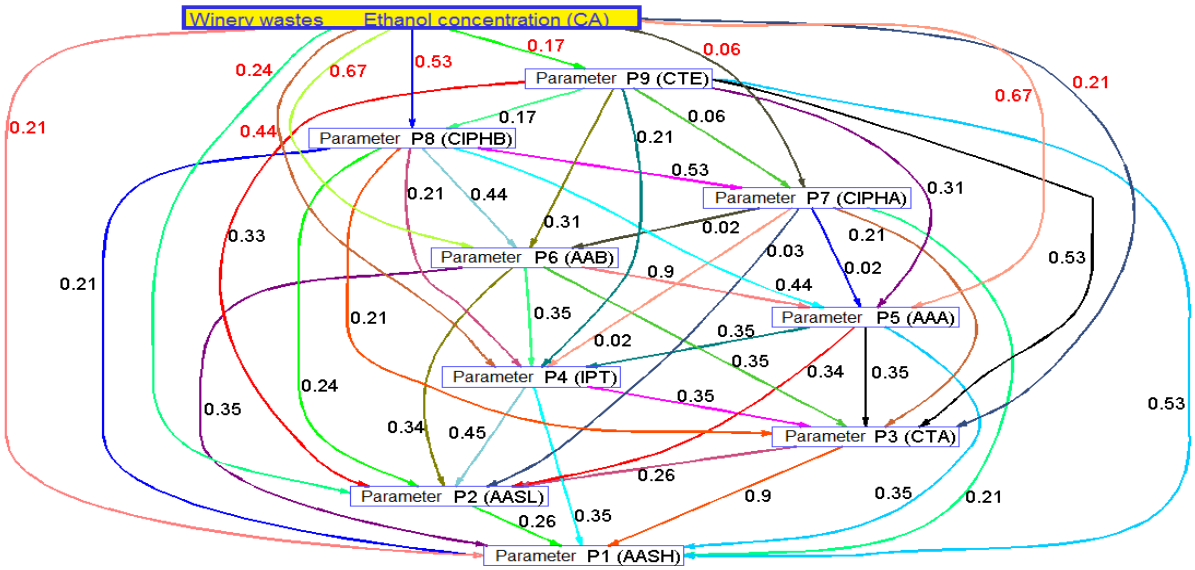

Figure 7. The graph with the results of informational analysis for winery waste.

The graph presents the factor of influence (the concentration of ethyl alcohol) at the top, and below this one, all the nine measured parameters are shown. The graph in fig. 7 highlights the fact that ethyl alcohol concentration influences to the greatest extent the P5 and P6 parameters (I = 0.67 bits). Next, in descending order of influence, the parameters P8 (I = 0.53 bits), P4 (s = 0.44 bits), P2 (I = 0.24 bits), P1 and P3 (I = 0.21 bit), P9 (I = 0.17 bit) and P7 (I = 0.06 bit). Therefore, in the case of winery waste, the concentration of ethyl alcohol has the greatest influence on the antiradical activity in the acidic environment (parameter P5) and on the antiradical activity in basic environment (P6), for both in equal measure; in contrast, the concentration of ethyl alcohol has the lowest influence on the ability of inhibition of hydrogen peroxide in the acidic environment (parameter P7). The graph in Fig. 7 also shows the dependencies between the parameters measured. For example, the largest mutual information is on the spring between P1 and P3 parameters, and the spring between the P5 and P6 (I = 0.9 bits). This means the dependence between the antioxidant activity of water-soluble substances (parameter P1) and the total content of anthocyanins (parameter P3) is higher than the one between the factor of influence CA (the concentration of ethyl alcohol) and the P5 and P6 parameters (s = 0.64 bits); the same conclusion also applies for the dependence between the antiradical activity in the acidic environment (parameter P5) and antiradical activity in the basic medium (P6).

The analysis of the trial data showed the existence of non-linear dependencies more or less pronounced; for this reason, the mathematical models are non-linear, i.e., polynomial models. It was appealed to the cubic Hermite polynomials. The Hermite polynomials are an important series of functions from orthogonal polynomials class as solutions of Hermite's differential equation. The general term of Hermite polynomials is used in probability theory [35]:

$$H_n(x) = (-1)^n e^{x^2/2} \frac{d}{dx^n} e^{-x^2/2} \quad (1)$$

The first 4 Hermite polynomials are:

$$H_0(x) = 1;\ H_1(x) = x;$$
$$H_2(x) = x^2 - 1; H_3(x) = x^3 - 3x \quad (2)$$

where H_n is a polynomial of degree *n*.

The mathematical models based on Hermite polynomials do not provide explicit analytic expressions. Instead, these models allow, even in the algorithm of calculation, the establishment of the parameters' values at any concentration of ethyl alcohol, in tabular form (in addition to the related graph) [37, 38].

Figure 8 presents the results of the polynomial models of dependence of the parameter P1 and the concentration ethyl alcohol C_a for winery waste. Taking in consideration the error values for each mathematical model (indicated in upper-left corner of the graph representation), it can be easily observed that only in the case of polynomial of degree 20 (fig. 8d), the modeling error for the nominal model is less than 2 % (value required for reasons of mathematical model precision); moreover, only in this case the uncertain models (upper and lower 95 %) covered all the experimental data. These observations may indicate the best mathematical model to be used for the optimization of antioxidants extraction from winery waste.

