NeoGiANT

The power of grape extracts: antimicrobial and antioxidant properties to prevent the use of antibiotics in farmed animals: 101036768

D2.3. Optimized grape extracts

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PROJECT INFORMATION

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4	VRI	Veterinary Research Institute	Czech Republic
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6	FUB	Freie Universität Berlin	Germany
7	FCUP	Universidade do Porto – Faculdade de Ciências	Portugal
8	ULL	Universidad de La Laguna	Spain
9	UNE	Asociación Española de normalización	Spain
10	JU	Jihočeská Univerzita	Czech Republic
11	CONICET	Consejo Nacional de Investigaciones Científicas y Técnicas	Argentina
12	ASAJA	Asociación Agraria de Jóvenes Agricultores	Spain
13	ATM	Anitom S.L	Belgium
14	i-GRAPE	i-GRAPE	Spain
15	CTA	Contactica S.L	Spain
16	NUS	Nutrition Science	Belgium
17	CZV	CZ VACCINES	Spain
18	LBE	LIFEBIOENCAPSULATION SL	Spain
19	BIAN	BIANOR BIOTECH	Spain
20	MAGA	MAGAPOR S.L.	Spain

DELIVERABLE DETAILS

Document Number:	D2.3
Document Title:	Optimized grape extracts
Dissemination level	PU – Public
Period:	PR1
WP:	WP2
Task:	Task 2.2
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Abstract:	This document corresponds to the Deliverable 2.3 Optimized grape extracts. It covers the optimization of the most critical experimental parameters at lab-scale to obtain the extracts with highest extraction efficiency and the corresponding report after formulation refinements.

Version	Date	Change
V1	26/09/2022	Initial version

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1 BACKGROUND

The first bioactive extracts were obtained by medium-scale ambient temperature (MSAT) procedure at lab-scale from the raw material (grape marc) in different Generally Recognised as Safe (GRAS) solvents and their hydro-organic mixtures (ethyl lactate/water, ethanol/water, propyleneglycol/water, dimethylformamide, dimethylsulfoxide and acetone), as it is described in D2.1.

Afterwards, a deep analytical characterization in terms of total polyphenolic content (TPC), antioxidant activity (AA), pH and individual polyphenols was carried out, showing that the $\sum$ bioactive compounds are similar in all extracts, with values between 111-187 mg L^{-1}, depending on the employed solvent. However, the profile for each polyphenolic group is slightly different, demonstrating their versatility for further applications. In addition, it was demonstrated that they comply with the maximum residue levels (MRLs) for fungicide residues, being safe for further use. These results are shown in D2.2.

As described in Task 2.2, the extracts with the highest antioxidant activity were produced at lab scale and sent directly to T2.5 (Development of solid-state products) and T2.6 (Development of specific formulations in liquid form) for the development of specific feed formulations, where they are being validated conveniently for aquatic animals, poultry and mammals. The extracts richest in bioactive compounds were sent to WP3 for assessing their antimicrobial activity, being the most promising ones optimized and then formulated at lab-scale.

2 OPTIMIZATION OF THE MOST CRITICAL MSAT EXPERIMENTAL PARAMETERS

As it is well known, polyphenols have a wide range of polarity and very different chemical characteristics, being the extraction of the different families a challenge. So the most critical experimental parameters affecting MSAT extraction procedure were optimized by design of experiments (DoE) statistical tools. Based on the previous studies, the extracts obtained in EtOH/water (50:50, v/v) showed the highest content of bioactive compounds (results in D2.2.). For this reason, this hydro-organic mixture was selected as extractant to perform the optimization of the experimental conditions. Other three parameters critical affecting extraction (i) sample size, (ii) extraction solvent volume and (iii) the ratio between sample-dispersant, were optimized. The studied levels for each parameter are summarized in ***Table D2.3.1***.

