CEN

WORKSHOP

AGREEMENT

CWA 18342

January 2026

ICS 67.080.01

English version

Guidelines for antioxidant assessment in extracts from an agri-food by-product: white grape marc

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Foreword

This CEN Workshop Agreement (CWA 18342:2026) has been developed in accordance with the CEN-CENELEC Guide 29 “CEN/CENELEC Workshop Agreements — A rapid way to standardization” and with the relevant provisions of CEN/CENELEC Internal Regulations — Part 2. It was approved by the Workshop CEN “NeoGiANT”, the secretariat of which is held by UNE, consisting of representatives of interested parties on 2026-01-02, the constitution of which was supported by CEN following the public call for participation made on 2025-08-22. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

The final text of this CEN Workshop Agreement was provided to CEN for publication on 2026-01-14.

Results incorporated in this CWA received funding from the European Union’s Horizon 2020 research and innovation programme NeoGiANT[1] under grant agreement No 101036768.

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[1] https://www.neogiant.eu/

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Introduction

The characterization of extracts of natural origin, and specifically from agri-food by-products, is challenging from an analytical point of view due to the wide variety of methodologies described in the literature and due to the large variety of matrices.

However, harmonization in this field is of great importance to establish unified criteria for the comparison of the analytical results obtained. In this sense, this CEN Workshop Agreement establishes a unified methodology not only to be applied to the extracts used in the project, obtained from a specific agri-food waste such as white grape marc, but also to those derived from other by-products of a similar nature.

This document specifies several analytical methodologies based on ultraviolet visible (UV-Vis) spectroscopy for the characterization of extracts obtained from an agri-food residue, white grape marc. The provided methods could be applied to extracts from other agri-food wastes and by-products, such as fruits or vegetables.

For the determination of the Total Polyphenolic Content (TPC) in the extracts, a UV-Vis microplate-based methodology using the Folin-Ciocalteu phenol reagent is detailed.

For the determination of the Antioxidant Activity (AA) of extracts, two different UV-Vis microplate-based methodologies using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as reagents are described.

This CEN Workshop Agreement is developed in the framework of NeoGiANT H2020 project. The main objective of NeoGiANT project is to develop and validate innovative natural formulations with antimicrobial and antioxidant properties from the valorisation of white grape marc, to be used in cattle, swine, poultry and farmed fish production, aiming to reduce the dependence on the use of antibiotics in livestock and aquaculture production.

1 Scope

This document aims to harmonize the most employed methodologies to determine Total Polyphenolic Content (TPC) and Antioxidant Activity (AA) in extracts from agri-food by-products.

Although these methodologies are derived within the framework of the NeoGiANT H2020 project, using white grape marc extracts, they can be extrapolated to extracts from agri-food industry, such as fruit or vegetable by-products.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document. For the purposes of this document, the following acronyms apply.

AA	Antioxidant Activity
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	2,2-diphenyl-1-picrylhydrazyl
GAE	Gallic acid equivalent
M	Molar
PTFE	Polytetrafluoroethylene
TPC	Total Polyphenolic Content
TRE	Trolox equivalent
UV-Vis	Ultraviolet-Visible

4 Determination of Total Polyphenolic Content by the Folin Ciocalteu method for UV-Vis microplate reader

4.1 Introduction

Clause 4 describes a methodology for the determination of Total Polyphenolic Content (TPC) in white grape marc extracts in a UV-Vis 96-well microplate reader through the Folin-Ciocalteu method.

4.2 Principle

The Folin–Ciocalteu reagent is composed by phosphomolybdic acid and phosphotungstic acid. In an alkaline medium, phenolic compounds (-OH groups and other reducing substances) donate electrons to the reagent, reducing the molybdenum (Mo^{6+}) and tungsten (W^{6+}) ions present in the complex. As a result, lower oxidation state oxides (Mo^{5+}, W^{5+}) are formed, generating an intense blue-coloured complex [1] (see Figure 1).

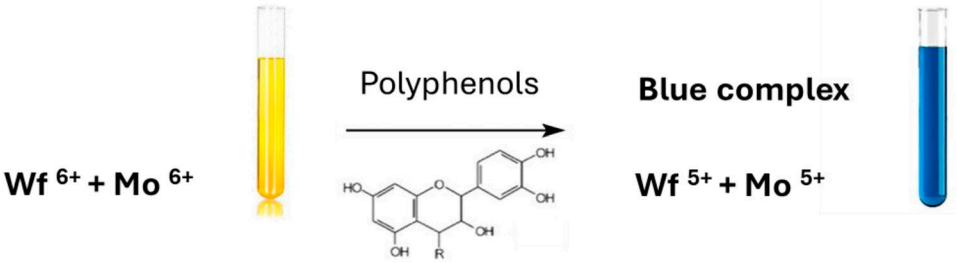

Figure 1 — Folin-Ciocalteu methodology principle

The intensity of the blue colour is proportional to the amount of reducing compounds present (mainly phenols) and their concentration is measured spectrophotometrically (UV-Vis), at wavelengths in the range: 760 nm to 765 nm.

