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Ultrasound-assisted extraction of active compounds from Beta vulgaris using deep eutectic solvents

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Research Article

Keywords: Ultrasound, Deep eutectic solvents, Beta vulgaris, Antioxidant activity

Posted Date: April 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1555350/v1

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Abstract

Deep eutectic solvents (DES) are emerging as green and sustainable solvents for the efficient extraction of bioactive compounds and with that have increasingly attracted attention as environmentally friendly alternatives of traditional organic solvents. In this study, aimed to investigate the application of ultrasound in the extraction of betanin from beets (*Beta vulgaris*) with DES. The optimization of extraction was performed using the response surface methodology. The time variable was relevant in the performance of the extractions. In this work, three DES Choline chloride: urea (CC:U), Choline chloride: glycerol (CC:G) and Choline chloride: Citric acid (CC: Ac) and were studied. The results demonstrated that DES were excellent solvents for extraction of betanin with superior efficiency to conventional extraction solvents. The concentration of betanin varied from 41.27 to 67.51 mg/100 g with CC-U 82.46 to 104.45 mg/100 g with CC-G and 50.06 to 111.93 mg/100 g with CC-Ac. The optimum point in the extraction of betanin using Choline chloride:Citric acid (CC:Ac) was 111.193 mg/100 g in 38 min and 44% DES in water in ultrasonic bath with temperature of 35 ºC, frequency of 37 kHz and amplitude of 100%W. The use of ultrasound is found to have significant improvement in the extraction efficiency of betanins obtained from beets. However, the higher content of betanins in the extracts did not translate into a greater active antioxidant capacity, as it may be related to the synergism of other compounds present in the beet extracts. This study is the first attempt to optimize the ultrasound parameters to extract *Beta vulgaris* betanin with eutectic solvents.

Introduction

The *Beta vulgaris* (Chenopodiaceae), popularly known as beet, is a vegetable that has attracted the interest of the population for the benefits provided to health (Jajja et al. 2014; Clifford et al. 2016; Mikolajczyk-Bator and Czapski 2017), due to the presence of bioactive compounds consisting of betalains, ascorbic acid, carotenoids, polyphenols, flavonoids and saponins (Chhikara et al. 2019).

Betalains are heterocyclic, nitrogenous and water-soluble compounds, and demonstrate bioactive potentials because of their high free radical scavenging activity (Slimen et al. 2018). In addition, betalains have therapeutic properties in preventing diseases such as hypertension, dyslipidemy, cancer, neurological disorders and vascular stenosis (Rahimi et al. 2019). Studies report that the betalains in beets are responsible for pigmentation, red beetroot powder could be an important ingredient in instant beverages for athletes, natural color enhancer for food products (Ng and Sulaiman 2018), nitrite or other colorant alternative in meat products (Sucu and Turp 2018). According to their chemical structure, these pigments can be subdivided into red-violet betacyananins or yellow betaxanthines. Among betacyanins, this work aimed to study betanin (CI Natural Red 33, E-number E162, betanidin-5-*O*-β-glucoside). Betanin is the only betalain approved for use in food and is almost entirely obtained from red beet cultures (Delgado-Vargas et al. 2000).

The choice of the method of extracting betanin is an important step to obtain the compound with the desired quality and quantity. It has been a challenge to find an extraction method that uses solvent

alternative to conventional volatile organics. Generally, the extraction procedures for bioactive compounds from plant species use volatile, toxic organic solvents, often with carcinogenic properties, presenting low extraction yields and harming the environment with the generated residues (Cunha and Fernandes 2018).

Seeking to solve these problems, several researchs have been carried out in the organic chemistry of natural products, developing solvent formulations that are less harmful to the environment, replacing conventional organic solvents in the extraction of bioactive substances from plant species (Belwal et al. 2018; Chanioti and Tzia 2018; Choi and Verpoorte 2019).

Green extraction processes have recently been developed as alternative procedures for the extraction of betanin, such as ultrasound (Ramli et al. 2014). Ultrasound is a key technology to achieve the goal of sustainable "green" chemistry, where its use can reduce extraction time, energy consumption, solvent and post-treatment compared to conventional procedures (Chemat et al. 2017; Alarcon-Rojo et al. 2019; Chen et al. 2019). Therefore, this medium has a better economic characteristic in the extraction procedures, with safety and quality to the product obtained and less environmental impact (Yasui et al. 2011).

