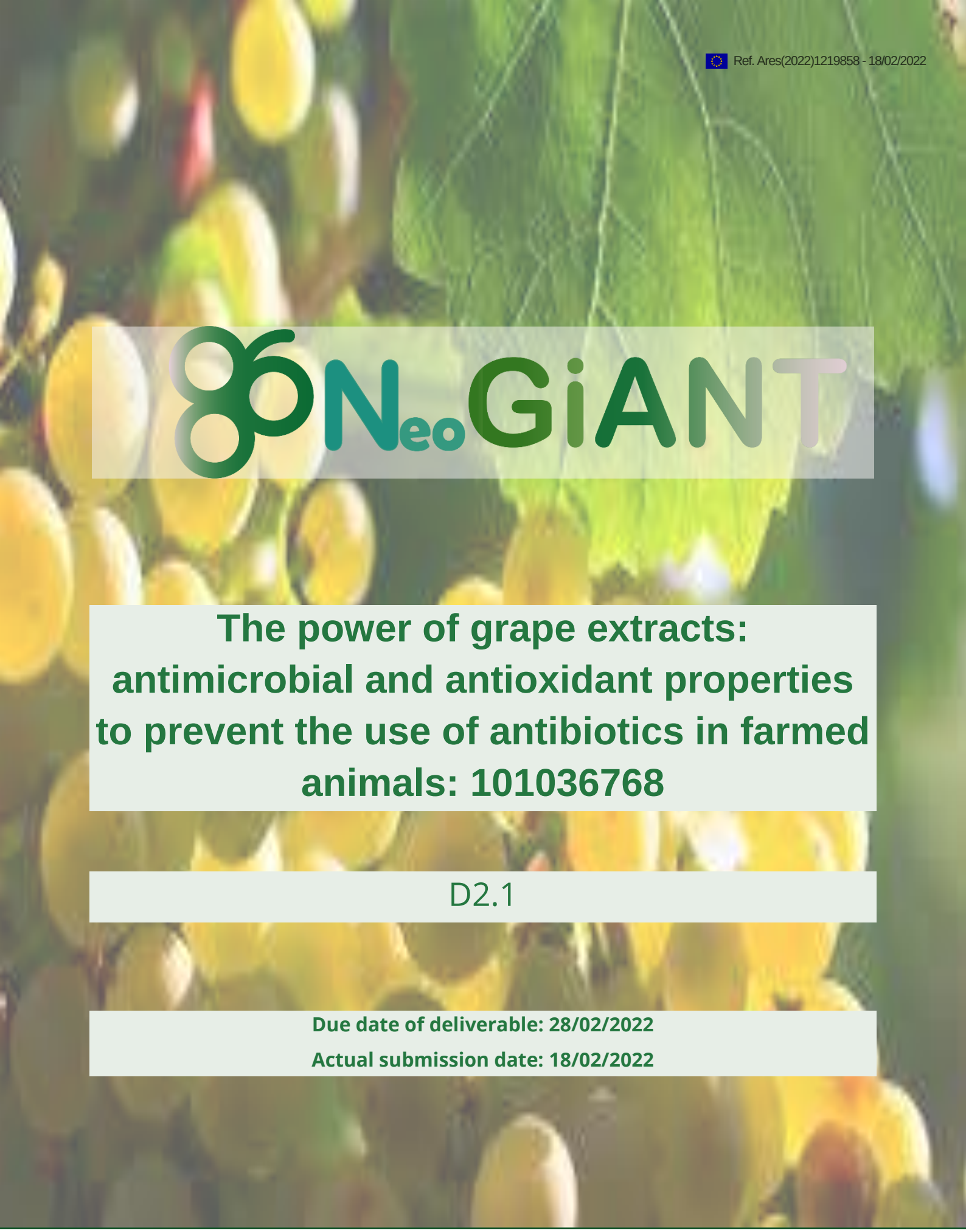
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The power of grape extracts: antimicrobial and antioxidant properties to prevent the use of antibiotics in farmed animals: 101036768