4. Conclusion

The experimental results and subsequent analysis lead to the following conclusions:

1. Winery waste is a valuable source of bioactive compounds with notable antiradical and antioxidant properties.
2. The concentration of ethyl alcohol in the extraction medium significantly affects both the extraction rate of bioactive compounds and all measured parameters:

- The concentration of ethyl alcohol most strongly influences antiradical activity in both acidic and basic environments, with the least effect on the inhibition of hydrogen peroxide in an acidic environment.
- The strongest correlations were observed between the antioxidant activity of water-soluble substances and the total anthocyanin content, as well as between antiradical activity in acidic conditions and antiradical activity in basic conditions.

3. The correlation analysis of experimental data allowed:

- determining the second order statistics (autocorrelation function, cross-correlation function, the correlation coefficient);
- assessing the correlation of experimental data, i.e. if they are independent or dependent and the character of dependence;
- estimating the non-linear character of the experimental series.

4. The information analysis of experimental data allowed:

- highlighting the dependencies between ethyl

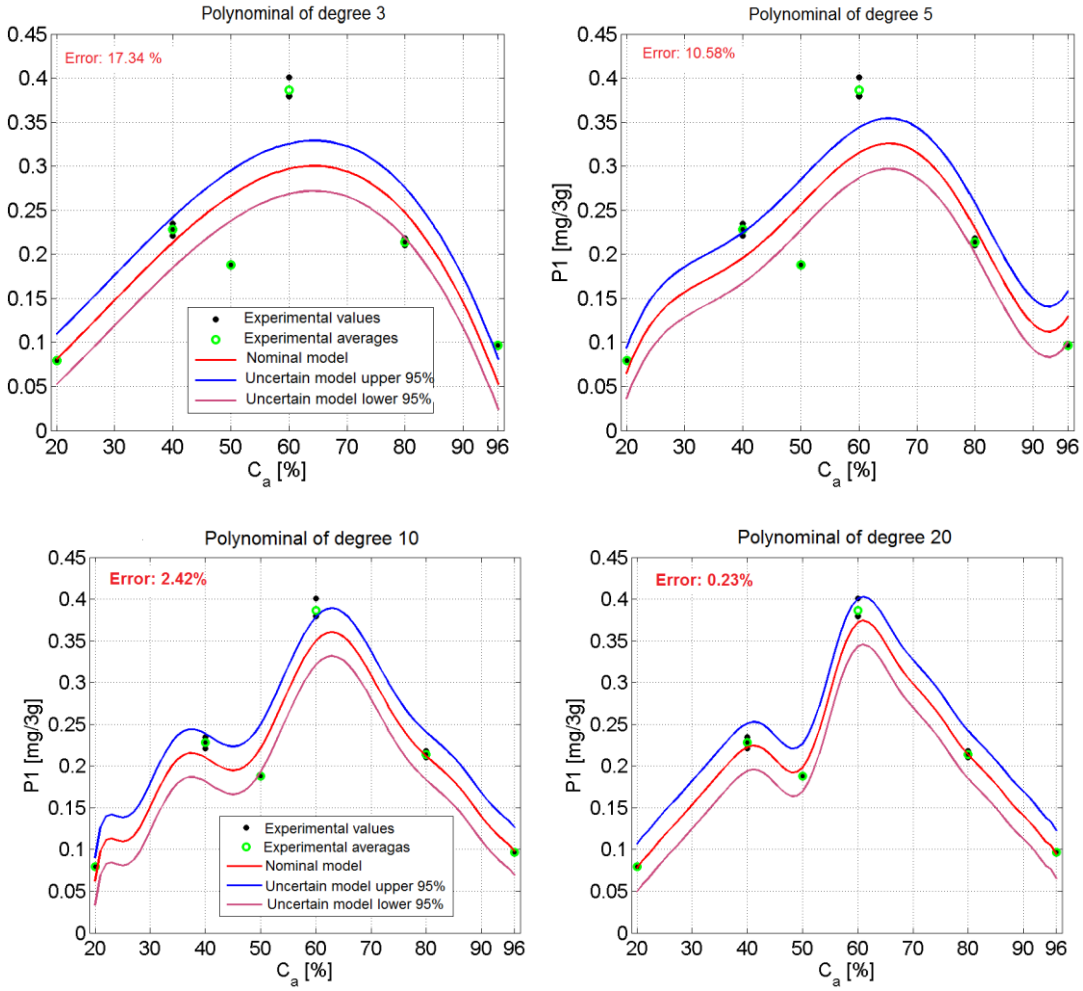

Figure 8. Mathematical models for winery waste, the P1 parameter values depending on the concentration of ethyl alcohol. Polynominal of degree 3; b) Polynominal of degree 5; c) Polynominal of degree 10; d) Polynominal of degree 20.

alcohol concentration and the parameters measured, by using graphs;

- highlighting the interdependencies between the parameters measured by using graphs;

The quantitative estimation of the influence of the ethyl alcohol concentration on the measured parameters, by using the mutual information;

- the quantitative estimation of the interdependencies between the measured parameters by using the mutual information.

5. The stochastic analysis of experimental data allowed:

- making prediction with a predictive horizon greater than the one offered by classical statistics, which gives a reliability of results;
- obtaining numerous accurate data on complementary to the experimental ones, which normally are fewer;
- obtaining reliable results in situations commonly encountered in practice i.e. when experimental data are not subject to the laws of distribution known in classical statistics.

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