Ultrasound assisted extraction of coloring of beetroot with traditional solvents were used by Sivakumar et al. (Sivakumar et al. 2009). The authors verified there is an influence of process parameters on the extraction. The use of ultrasound is found to have significant improvement in the extraction efficiency of colorant obtained from beetroot. On the experiments it has been found that a mixture of 1:1 ethanol-water with 80 W ultrasonic power provided better yield and extraction efficiency (Sivakumar et al. 2009).

Deep eutectic solvents (DES) are a class of "green" solvents, prepared from eutectic mixtures (Cunha and Fernandes 2018; Lee et al. 2019). They are prepared by mixing two or more components, a hydrogen bonding receptor (HBR) and a hydrogen bonding donor (HBD) in different proportions (Abbott et al. 2003; Kucan and Rogosic 2019). The most common DES are formed using choline chloride, which is a non-toxic quaternary ammonium salt, such as HBR, together with natural uncharged compounds, such as alcohols, amines, carboxylic acids, sugars and vitamins, such as DLH (Ruesgas-Ramon et al. 2017). These compounds have good prospects for wider use in green extraction technologies (Belwal et al. 2018; Zainal-Abidin et al. 2017; Procentese et al. 2018).

There are no reports in the literature on optimization betanin from *Beta vulgaris* extraction with DES and assisted by ultrasound. In this context, the originality of the present study consists in developing and optimizing of betanin extraction method using DES and ultrasound using the response surface methodology aiming at higher extraction yield.

Material And Methods

Instruments and Chemical

Chemicals

Choline chloride, citric acid, glycerol, urea, acetic acid, acetonitrile, phosphate buffer, potassium ferrocyanide, trichloroacetic acid, iron (III) chloride, butylated hydroxytoluene (BHT), 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), ethanol and betanin standard were acquired by Sigma Aldrich.

Plant Material

Fresh beets (*Beta vulgaris*) were purchased at an organic products fair in Viçosa, Minas Gerais, Brazil. The Genetic Patrimony/CTA of the *Beta vulgaris* was registered in SisGen No. A61E5D4. 100 g of beet were weighed, cleaned, crushed using a food processor) and subjected to the lyophilization process to obtain a fine powder that was packed in plastic bottles in a desiccator at room temperature.

Des Preparation

Three deep eutectic solvents were prepared. The combination of choline chloride was used as HBR and citric acid, glycerol and urea as HBD. The DES preparation was based on the methodology described by Zhao et al. (2015). Initially, choline chloride was weighed in a beaker and then HBD, according to the previously established molar ratios in Table 1. With the aid of a glass stick, the reagents were homogenized. The mixture was placed in a glycerin bath previously stabilized at 70 ºC and the bath rotation was fixed at 300 rpm. The eutectic mixture was kept in the bath under magnetic stirring until the formation of a clear and homogeneous liquid. Finally, the DES was removed from the bath, stored in the amber bottle in the desiccator.

Table 1

Sample name	Component 1	Component 2	Molar ratio
CC-Ac	Choline chloride	Citric acid	1:2
CC-G	Choline chloride	Glycerol	1:2
CC-U	Choline chloride	Urea	1:2

Ultrasound-assisted Extraction (Uae)

Ultrasonic extraction experiments were performed using ultrasonic probe (Elma Sonics P180H) with temperature controlled at 35 ºC, frequency of 37 kHz and amplitude of 100% W. The material was filtered, and an aliquot was analyzed by High Performance Liquid Cromatography (HPLC).