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PROJECT INFORMATION

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

Acronym: NeoGiANT

Call: H2020-LC-GD-2020-4

Topic: LC-GD-6-1-2020

Start date: 1st October 2021

Duration: 48 months

List of participants:

No.	Acronym	Participant organisation name	Country
1 (Coord)	USC	Universidade de Santiago de Compostela	Spain
2	MRI	Moredun Research Institute	United Kingdom
3	IBPRS	Instytut Biotechnologii Przemysłu Rolno-Spożywczego im. prof. Wacława Dąbrowskiego	Poland
4	VRI	Veterinary Research Institute	Czech Republic
5	MATE	Nemzeti Agrárkutatási és Innovációs Központ	Hungary
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

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DELIVERABLE DETAILS

Document Number:	D2.1
Document Title:	First grape extracts & characterization reports (Lab-scale)
Dissemination level	PU – Public
Period:	PR1
WP:	WP2
Task:	Task 2.1
Author:	UNIVERSITY OF SANTIAGO DE COMPOSTELA (USC)
Abstract:	This document corresponds to the Deliverable 2.1 First grape extracts & characterization reports (Lab-scale). It covers the description of the methods used to obtain the first extracts at lab-scale, as well as their physic-chemical characterization (pH, density), total polyphenolic index (TPI) and antioxidant activity (AA).

Version	Date	Change
V1	17/02/2022	Initial version

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1 ANALYSIS OF THE RAW MATERIAL (GRAPE MARC)

The starting plant material for obtaining the bioactive extracts that will form the basis of the NeoGiANT project is white grape marc. This by-product is generated after the winemaking process of white grapes (*Vitis vinifera* var. Albariño) grown in the Rías Baixas Designation of Origin (Galicia, NW Spain) for the production of high-quality white wines (2021 harvest). Like any other non-organic crop, wine grapes are treated with plant-protection products to prevent and cure potential pests that may affect them, particularly fungal pests. The residues of such phytosanitary products that can be detected in wine grapes are regulated and the maximum residue limits (MRLs) are presented in ***Table D2.1.1***. Given the way in which white wine is vinified, the grape marc obtained is very similar to the original grapes, with the exception of the squeezed must that follows the process to become wine.

This is why, to ensure the safety of the starting material, an analysis of the possible fungicide residues that the NeoGiANT grape marc may contain was carried out, to confirm that they are below the legal limits (although we remind that the limits are for grapes and not for grape marc, which will be a material with a more concentrated content by definition). The grape marc extraction process was designed for the extraction of polyphenols and other bioactive compounds. Thus, problems with plant-protection products' residues in the NeoGiANT extracts are not expected. However, in any case, the levels of these compounds will be carefully controlled throughout the process.

To guarantee the absence of unwanted compounds such as fungicides in the raw material and therefore, in the further obtained extracts, the raw material was submitted to a previously validated analytical methodology in the LIDSA group. Grape marc was extracted with ethyl acetate and analysed by gas chromatography-tandem mass spectrometry (GC-MS/MS). The 17 target fungicides selected are the most widely employed in vineyards. The detected concentration, expressed as ng g^{-1} (dry weight) is shown in ***Table D2.1.1***. The obtained values were compared with the MRLs established by the EU legislation [1] for wine grapes.

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Table D2.1.1. Target fungicides, CAS identification number, concentration (ng g^{-1}) found in the raw material and MRLs (ng g^{-1}) established by the European Regulation [1].

FUNGICIDE	CAS	CONCENTRATION (ng g^{-1})	MRLs in wine grapes (ng g^{-1})
Metalaxyl	57837-19-1	34 ± 6	1000
Cyprodinil	121552-61-2	1760 ± 169	3000
Tolylfuanid	731-27-1	n.d	20
Procymidone	32809-16-8	n.d	10
Folpet	133-07-3	n.d	20000
Fludioxonil	131341-86-1	377 ± 67	4000
Kresoxim-methyl	143390-89-0	n.d	1500
Iprovalicarb (E+Z) [a]	140923-17-7	740 ± 102	2000
Myclobutanil	88671-89-0	65 ± 9	1500
Trifloxystrobin	141517-21-7	104 ± 33	3000
Benalaxyl	71626-11-4	n.d	300
Fenhexamid	126833-17-8	4629 ± 1552	15000
Tebuconazole	80443-41-0	215 ± 47	1000
Pyraclostrobin	175013-18-0	68 ± 27	2000
Azoxystrobin	131860-33-8	n.d	3000
Dimethomorph (E+Z) [a]	113210-97-2	2046 ± 347	3000

[a] Sum of both isomers, n.d: not detected (lower than the limits of detection, LODs, of the employed methodology that were in all cases lower than 1 ng g^{-1}).