4.3 Reagents

— Milli-Q® water[2] (resistivity of 18,2 MΩ·cm).

— Folin-Ciocalteu's phenol reagent (2M with respect to acid). Dilution 10 % (v/v) in Milli-Q® water (10 mL of reagent to 100 mL of Milli-Q® water in a 100 mL volumetric flask).

— Sodium carbonate anhydrous (purity > 99,5 %). Dilution 7,5 % (m/v) in Milli-Q® water (weight 7,5 g of anhydrous sodium carbonate and dissolve it to close to 100 mL of Milli-Q® water in a 100 mL beaker under magnetic stirring). It is important to note that the reaction is exothermic, so it is recommended to first dissolve the mixture in a beaker and then transfer it to a volumetric flask and fill it up with Milli-Q® water to 100 mL once it has cooled to room temperature.

— Gallic acid anhydrous (purity > 99 %). Stock solution of 1000 mg L^{-1} in Milli-Q® water (weight 10 mg of gallic acid and dilute it to 10 mL of Milli-Q® water in a 10 mL volumetric flask).

4.4 Apparatus

— Spatula.

— Analytical balance. Capacity to weigh to an accuracy of ± 0,0001 g.

— Pipettes to cover the volume range for the solutions.

— Volumetric flasks (5 mL, 10 mL, 100 mL).

— Beaker (100 mL).

— Polytetrafluoroethylene (PTFE) syringe filters (0,22 µm).

— Magnetic stirrer.

— Vortex mixer.

[2] Milli-Q® is an example of a suitable product available commercially. This information is given for the convenience of users of this CEN Workshop Agreement and does not constitute an endorsement by CEN of this product.

— Glass vials (or Eppendorf).

— 96-well microplate (polystyrene with optical transparency).

— Spectrophotometer set at 760 nm and able to accommodate 96-well microplates.

4.5 Samples

At least 5 mL of the samples (grape marc liquid extracts) will be kept at −20 °C in a glass vial (or Eppendorf) and protected from light until their analysis.

4.6 Experimental procedure

4.6.1 Calibration curve

Gallic acid calibration curve is prepared in Milli-Q® water from the initial stock solution (see subclause 4.3), following the dilutions in Table 1.

Table 1 — Standard solutions to carry out the calibration curve of gallic acid

Concentration level	Gallic acid concentration (mg L^{-1}) [a]	Gallic acid stock solution volume (µL)	Milli-Q® water volume (µL)	Total volume (µL)
1	20	20	980	1 000
2	40	40	960	1 000
3	60	60	940	1 000
4	80	80	920	1 000
5	100	100	900	1 000
6	120	120	880	1 000
7	140	140	860	1 000
8	160	160	840	1 000

[a] Linear range.

Then, 20 µL of each concentration level are placed (by triplicate) on the 96-well microplate as shown in Figure 2.

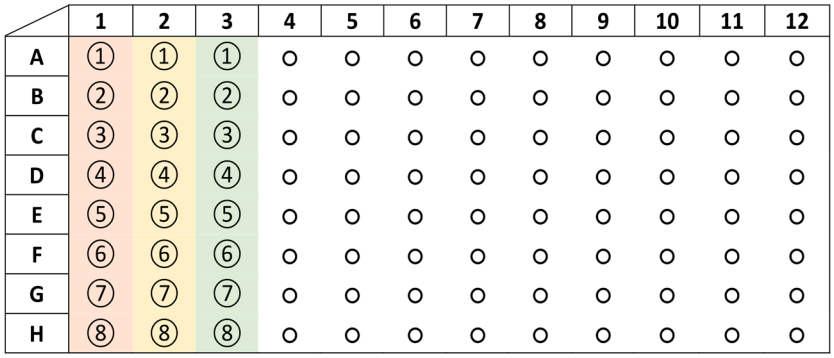

Figure 2 — Representation of the 96-well microplate for the gallic acid calibration curve preparation

Afterwards, 100 µL of the Folin-Ciocalteu 10 % aqueous dilution and 80 µL of the sodium carbonate 7,5 % aqueous solution (See preparation in subclause 4.3) are added. It is mandatory to add the reagents in this order for the reaction to take place.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 30 min. Afterwards, absorbance at 760 nm is measured.

4.6.2 Sample preparation

The samples (grape marc extracts in liquid form) are homogenized at room temperature in a vortex and filtered through 0,22 µm PTFE filters. Then, different dilutions (1:50, v/v; 1:100, v/v; 1:200, v/v) are prepared in Milli-Q® water as shown in Table 2.

