Appendix

Compressed fluid-based technologies for downstream isolation of bluish anthocyanin-derived pigments obtained from blueberry surplus

Ana N. Nunes[ab*], Joana Oliveira[c], Maria Rosário Bronze[abd], Ana A. Matias[a]

[a] iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901 Oeiras, Portugal

[b] ITQB, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. Da República 2780-157 Oeiras, Portugal

[c] REQUIMTE, Laboratório Associado para a Química Verde, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, S/N, 4169-007 Porto, Portugal

[d] FFULisboa, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003, Lisboa, Portugal

[*]annunes@ibet.pt

Number of Pages: 8

Number of Tables: 3

Compressed fluid-based separation process

Table A1. Compressed fluid-based separation process conditions applied and respective density of the CO_2 + ethanol binary systems.

Exp Name	Pressure (bar)	Temperature (°C)	Ethanol content (wt. %)	x CO_2	x EtOH	CO_2 + EtOH density (Kg/m³)
N1	100	40	20	0.81	0.19	796 (1)
N2	500	40	20	0.81	0.19	~956[a] (2)
N3	100	60	20	0.81	0.19	~779[b] (2)
N4	500	60	20	0.81	0.19	~912[c] (2)
N5	100	40	40	0.61	0.39	827 (1) (2)
N6	500	40	40	0.61	0.39	nf
N7	100	60	40	0.61	0.39	nf
N8	500	60	40	0.61	0.39	nf
N9	100	50	30	0.71	0.29	760
N10	500	50	30	0.71	0.29	~922[d] (2)
N11	300	40	30	0.71	0.29	~914[e] (2)
N12	300	60	30	0.71	0.29	~855[f] (2)
N13	300	50	20	0.81	0.19	882 (3) (2)
N14	300	50	40	0.61	0.39	nf
N15	300	50	30	0.71	0.29	873 (3)
N16	300	50	30	0.71	0.29	873 (3)
N17	300	50	30	0.71	0.29	873 (3)

[a] x EtOH=0.203, 45 °C, 450 bar; [b] x EtOH=0.203, 65 °C, 150 bar; [c] x EtOH=0.203, 65 °C, 450 bar; [d] x EtOH=0.271, 55 °C, 450 bar; [e] x EtOH=0.271, 45 °C, 300 bar; [f] x EtOH=0.271, 65 °C, 300 bar; nf – not found

Experimental design and statistical analysis

Compressed fluid separation process parameters optimization

When compressed fluids are applied as a solvent, various experimental factors need to be considered and optimized for each investigated system. A systematic approach was necessary to effectively address the expected process factors, intricate interactions, and the desired high yield and content.

Building upon previous studies on anthocyanins, which are the precursors for the formation of portisins, as well as preliminary experimental findings and instrumental limitations, some factors were kept constant to simplify the task and reduce the complexity of the parameter space explored, namely the amount of RM2, the CO_2+ethanol flow rate, and extraction time.

The amount of RM2 was maintained constant due to limitations in the available extract quantity, ensuring consistency in sample preparation across experiments. The flow rate was also kept

constant to ensure that we operated above the minimum limit of both pumps (CO_2 and co-solvent), guaranteeing reproducible extraction conditions. Additionally, it was not feasible to explore higher flow rates as the quantity of RM2 tested was limited. Regarding the fixed extraction time, the first experiment of central composite face design (N1) indicated that 35 minutes were required until target compounds extraction reached saturation. Subsequently, this time was fixed for all the subsequent extraction experiments to avoid introducing an uncontrollable variable. Keeping the extraction time constant ensured consistency in the extraction process, allowing for a fair comparison of results and minimizing the risk of variability introduced by different extraction times.

A response surface methodology was used to evaluate the influence of process conditions on the compressed fluid separation process of portisins from a complex natural extract. Several factors are known to affect the portisins content, as well as extraction yield and portisins content. Among all the potential factors, the effect of the pressure, temperature, and ethanol content in the compressed fluid mixture, in extraction yield, portisins yield, and portisins content were studied (Manuscript Table 1).

The selection of operating parameters ranges, including pressure (100-500 bar) and temperature (40-60 °C), was based on existing literature on the application of compressed fluids for anthocyanin recovery (4–12). Also, to achieve efficient separation of portisins, which are medium polar compounds, a biocompatible polar co-solvent, namely ethanol, was added to the dense CO_2. Different ethanol concentrations in the compressed fluid mixture (20-40 wt.%) were investigated for the portisins separation.