Hplc Analysis

The extracts were analyzed using High Performance Liquid Chromatography (HPLC) with detection of the analyte by ultraviolet (UV) for quantification of betanin, using the Shimadzu liquid chromatograph model LC10AD. Elution was carried out through a gradient between two mobile phases: Phase A: water with 0.5% v/v acetic acid and Phase B: 40% v/v acetonitrile/water with 0.5% v/v acid acetic. The analysis was performed with a linear gradient 5–95% B over 40 min, Flow rate: 1 mL min^{-1} and temperature of approximately 23°C. The samples were monitored by UV-Vis absorption at 536 nm. For the quantification of betanin, an external standard curve was used from the injection of the standard in different concentrations. For the analytical curve, the betanin standard (Sigma-Aldrich) was used. The analytical curve was prepared from successive dilutions of the 1.0 mg mL^{-1} stock solution. The concentrations used to construct of analitical curve were 1.0; 0.8; 0.6; 0.4; 0.2; 0.1; 0.05; 0.0125 and 0.00625 mg mL^{-1}. For the quantification of betanin, the equation of the line $y = 204868x + 236.46$ and correlation coefficient $r^2 = 0.9997$ were obtained.

Extraction And Concentration Of Betalain Pigment

In a test tube, lyophilized beet powder (0.1 g) was added together with DES and water in the proportions described in Table 2. Subsequently, the tubes were subjected to extraction by ultrasound. The extracted solution was filtered and immediately subjected to HPLC analysis.

Table 2

Test	Coded variable		Original variable	
	X1	X2	t (min)	Conc. DES in water (%)
1	-1	-1	16	10
2	+ 1	-1	60	10
3	-1	+ 1	16	30
4	+ 1	+ 1	60	30
5	$-\sqrt{2}$	0	7	20
6	$+\sqrt{2}$	0	69	20
7	0	$-\sqrt{2}$	38	6
8	0	$+\sqrt{2}$	38	44
9	0	0	38	20
10	0	0	38	20
11	0	0	38	20
12	0	0	38	20
13	0	0	38	20
X1 = time variable (min.); *X2* = solvent concentration variable (%)				

Experimental Design

The optimization of extraction was performed using the response surface methodology. A central composite planning with two factors was used, consisting of four factorial tests obtained from the combination of levels + 1 and − 1, five repetitions at the central point denoted by the levels (0.0) and four tests on the axial points obtained from the combinations of the levels (± $\sqrt{2}$, 0) and (0, ± $\sqrt{2}$), with time (X1; min) and the proportion of deep eutectic solvent in water (X2; %: v/v) as the independent variables.

Mathematical Model

The response surface models were adjusted, generating second order polynomial equations. The response function (Y) was divided into the linear, quadratic and interaction components in the equation:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_{11} + b_{22}x_{22} + b_{12}x_1x_2$$

where: b_0 is the constant coefficient, b_1 and b_2 are the linear coefficients, b_{11} and b_{22} are the quadratic coefficients and b_{12} is the interaction coefficient, x represents the independent variables or factors, and Y represents the dependent variable or response. The regression coefficients were obtained using multiple linear regression (RLM), using the least squares method. Such coefficients were statistically evaluated for their significance and interpreted according to their importance in the system.

Statistical analysis

The extraction optimization was performed using the response surface methodology. A central composite design with two factors was used, consisting of four factorial trials obtained from the combination of levels + 1 and − 1, five repetitions at the central point denoted by levels (0.0). Measured responses (dependent variable) were expressed as mg of betanin present in 100 g of lyophilized beetroot (mg/100 g).

All data analysis in this work was performed in StatSoft Statistica version 7 (www.statsoft.com) (StatSoft Inc., 2004).

Analysis of variance (ANOVA) was used to verify the validity of the adjusted models. The response surface plots were obtained using the values estimated by the fitted models. The F-test for the lack of adjustment was made when evaluating the adjusted models. Student's t-test was used to test the significance of the regression coefficients. All calculations were performed using the Statistica 7.0 software (www.statsoft.com) (StatSoft Inc., 2004). The datasets generated analysed during the current study are available in the Mendeley Data, repository, doi: 10.17632/vnfm2mv55x.1.

Antioxidant Activity (Dup: Abstract ?)