Table 2 — Data to carry out the sample dilutions

Code	Dilution factor (v/v)	Sample volume (µL)	Milli-Q® water volume (µL)	Total volume (µL)
A	50	20	980	1 000
B	100	10	990	1 000
C	200	5	995	1 000

20 µL of each dilution (A, B, C) are placed (by triplicate) on the 96-well microplate as shown in Figure 3.

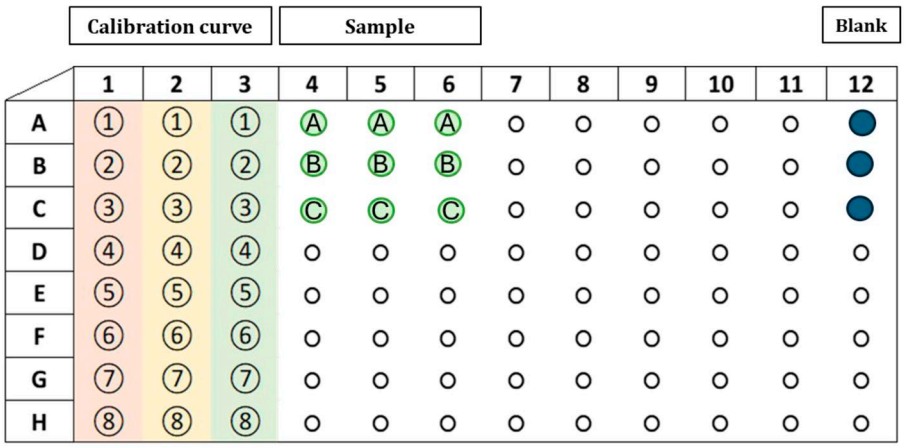

Figure 3 — Representation of the 96-well microplate for the sample and blank preparation

Then, 100 µL of the Folin-Ciocalteu 10 % aqueous dilution and 80 µL of the sodium carbonate 7,5 % aqueous solution (See preparation in subclause 4.3) are added. It is mandatory to add the reagents in this order for the reaction to take place.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 30 min. Then, absorbance at 760 nm is measured.

4.6.3 Blank preparation

To avoid overestimating the results, a blank sample (by triplicate) is performed with each batch of samples as follows: 20 µL of Milli-Q® water is placed (by triplicate) on the 96-well microplate as it is also depicted in Figure 3. Then, 100 µL of the Folin-Ciocalteu 10 % aqueous dilution and 80 µL of the sodium carbonate 7,5 % aqueous solution (see preparation in subclause 4.3) are added. It is mandatory to add the reagents in this order for the reaction to take place.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 30 min. Then, absorbance at 760 nm is measured.

4.7 Quantification

The gallic acid calibration curve (see subclause 4.6.1) is:

Calibration curve: $y = a + bx$

where y: absorbance (sample – blank); a: intersection; b: slope; x: gallic acid concentration.

TPC results are expressed in milligrams of gallic acid equivalents per Liter of sample (mg GAE L^{-1}).

$$\text{TPC}\left(\text{mg GAE L}^{-1}\right) = \frac{\left(\text{Abs}\left(\text{Sample} - \text{Blank}\right) - \text{a}\right) * V_{TOT}}{\text{b} * V_{SAMP}}$$

where V_{TOT}: final volume of the dilution; V_{SAMP}: sample volume (see Table 2).

4.8 Precision

4.8.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a day (24 h), should not exceed 5 % (expressed as coefficient of variation).

4.8.2 Intralaboratory reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within different days (>24 h), should not exceed 8 % (expressed as coefficient of variation).

5 Determination of Antioxidant activity by the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method for UV-Vis microplate reader

5.1 Introduction

Clause 5 describes a methodology for the determination of Antioxidant Activity (AA) in white grape marc extracts in a UV-Vis 96-well microplate reader through the ABTS method.

5.2 Principle

The cationic radical ABTS•+ is generated by oxidising ABTS with an oxidising agent (commonly potassium persulphate). This radical has an intense blue-green colour and shows strong absorption bands in the ranges 640 nm to 660 nm, 730 nm – 750 nm and 820 nm – 840 nm, depending on the solvent used. When a compound with antioxidant capacity is added, it donates electrons or hydrogen atoms to the ABTS•+ radical, reducing it back to its stable ABTS form, resulting in a colour intensity decrease, that is proportional to the antioxidant power of the sample [2] (see Figure 4).