Table D2.3.1. Extraction parameters and factor levels optimized

EXPERIMENTAL PARAMETERS	CODE	LOW	HIGH
Grape marc (g)	A	20	200
Extract volume (mL)	B	10	250
Ratio dispersant/sample (g/g)	C	0.5	2

A composite center surface matrix design employing as response the TPC and AA was carried out. The design matrix comprising 23 trials, as well as the TPC and AA values for each experiment, are shown in ***Table D2.3.2.***

Table D2.3.2. Design matrix and results for TPC and AA

ASSAY	A	B	C	TPC (mg GAE L^{-1})	AA (mmol TRE L^{-1})
1	60	130	1.25	3016	23
2	60	130	1.25	2998	19
3	36	57	1.71	3679	27
4	36	203	0.79	2550	19
5	84	203	1.71	2677	17
6	84	57	0.79	3128	18
7	60	130	1.25	2793	13
8	84	57	1.71	3727	21
9	60	130	1.25	2829	27
10	36	203	1.71	2068	18
11	84	203	0.79	2271	18
12	36	57	0.79	1634	13
13	60	130	1.25	2319	18
14	60	130	1.25	2791	23
15	60	130	2.00	3714	31
16	60	10	1.25	3304	26
17	60	250	1.25	1642	12
18	60	130	0.50	2321	20
19	20	130	1.25	2009	19
20	100	130	1.25	3508	28
21	150	150	2	3393	25
22	200	100	1.25	5918	44
23	165	75	1.70	4140	28

To easily visualize the influence of the factors for the TPC, its Pareto chart is shown in ***Figure D2.3.1***. In these plots, and for each factor or interaction, the length of each bar is proportional to the absolute value of its associated standardized effect by its standard error. The vertical line represents the statistical significance bound at the 95% confidence level. P<0.05 denotes statistically differences.

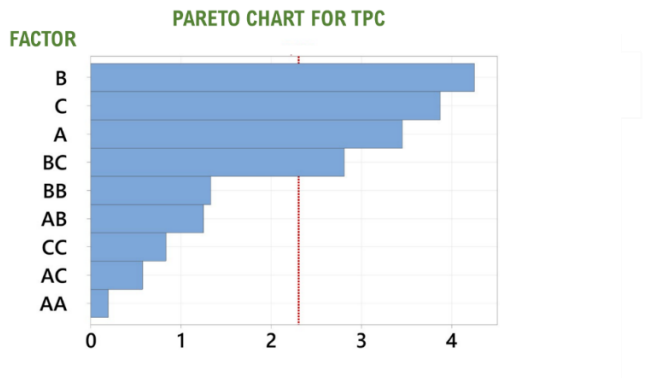

Figure D2.3.1. Pareto plot to visualize the influence of the studied parameters in the TPC values.

As can be seen in ***Figure D2.3.1***, the TPC values showed clear differences between the different experiments. The three studied parameters were statistically significant: (i) sample size (A, p=0.009), (ii) the extraction solvent volume (B, p=0.003) and (iii) the ratio between sample-dispersant (C, p=0.005), as well as the interactions between factors B and C (p=0.023). On the other hand, the AA was only statistically significant for the interaction AC (p=0.023).

The response surface plots for the 3 studied parameters are depicted in Figure ***D2.3.1a*** (for TPC results) and ***Figure D2.3.1b*** (for AA results).