The reducing power of betanin extracts was determined according to the method of Yen and Chen (Yen and Chen 1995) To 1.0 mL aliquot of each sample was transferred to 25 mL test tubes. To these aliquots were added: 2.5 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.6) and 2.5 mL of 1% m/v potassium ferrocyanide ($K_3[Fe(CN)_6]$). The mixture was incubated at 45 ^oC for 20 min 2.5 mL of 10% w/v trichloroacetic acid was added to the solution in the test tube, with subsequent stirring. A 2.5 mL volume of the mixture was transferred to another test tube, in which 2.5 mL of Milli-Q water and 0.5 mL of 0.1% w/v $FeCl_3$ were added, with stirring. The absorbance reading was performed at 700 nm. The readings were performed in triplicate and in this test the absorbance of the 2,6-di-*tert*-butyl-4-methylphenol (BHT) standard was used as 100% of activity. The free radical scavenging activity (ASRL) 2,2-Diphenyl-1-picryl-hydrazil (DPPH) from beet extracts was performed according to the methodology described by Hatano et al. (Hatano et al. 1988) with modifications. In 4 mL of the sample, 1 mL of 0.5 mmol L^{-1} DPPH was added, equally diluted in ethanol. The mixture was packed in an amber test tube and stirred. After 30 min, the absorbance was read at 517 nm.

Results And Discussion

Effects of operational variables on the extraction of betanin

The experimental values of betanin content for each set of combinations of variables and DES used in this study are presented in Table 3.

Table 3
Effect of the experimental conditions on the yields of betanin (mg/100 g)

Test	Original variables Deep eutectic solvents				
	Time (min)	Solvent concentration (%)	CC-Ac	CC-G	CC-U
1	16	10	80.01	96.99	41.84
2	60	10	51.68	90.72	52.49
3	16	30	72.72	94.09	55.87
4	60	30	88.36	104.45	67.51
5	7	20	105.70	90.43	59.89
6	69	20	50.06	102.26	62.14
7	38	6	61.58	82.93	51.11
8	38	44	111.93	82.46	60.75
9	38	20	86.66	92.18	50.58
10	38	20	89.06	92.90	43.11
11	38	20	87.75	92.85	41.27
12	38	20	90.95	91.62	42.44
13	38	20	88.43	93.66	42.39

The concentration of betanin varied from 41.27 to 67.51 mg/100 g with CC-U 82.46 to 104.45 mg/100 g with CC-G and 50.06 to 111.93 mg/100 g with CC-Ac. In extractions with CC-Ac, the highest concentration obtained was 111.93 mg/100 g in 38 min. With the highest percentage of solvent of 44%, the lowest concentration obtained was 50.06 mg/100 g, with no relation to the lowest percentage of solvent used. However, the time in contact with the ultrasonic bath was 69 min. In the extractions with CC-G, the highest concentrations were 104.45 and 102.26 mg/100 g, both with the longest extraction times, as well as for the tests with CC-U, which also obtained the highest concentrations with the longest extraction. There is also a good repetition at the central point, less low error in the estimation of the coefficients.

Response Surface Analysis

To maximize extraction conditions for beet betanin, a regression analysis was performed on the results and a polynomial equation was derived using the significant values of the regression coefficients estimated in Table 4. The adequacy of the model to compare the experimental and predicted values was verified using ANOVA values, which were statistically acceptable with a 95% confidence level. Figure 1 (a, b c) three-dimensional plots were constructed to show the variation in betanin extraction as a function of time and concentration of extractors.

Table 4
Regression coefficients

Estimated values of the coefficients for the different extractors and their errors						
	CC-Ac		CC-G		CC-U	
Coefficient	Value	Error	Value	Error	Value	Error
b_0 (average)	92.3	± 2.00	96.9	± 1.92	136	± 9.33
b_1 (time)	-0.417	± 0.109	-0.744	± 0.0602	-2.71	± 0.292
b_2 (conc.)	Not signif.	-	0.574	± 0.109	-3.62	± 0.529
b_{11} $(time)^2$	-0.0143	± 0.00127	0.00637	± 0.000621	0.0223	± 0.00301
b_{22} $(conc.)^2$	-0.0163	± 0.00229	-0.0262	± 0.00171	0.0406	± 0.00829
b_{12} (time)x(conc.)	0.0494	± 0.00287	0.0189	± 0.00176	0.0466	± 0.00854
Significance at $p < 0.05$						

In the analysis of the regression equation for the CC-Ac extractor, Table 4, it appears that the extractor time and concentration, when interpreted in isolation, contribute negatively to the response when its levels are increased. In the joint interpretation of the variables, it is observed that there is a synergistic interaction causing an increase in the response. This fact can best be verified by means of the surface graph concentration versus time shown in Fig. 1a, which provides the contours of the responses, obtained through the predicted values of the adjusted models, where it is possible to observe the effect of the interaction between the variables. It can be concluded that when the levels of the variables are increased simultaneously, the maximum region of extraction of betanin occurs around the proportion of solvent in 45% and time between 40 and 60 min.