To achieve the balance between the number of experimental runs and the acquired knowledge, a central composite face design was selected, which combined a full factorial design with star points placed on the faces of the sides. Central replicate points were included to assess the experimental variability, uncertainty of results, and the goodness of fit of the mathematical model.

Extraction yield, portisins yield, and portisins content resulting from the experiments performed according to the experimental conditions defined by the CCFD were analysed by using the Modde v.12 (Umetrics, Umeå, Sweden) software. The statistical tests, including the adjustments of the design model and factors effects, were considered to be significant when the resulting *p*-value was lower than the predefined α = 0.05. Factors with p-values slightly above 0.05 were carefully evaluated to ensure that their exclusion did not compromise the overall accuracy and reliability of the model. It is better to accept factors with values higher than 0.05 rather than to

take the chance of missing important information. It is worth noting that both portisins yield and portisins content presented positive skewness, which indicated the need for a log transformation prior to statistical analysis.

The underlying two-factor polynomial models include linear, factor interactions as well as quadratic terms as depicted by Eq. A1. In this equation, A, B, and C represent the independent variables, i.e. the pressure, temperature, and ethanol content in the compressed fluid mixture.

$$Y = a + b_1A + b_2B + b_3C + + b_{12}AB + b_{13}AC + b_{23}BC + c_1A^2 + c_2B^2 + c_3C^2$$
(Eq. A1)

The model coefficients (a, b_x, and c_x) were estimated by multivariate linear regression and their significance was assessed after performing the corresponding ANOVA (Table A2).

Table A2. Linear and quadratic effects and respective significance levels (p) of the tested variables [factors: pressure (A), temperature (B), and ethanol content in the compressed fluid mixture (C)] and interactions on extraction yield, portisins yield, and portisins content in the extract.

	Extraction yield		Portisin yield		Portisin content	
	Coeff. SC	p value	Coeff. SC	p value	Coeff. SC	p value
Constant	25.1	1.41E-09	-0.46	2.01E-04	1.41	3.57E-07
A	0.9	4.62E-01	0.08	4.18E-01	0.14	9.88E-02
B	0.3	8.37E-01	-0.08	3.95E-01	-0.04	6.54E-01
C	7.9	8.15E-05	0.63	1.60E-05	0.45	2.22E-04
AB	-3.4	1.41E-02	-0.16	7.05E-02		
AC	6.2	2.78E-04			-0.21	1.47E-02
BC					-0.13	8.55E-02
C²					-0.19	7.62E-02

The response surfaces fitted to the extraction yield, portisins yield, and portisins content (Manuscript Figure 2) can be described using a polynomial model as a function of pressure (A), temperature (B), and ethanol content in the compressed fluid mixture (C). In these response surface, the non-significant effects (Table A2) were removed from the complete model (Eq. A1) giving origin to the simplified models described in Eq. A2, Eq. A3, and Eq. A4.

$$Extraction\ yield, wt.\% = \ 25.1 + 0.9A + 0.3B + 7.9C - 3.4AB + 6.2AC \tag{Eq. A2}$$

$$Portisins\ yield, wt.\% = -\ 0.46 + 0.08A - 0.08B + 0.63C - 0.16AB \tag{Eq. A3}$$

$$Portisins\ content, \frac{mg_{portisin}}{g_{extract}} = \ 1.41 \mp 0.14A - 0.04B + 0.45C - 0.21AC - 0.13BC - 0.19C^2 \tag{Eq. A4}$$

Table A3. ANOVA analysis for extraction yield, portisins yield, and portisins content using different compressed fluid separation conditions.

Extraction yield	DF	SS	MS (variance)	F	*p* value	SD
Total	16	12119	757			
Constant	1	10050	10050			
Total corrected	15	2069	138			11.74
Regression	5	1839	368	15.96	0.000	19.18
Residual	10	230	23			4.80
Lack of Fit (Model error)	9	205	23	0.90	0.679	4.78
Pure error (Replicate error)	1	25	25			5.02
	N = 16	Q^2 =	0.627	Cond. no. =	1.1	
	DF = 10	R^2 =	0.889	RSD =	4.8	
	Comp. = 1	R^2 adj. =	0.833			