As can be seen in ***Table D2.1.1.***, several fungicides were detected in the raw material but, in all cases, they were well below the established MRLs for wine grapes showing that the raw material can be safely employed to obtain extracts.

2 EXPERIMENTAL PROCEDURE TO OBTAIN FIRST GRAPE EXTRACTS AT LAB-SCALE

First grape extracts at lab-scale were obtained by LIDSA-USC employing an environmentally friendly patented procedure based on Medium-Scale Ambient Temperature system (MSAT) [2,3]. This procedure allows obtaining representative volume of extracts for these preliminary lab-scale experiments. A schematic representation of the MSAT- based experimental procedure is depicted in ***Figure D2.1.1.***

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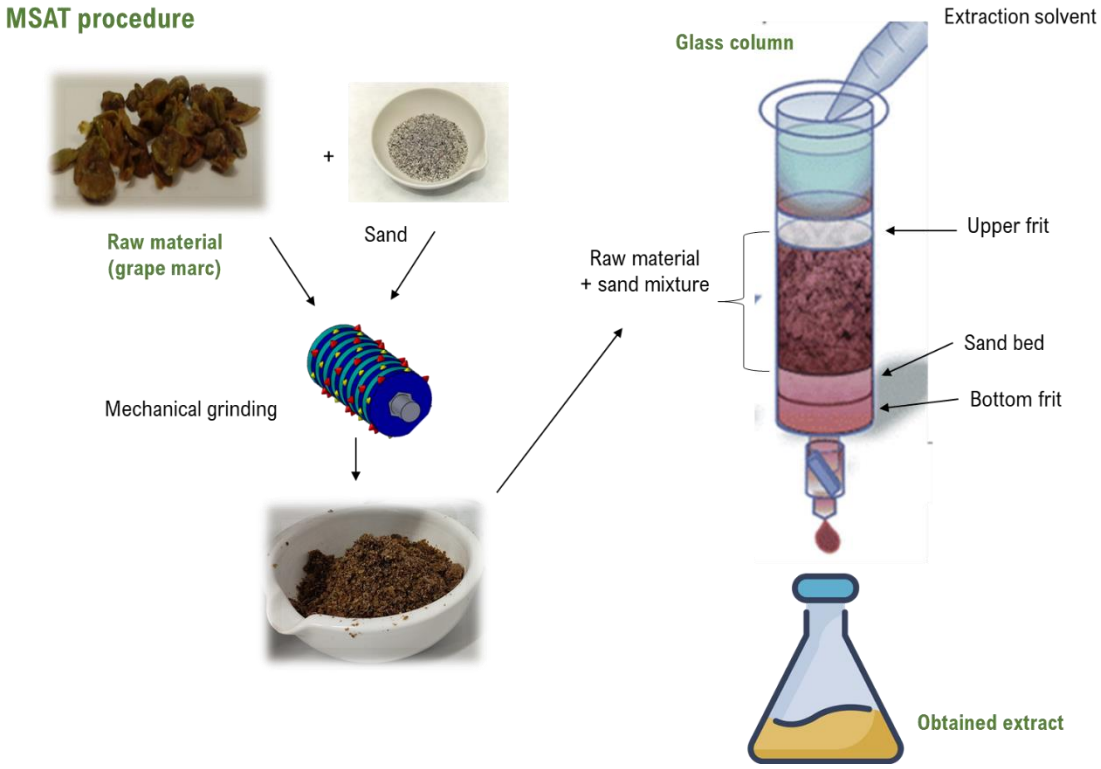


Figure D2.1.1. Schematic representation of the MSAT experimental procedure.