Considering the CC-G extractor, Table 4, a behavior similar to that of CC-Ac is observed with respect to the extraction time, since the isolated action of time does not promote an increase in extraction. The high value of the coefficient b_2 (concentration) when analyzed in isolation contributes to increase the response, however this increase in the response only occurs for lower levels of the variable, for higher concentration values the response tends to fall due to the negative value of b_{22} $(concentration)^2$. The

effects of the variables, when interpreted together, allow to observe synergistic interaction promoting an increase in the response. Fig. S1 provides a visualization of these effects. When the levels of the time and concentration variables are increased simultaneously, it is observed that the proportions between the variables that produce the best extractions are: 20% < concentration < 40% and 60 min. < time < 69 min, obtaining extractions between 100 and 110 mg/100 g.

The acidity/basicity of the medium compromises the stability of the pigment. Extracts containing betaninas are generally relatively stable from pH 3 to 7 (Shiozer and Barata, 2007). It is observed that water is the best extractor solvent due to its high polarity, as Stated by Stintzing (2008) that betalains are soluble in water. Betacyanins precipitate in acidic medium, and result in betaxanthins (Delgado-Vargas et al. 2000), as can be observed that the extraction performed with acidic solvents were inefficient compared to solvents with neutral characteristics. It is observed that by adding other solvents to the water the polarity is modified, thus increasing the concentration of extracted betanin, such as extractions with ethanol and ethanol/water 50%. In the extracts obtained with the SEP, ChCl:Ac showed higher betanin concentration, due to the pH of the extractor solution, which stabilized the compound, while SEP ChCl:G presented close values. The ChCl:U SEP, extracted in a smaller amount compared to the other SEP's, due to its basicity.

The use of the CC-U extractor produced an unusual result (Table 4), since the linear coefficients b_1 (time) and b_2 (concentration) showed high and negative values, showing the low extraction values around the central point. An increase in extraction is observed when moving away from the central point, described by the quadratic coefficients and by the synergistic interaction between the variables. The behavior of the system can be better understood by the graphical analysis of Fig. 1c, a minimum of extraction is observed in the central region of the experimental space followed by a small increase towards the limits of the investigated levels. Thus, the best results provided by the equation are found around the points defined by (16 min, 10%) and (16 min, 30%).

Antioxidant Activity

In the present study, the stable free radical DPPH and the reducing power were used to investigate the potential antioxidant properties of the extracts obtained by ultrasound. For the extracts, the following order of antioxidant activity was observed: CC-G > CC-U > CC-Ac.

In the results obtained using the lowest concentration of extract with DES CC-G, the highest antioxidant capacity was observed both in the test of elimination of the radical DPPH with a value of 77.05 ± 0.08, and in the test of inhibition of lipid oxidation with a value of 63.86 ± 0.12.

The extracts with the DES CC-U and CC-Ac. exhibited similar antioxidant capacity, with a value of 51.75 ± 0.05 and 51.00 ± 0.02, respectively for elimination of DPPH radicals and values of 57.72 ± 0.13 and 60.17 ± 0.03 in the reducing power.

Pearson's correlation coefficient for betanin in DPPH had $r = 0.93$ and with the reducing power of $r = 0.81$. The highest correlation coefficient was betanin compared to DPPH, showing a very strong correlation ($0.8 < r < 1$), according to Pearson's correlation concepts. As for Pearson's correlation for betanin at the reducing power r = 0.81, it was considered a strong correlation. These results show the influence of betanin on antioxidant activity in both methods. However, it is possible that the activity of the antioxidant potential shown is not only due to betanin, but may be related to the synergism of other compounds present in the beet extracts used in the present study, which are likely to contribute to the radical scavenging activity of beet extracts.