Portisin yield	DF	SS	MS (variance)	F	*p* value	SD
Total	17	12.21	0.72			
Constant	1	3.63	3.63			
Total corrected	16	8.58	0.54			0.73
Regression	4	7.00	1.75	13.33	0.000	1.32
Residual	12	1.57	0.13			0.36
Lack of Fit (Model error)	10	1.54	0.15	10.01	0.094	0.39
Pure error (Replicate error)	2	0.03	0.01			0.12
	N = 17	Q^2 =	0.625	Cond. no. =	1.1	
	DF = 12	R^2 =	0.816	RSD =	0.4	
	Comp. = 1	R^2 adj. =	0.755			

Portisin content	DF	SS	MS (variance)	F	*p* value	SD
Total	17	31.79	1.870			
Constant	1	25.61	25.609			
Total corrected	16	6.18	0.386			0.62
Regression	6	5.17	0.862	8.55132	0.002	0.93
Residual	10	1.01	0.101			0.32
Lack of Fit (Model error)	8	0.99	0.124	16.414	0.059	0.35
Pure error (Replicate error)	2	0.01	0.007			0.09
	N = 17	Q^2 =	0.512	Cond. no. =	2.8	
	DF = 10	R^2 =	0.837	RSD =	0.3	
	Comp. = 1	R^2 adj. =	0.739			

The values for R^2 of these models suggest a good agreement between the experimental data and the values predicted by the model for the extraction yield, portisins yield, and portisins content. About 89 %, 82 %, and 84 % of the observed overall variance concerning the extraction yield, portisins yield and portisins content respectively, are explained by these models (Table A3). The reproducibility of the model to the extraction yield, portisins yield, and portisins content was 92 %, 97 %, and 98% respectively, considering the center points.

References

1. Kato M, Kodama D, Ono T, Kokubo M. Volumetric Properties of Carbon Dioxide + Ethanol at 313.15 K. J Chem Eng Data. 2009;54(10):2953–6.

2. Zhu T, Gong H, Dong M. Density and Viscosity of CO 2 + Ethanol Binary Systems Measured by a Capillary Viscometer from 308 . 15 to 338 . 15 K and 15 to 45 MPa. J Chem Eng Data. 2020;65(8):3820–33.

3. Pohler H, Kiran E. Volumetric Properties of Carbon Dioxide + Ethanol at High. J Chem Eng Data. 1997;42(2):384–8.

4. Garcia-Mendoza M del P, Espinosa-Pardo FA, Baseggio AM, Barbero GF, Maróstica Junior MR, Rostagno MA, et al. Extraction of phenolic compounds and anthocyanins from juçara (Euterpe edulis Mart.) residues using pressurized liquids and supercritical fluids. Journal of Supercritical Fluids. 2017;119:9–16.

5. Paes J, Dotta R, Barbero GF, Martínez J. Extraction of phenolic compounds and anthocyanins from blueberry (Vaccinium myrtillus L.) residues using supercritical CO2 and pressurized liquids. Journal of Supercritical Fluids. 2014;95:8–16.

6. Otero-Pareja MJ, Casas L, Fernández-Ponce MT, Mantell C, De La Ossa EJM. Green extraction of antioxidants from different varieties of red grape pomace. Molecules. 2015;20(6):9686–702.

7. Seabra IJ, Braga MEM, Batista MT, De Sousa HC. Effect of solvent (CO2/ethanol/H2O) on the fractionated enhanced solvent extraction of anthocyanins from elderberry pomace. Journal of Supercritical Fluids. 2010;54(2):145–52.

8. Babova O, Occhipinti A, Capuzzo A, Maffei ME. Extraction of bilberry (Vaccinium myrtillus) antioxidants using supercritical/subcritical CO2 and ethanol as co-solvent. Journal of Supercritical Fluids. 2016;107:358–63.

9. Tamkutė L, Liepuoniūtė R, Pukalskienė M, Venskutonis PR. Recovery of valuable lipophilic and polyphenolic fractions from cranberry pomace by consecutive supercritical CO2 and pressurized liquid extraction. Journal of Supercritical Fluids. 2020;159.

10. Sainz Martinez A, Kornpointner C, Haselmair-Gosch C, Mikulic-Petkovsek M, Schröder K, Halbwirth H. Dynamic streamlined extraction of iridoids, anthocyanins and lipids from haskap berries. Lwt. 2021;138(November 2020).

11. Serra AT, Seabra IJ, Braga MEM, Bronze MR, De Sousa HC, Duarte CMM. Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds. Journal of Supercritical Fluids. 2010;55(1):184–91.

12. Kühn S, Temelli F. Recovery of bioactive compounds from cranberry pomace using ternary mixtures of CO_2 + ethanol + water. Journal of Supercritical Fluids. 2017;130(March):147–55.