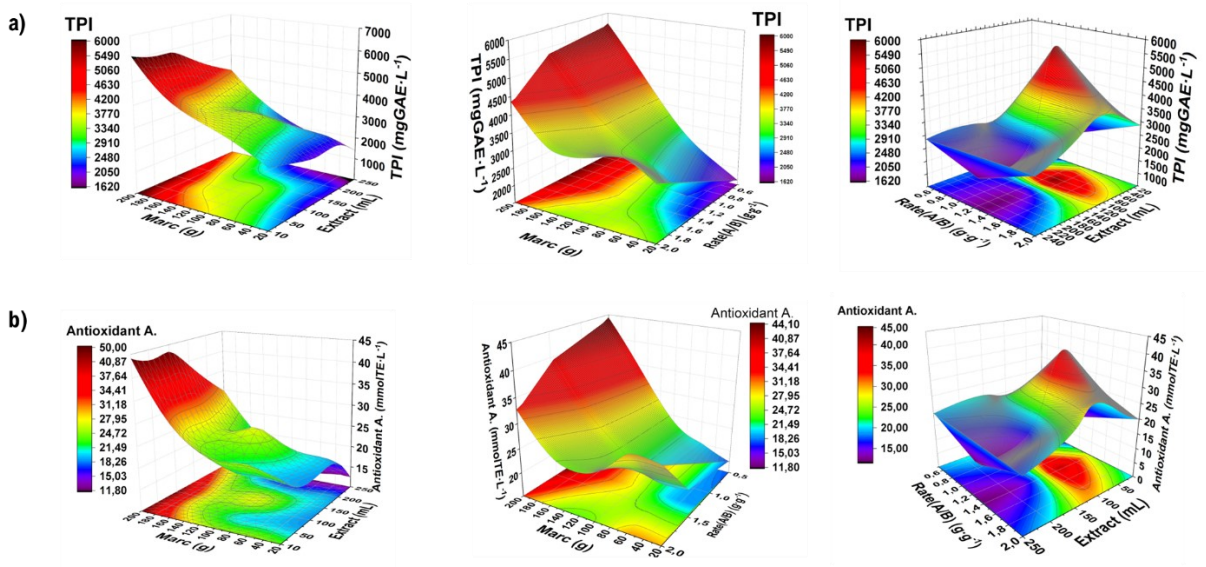

Figure D2.3.2. Response surface plots for the evaluated parameters

After the statistical study employing advanced statistical tools, the optimized conditions involve the use of 200 g of grape marc, in a proportion 1:25 sample/dispersant, and employing 100 mL of solvent.

3 OPTIMIZATION OF THE EXTRACTION SOLVENT

All previous experiments were performed with EtOH/water (50:50, v/v) but solvent is crucial to modulate the polarity profile of the bioactive compounds present in the ready-to-use extracts, as was demonstrated in D2.1 and D2.2. For this reason, once the experimental parameters were optimized, the optimization was extended to the other GRAS solvents that showed the best results in the previous experiments in WP2 and WP3 tasks, including ethyl lactate and propyleneglycol as well as their mixtures in different proportions with water, as can be seen in ***Table D2.3.3***.

Table D2.3.3. Tested GRAS solvents and their hydro-organic mixtures. Obtained pH values.

SOLVENT	%	pH
Water	100	4.31
Ethanol	25	4.44
	50	4.62
	75	4.72
	100	4.96
Ethyl lactate	25	4.22
	50	4.11
	75	4.62
	100	4.73
Propylene glycol	25	4.42
	50	4.47
	75	4.58
	100	4.63

pH was measured, showing values ranging between 4.11 and 4.96. Other indexes such as TPC, AA and IC50 were measured for all the tested solvents and mixtures, showing a correlation between TPC and AA. Results are summarized in ***Figure D2.3.3***.