Conclusion

This study provided an example on the use of natural DES solutions to replace volatile organic solvents and enable efficient betanin extraction through ultrasound-assisted and antioxidant capacity assessment. It is suggested that the DES solution containing extracts has the potential to be safely applied in the cosmetic and pharmaceutical areas without additional product isolation steps.

Declarations

Acknowledgments We thank the following for grant and scholarship financial support: the Research Support Foundation of the State of Minas Gerais – FAPEMIG and the National Council for Scientific and Technological Development – CNPq (A.J.D., M.H.S., and P.C.S.) and Coordination for the Improvement of Higher Education Personnel (CAPES, Finance Code 001) and fellowship (A.C.S.D., and D.E.B.).

CRediT authorship contribution statement A.C.S.D., A.J.D., M.H.S performed financial resources, experimental part, characterization of compounds, wrote and reviewed the manuscript; C.R., R.C.S.S. performed the statistical analysis, analyzed the results, and wrote part of this manuscript; D.E.B., P.C.S. performed the antioxidant, analyzed the data and reviewed the manuscript.

Funding This work was supported by the Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – research fellowships and financial resources (A.J.D., M.H.S., P.C.S.), Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) (A.J.D, M.H.S., finnancial resources) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)-research fellowships (A.C.S.C., and R.C.S.S., and D.E.B.).

Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Datasets The datasets generated analysed during the current study are available in the Mendeley Data, repository, doi: 10.17632/vnfm2mv55x.1.

Demuner, Antonio (2022), “Ultrasound-assisted extraction of active compounds of Beta vulgaris”, Mendeley Data, V1, doi: 10.17632/vnfm2mv55x.1

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Figures

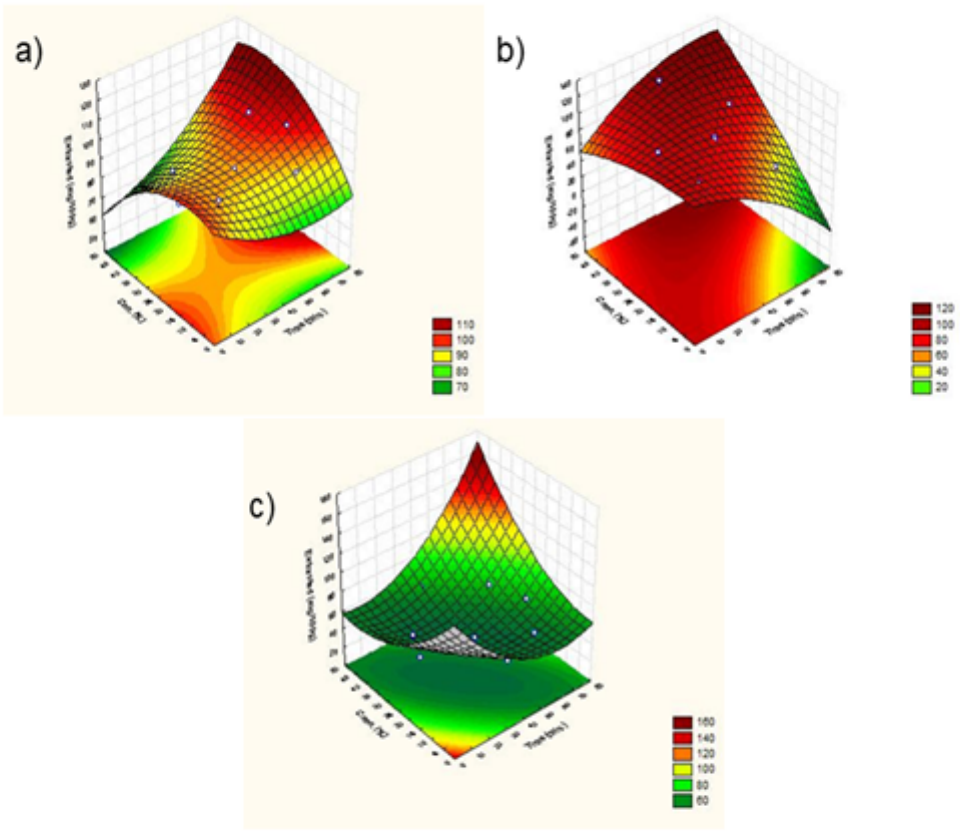

Figure 1

Legend not included with this version

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