The NeoGiANT project foresees the development of a wide range of applications for grape marc extracts, including formulations for animal feed, therapeutics and preservatives. Based on the investigations performed over last years in the research group LIDSA to obtain extracts from raw materials of natural origin, the extracting solvent is a key factor in the process, as it will allow modulation of both the qualitative and quantitative profile of the bioactive components of the different extracts. Hence, in these preliminary experiments at lab-scale, different solvents were tested.

2.1 Tested solvents

The used solvent to obtain extracts is crucial to modulate the polarity profile of the bioactive compounds present in the ready-to-use extracts. The solvents selection was assessed based on further WP2 tasks (2.2). Since a deep analytical characterization is planned to evaluate the presence of bioactive compounds, mainly polyphenols (most of them are polar or semi-polar compounds) as well as to guarantee the absence of unwanted compounds such as pesticides (most of them present low polarity) in the extracts, solvents presenting a broad range of

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polarity, including hydro-organic mixtures have been proposed to obtain the first extracts at lab-scale.

The initial proposal included the study of at least three solvents (task 2.1), namely ethyl lactate, propylene glycol and ethanol and their respective hydro-organic mixtures, all of them Generally Recognized as Safe (GRAS). After meeting with the USC formulations team, it has been decided to include other solvents in these preliminary studies, to have a full range of knowledge.

In this way, classical solvents such as dichloromethane, dimethyl sulfoxide, and dimethylformamide have been also evaluated, and other GRAS solvents as acetone were also tested (***Table D2.1.2***.)

Table D2.1.2. Tested organic solvents and their proportions in hydro-organic mixtures to obtain the first extracts at lab-scale

TESTED ORGANIC SOLVENTS TO OBTAIN FIRST EXTRACTS AT LAB-SCALE	
SOLVENT	**PROPORTION**
Ethyl lactate/water	50:50 (v/v)
Ethanol/water	50:50 (v/v)
Propylene glycol/water	50:50 (v/v)
Dimethylformamide	100%
Dimethylsulfoxide	100%
Acetone	100%
Dichloromethane	100%

2.2 Optimization of sample size to solvent ratio

It has been demonstrated that the sample size/solvent ratio (even if the proportion is kept constant) is one of the experimental parameters that highly affects the presence of bioactive compounds in the final extract. In this way and based on the LIDSA group background in obtaining extracts rich in bioactive compounds, the initial sample (grape marc) size was kept constant at 20 g and different volumes were considered for each one of the solvents presented in ***Table D2.1.2***. The obtained extracts at lab-scale can be seen in ***Figure D2.1.2***.

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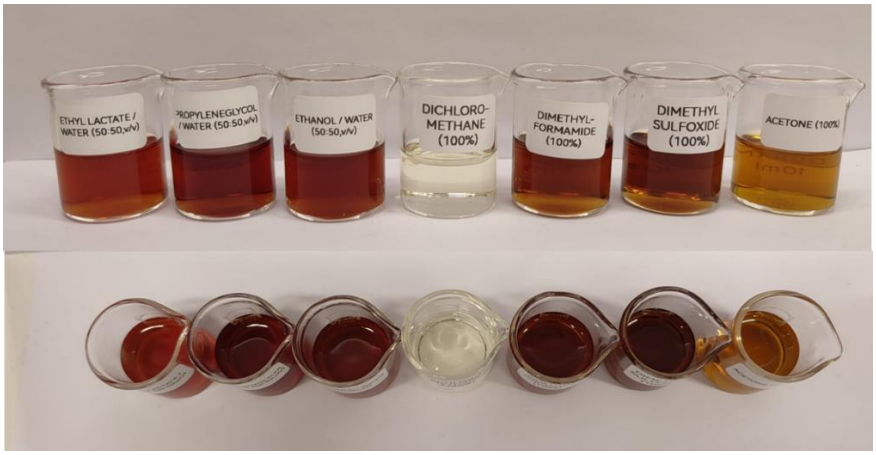


Figure D2.1.2. First lab-scale extracts obtained for the tested organic solvents and their proportions in hydro-organic mixtures.