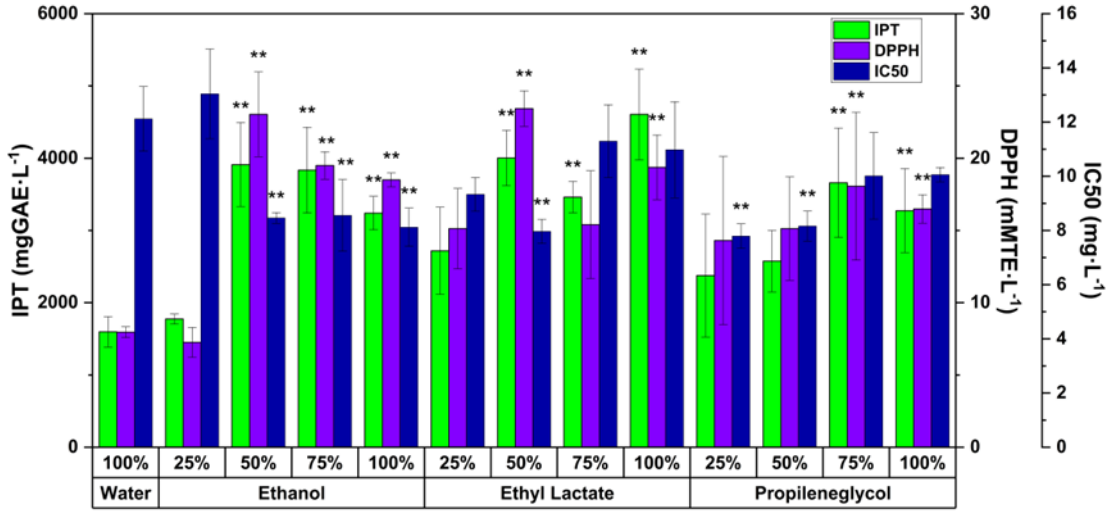

Figure D2.3.3 Response for TPC, AA and IC50 depending on the tested solvents. ** indicates statistical differences ($p<0.001$).

As can be seen, it is evident that in all cases there is a correlation between the TPC and the AA, indicating that the polyphenolic compounds are the main cause of the free radical inhibitory power of the white grape marc extract. Extract in ethyl lactate/water (50:50, v/v) showed the highest bioactivity values (for the three parameters evaluated): TPC (4003 mgGAE L^{-1}), AA (23 mmolTRE L^{-1}) and IC50 (8 mg L^{-1}). Ethanol/water (50:50, v/v) showed similar results. It was also observed that, in general, when the percentage of water increased, the responses were lower.

4 INDIVIDUAL POLYPHENOLIC CONTENT

All the obtained extracts were analyzed by LC-MS/MS to quantify the concentration of individual polyphenols. Results, expressed as mg L^{-1} for the ∑ of bioactive polyphenols are summarized in ***Table D2.3.4***. The individual concentration for each compound is shown in Annex 1.

Table D2.3.4. Concentration (mg L^{-1}) for the ∑ of bioactive polyphenols

SOLVENT	%	∑ bioactive polyphenols
Water	100	157 ± 2
Ethanol	25	115 ± 9
	50	**265 ± 51[a]**
	75	209 ± 10
	100	233 ± 11
Ethyl lactate	25	161 ± 40
	50	**252 ± 33[a]**
	75	168 ± 1
	100	200 ± 41
Propylene glycol	25	142 ± 38
	50	144 ± 7
	75	201 ± 29
	100	174 ± 5
LSD test		p<0.025

[a] Upper significant value

Results depicted in ***Table D2.3.4***, show that the highest concentrations for the ∑ of bioactive polyphenols (values in bold) were obtained with the EtOH/water (50:50, v/v) extract (265 mg L^{-1}), and the extract obtained in ethyl lactate with the same proportion of water (252 mg L^{-1}). For both solvents, statistically significant differences were obtained depending on the proportion of water employed. On the other hand, the extracts obtained with propyleneglycol did not show significant differences on the ∑ of bioactive polyphenols although obtained values are also satisfactory and they could be used for further applications, as well.

5 CONCLUSIONS

- ✓ The main critical experimental parameters affecting MSAT procedure at lab scale, sample size, ratio sample/dispersant, extraction solvent and volume were optimized by response surface matrix (RSM) design.
- ✓ Results showed that the use of 200 g of grape marc, its ratio with a dispersant medium (1.25, g/g) and 100 mL of solvent results on the best extraction efficiency in terms of TPC and AA.
- ✓ Ethyl lactate/water (50:50, v/v) and ethanol/water (50:50, v/v) provided the highest responses for the tested bioactivity parameters (TPC, AA and IC50). The ethyl lactate based-extract showed the highest extraction of the flavonols kampferol and quercetin, and their glycoside derivatives, whereas the ethanolic was more enriched in flavanols and procyanidins.
- ✓ In any case, all of them showed satisfactory TPC, AA, IC50 and individual polyphenolic content, therefore they could be used in further WPs and tasks, depending on the final applications.

6 ANNEX 1

The individual concentration (mg L^{-1}) of the detected polyphenols is shown in the Table presented below.

SOLVENT	%	Gallic acid	Catechin	Epicatechin	Epigallocatechin gallate	Epicatechin gallate	Quercetin-3-glucuronide	Rutin	Quercetin-3-glucoside	Quercetin	Kaempferol	Caftaric acid	Myricetin	∑ procyanidines (B1, B2, C1, C2)
Water	100	3.7 ± 0.1	30 ± 2	22 ± 1	nd	2.2 ± 0.3	5.5 ± 0.6	1.8 ± 0.2	5.7 ± 0.7	nd	nd	2.07 ± 0.04	nd	84 ± 3
Ethanol	25	5.3 ± 0.9	25 ± 1	21 ± 2	0.247 ± 0.002^{H}	2.1 ± 0.5	5.1 ± 0.3	0.7 ± 0.1	5.6 ± 0.5	0.09 ± 0.06	nd	0.581 ± 0.003	nd	49 ± 6
	50	5 ± 1	42 ± 9	30 ± 6	0.324 ± 0.007^{H}	16 ± 2	14 ± 2	2.3 ± 0.3	20 ± 3	0.52 ± 0.01	nd	0.8 ± 0.3	0.024 ± 0.006	134 ± 27
	75	4 ± 1	49 ± 2	34 ± 1	0.302 ± 0.001^{H}	16 ± 1	12 ± 2	1.3 ± 0.3	14 ± 4	1.34 ± 0.01	0.03 ± 0.01	0.5 ± 0.1	nd	77 ± 2
	100	3.0 ± 0.1	62 ± 9	40 ± 7	0.368 ± 0.004^{H}	18 ± 1.	12 ± 3	1.7 ± 0.1	16 ± 5	0.9 ± 0.4	nd	0.4 ± 0.1	nd	77 ± 1
Ethyl lactate	25	10 ± 3	39 ± 18	22 ± 10	nd	4 ± 2	10 ± 2	0.4 ± 0.1	8.5 ± 0.4	1.9 ± 0.1	0.07 ± 0.01	Nd	nd	66 ± 14
	50	6.8 ± 0.6	59 ± 7	24 ± 2	0.255 ± 0.009^{H}	14 ± 2	22 ± 4	0.5 ± 0.1	26 ± 5	5.4 ± 0.9	1.0 ± 0.53^{H}	nd	0.061 ± 0.007	93 ± 19
	75	3 ± 1	59 ± 1	21 ± 1	0.249 ± 0.001^{H}	15 ± 1	15 ± 2	1.34 ± 0.01	18 ± 1	0.99 ± 0.06	nd	nd	nd	35 ±
	100	2.5 ± 0.1	83 ± 37	24 ± 9	0.262 ± 0.003^{H}	18 ± 1	16 ± 5	1.50 ± 0.08	17 ± 7	0.7 ± 0.2	nd	nd	nd	37 ± 8
Propyleneglycol	25	8.8 ± 0.3	34 ± 16	26 ± 11	0.247 ± 0.002^{H}	2 ± 1	6 ± 2	0.52 ± 0.08	6 ± 2	0.11 ± 0.02	nd	0.8 ± 0.2^{L}	nd	58 ± 11
	50	10 ± 2	31 ± 4	25 ± 3	0.255 ± 0.008^{H}	4 ± 2	8 ± 2	0.80 ± 0.01	6.7 ± 0.4	0.35 ± 0.07	nd	1.1 ± 0.4^{L}	nd	58 ± 2
	75	11 ± 2	40 ± 9	30 ± 3	0.268 ± 0.002^{H}	8 ± 1	11 ± 1	0.63 ± 0.08	11 ± 1	1.2 ± 0.5	nd	1.4 ± 0.7	nd	85 ± 12
	100	5.7 ± 0.6	40 ± 4	31 ± 2	0.275 ± 0.007^{H}	12 ± 1	8 ± 1	0.76 ± 0.07	8.3 ± 0.8	1.2 ± 0.1	nd	0.8 ± 0.2	nd	65 ± 1

nd: not detected (lower that the limits of detection, LODs, of the employed methodology that were in all cases lower than 0.005 mg L^{-1})

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