3 PRELIMINARY CHARACTERIZATION OF THE OBTAINED EXTRACTS AT LAB-SCALE

The basic physic-chemical parameters pH and density were assessed for all the obtained extracts at lab scale. In addition, spectrophotometric indexes, such as the total polyphenolic index (TPI) and the antioxidant activity (AA) were evaluated.

Plant polyphenols (target compounds) are natural secondary metabolites produced in plant cells that exhibit a remarkably diverse range of bio-physicochemical properties. They have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores, protection against solar radiation, as well as plant reproduction, nutrition, and growth [4].

The polyphenols content of the extracts (TPI) was determined by the Folin-Ciocalteau spectrophotometric method described by Singleton and Rossi [5]. The extracts are made to react with Folin&Ciocalteu's reagent in the dark. The reagent contains W^{+6} and Mo^{+6} salts which are reduced to +3 oxidation states by reacting with the polyphenols. This causes a change in the colour of the solution that can be measured by UV-Vis spectrophotometry at 760 nm wavelength. The procedure relies on the transfer of electrons in alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds in the sample (grape marc extract). Results are expressed as mg Gallic acid equivalents (GAE) per L of extract.

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The antioxidant activity (AA) was evaluated employing the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) method described by Brand-Williams [6]. The DPPH assay is used to predict antioxidant activities by using a stable free radical. The assay is based on the measurement (at 515 nm wavelength) of the scavenging capacity of antioxidants towards DPPH, and therefore determinates free radical scavenging capacity of the extract. The DPPH reagent (violet in its oxidised form) provides free radicals to the reaction which can be trapped by the polyphenols present in the samples, resulting in a colour change to yellow. Trolox®, a water-soluble synthetic analogue of vitamin E, is used as the reference antioxidant. Results are expressed as mmol Trolox equivalents (TRE) per L of extract.

TPI and AA were assessed for all obtained lab-scale extracts. First results revealed that the extraction efficiency was highly affected by the raw material grinding process (TPI values ranged between 390-1860 mg GAE L^{-1} and AA between 3.0-8.5 mmol TRE L^{-1}). To improve the extraction efficiency, the initial manual sample disruption was substituted by a mechanical procedure (intensive treatment), allowing a reduction on the sample size and then, enhancing the extraction efficiency employing lower solvent volume.

The final experimental conditions involve the use of 20 g of raw material (grape marc), and 50 mL of solvent or hydro-organic mixture. The obtained results for TPI and AA, as well as for other physic-chemical parameters such as pH or density are summarized in ***Table D2.1.3***.

Table D2.1.3. Physic-chemical characterization of the first lab-scale extracts: pH, density, TPI and AA.

Solvent	pH	Density (g mL^{-1})	TPI (mg GAE L^{-1})	AA (mmol TRE L^{-1})
Ethyl lactate/water	4.19	1.022	2879 ± 31	29.9 ± 0.6
Ethanol/water	5.08	0.938	2204 ± 7	22.6 ± 2.2
Propylene glycol/water	5.00	0.994	2163 ± 4	22.6 ± 0.4
Dimethylformamide	7.36	0.969	3986 ± 10	39.3 ± 2.3
Dimethylsulfoxide	7.99	1.131	3381 ± 64	27.2 ± 1.8
Acetone	5.85	0.852	2614 ± 20	29.5 ± 0.2
Dichloromethane	4.80	1.285	n.c	n.c

n.c. Not calculated since dichloromethane is not adequate to perform TPI and AA analysis

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A graphical comparison of the obtained results for TPI and AA analysis for all the extracts is depicted in Figure ***D2.1.3***.

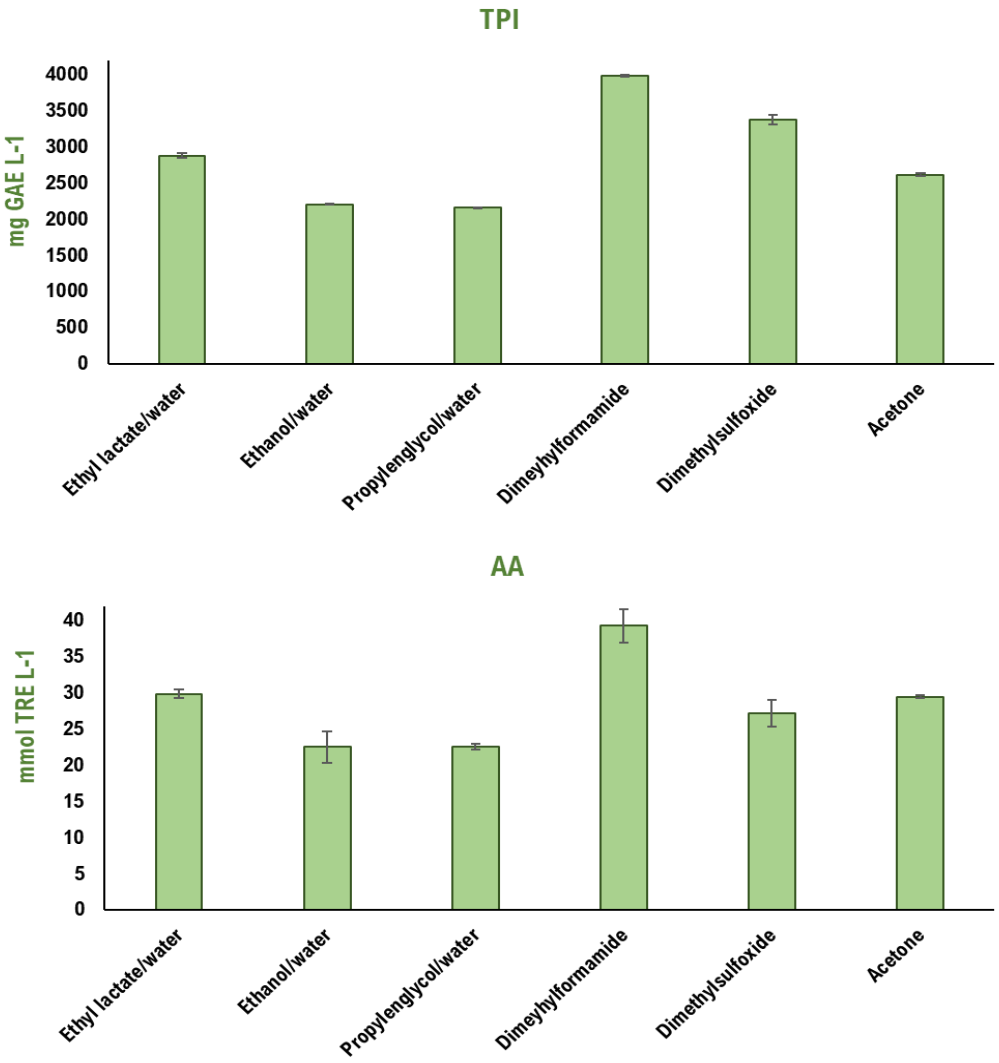


Figure D2.1.3. TPI and AA values for the extracts obtained at lab-scale.

Satisfactory TPI and AA values were obtained for all obtained lab-scale extracts. Therefore, all of them are suitable for further experiments and they will be deeply characterized to identify and quantify bioactive molecules (polyphenols) and to evaluate the absence of unwanted compounds such as fungicides (Task 2.2.).

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4 CONCLUSIONS

- ✓ Grape marc from *Vitis vinifera* var. Albariño (2021 harvest) complies with the MRLs for fungicides and is, therefore, apt to become the raw material for the NeoGiANT extracts.
- ✓ Different GRAS solvents and their hydro-organic mixtures were selected based on their further application to obtain the first extracts at lab-scale.
- ✓ Several MSAT experimental parameters (sample size, grinding procedure and solvent volume) were optimized to obtain the highest extraction efficiency.
- ✓ Results in terms of TPI and AA showed that all the obtained extracts at lab-scale are suitable for further experiments.

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