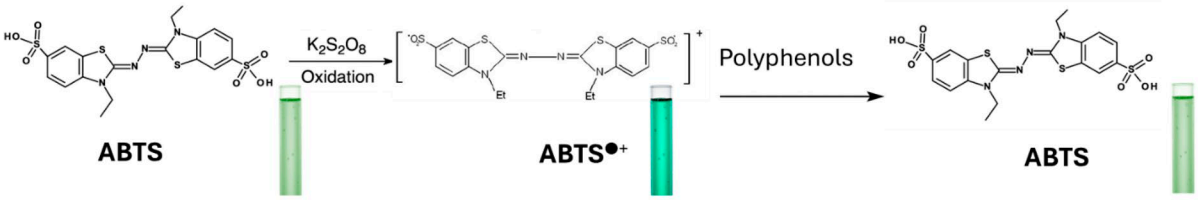

Figure 4 — ABTS methodology principle

5.3 Reagents

— Milli-Q® water (resistivity of 18,2 MΩ·cm).

— Methanol (purity > 99,9 %).

— ABTS reagent: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (purity > 98 %). 7 mM solution in Milli-Q® water (weight 0,0960 g of ABTS reagent and dilute it to 25 mL in a 25 mL volumetric flask. Keep the solution at 4 °C and protected from light.

— Potassium persulfate (purity > 99 %). 2.45 mM solution in Milli-Q® water (weight 0.0660 g and dilute it to 100 mL in a 100 mL volumetric flask). Keep the solution at 4 °C and protected from light.

— Trolox reagent: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (purity > 99 %). 4 mM stock solution in methanol (weight 10 mg of Trolox reagent and dilute it to 10 mL in a 10 mL volumetric flask).

— ABTS•+ radical solution. 5 mL of the ABTS 7 mM solution and mix with 5 mL of the potassium persulfate 2,45 mM solution in a 10 mL glass amber vial. The mixture must be kept refrigerated (4 °C) and protected from light. It must be freshly prepared every 3 days and its absorbance should be greater than 0,700 (it is recommended to check it before each use).

5.4 Apparatus

— Spatula.

— Analytical balance. Capacity to weigh to an accuracy of ± 0,0001 g.

— Pipettes to cover the volume range for the solutions.

— Volumetric flasks (5 mL, 10 mL, 25 mL, 100 mL).

— Polytetrafluoroethylene (PTFE) syringe filters (0,22 µm).

— Glass vials (or Eppendorf).

— Vortex mixer.

— 96-well microplate (polystyrene with optical transparency).

— Spectrophotometer set at 748 nm and able to accommodate 96-well microplates.

5.5 Samples

At least 5 mL of samples (liquid grape marc extracts) will be kept at −20 °C in a glass vial (or Eppendorf) and protected from light until their analysis.

5.6 Experimental procedure

5.6.1 Calibration curve

Trolox calibration curve is prepared in methanol from the initial stock solution (see subclause 5.3), following the dilutions in Table 3.

Table 3 — Standard solutions to carry out the calibration curve of Trolox

Concentration level	Trolox concentration (mmol L^{-1})[a]	Trolox stock solution volume (µL)	Methanol volume (µL)	Total volume (µL)
1	0,008	10	4 990	5 000
2	0,016	20	4 980	5 000
3	0,032	40	4 960	5 000
4	0,064	80	4 920	5 000
5	0,096	120	4 880	5 000
6	0,128	160	4 840	5 000
7	0,144	180	4 820	5 000
8	0,160	200	4 800	5 000
[a] Linear range.				

Then, 50 µL of each concentration level are placed (by triplicate) on the 96-well microplate as follows in Figure 5.

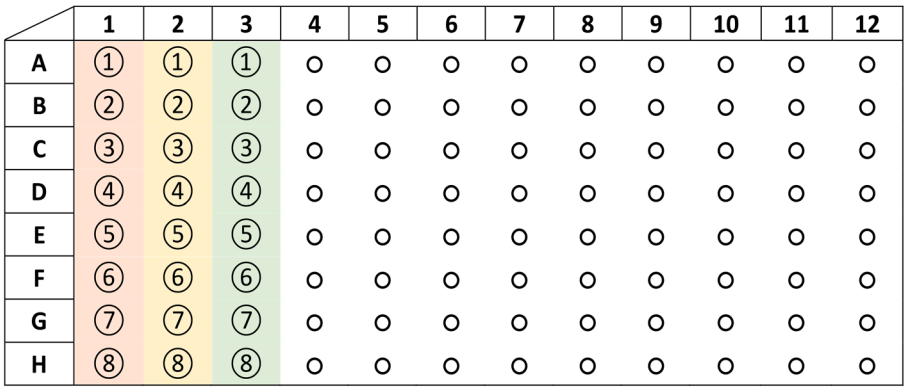

Figure 5 — Representation of the 96-well microplate for the Trolox calibration curve preparation

Afterwards, 200 µL of the ABTS•+ radical solution (see subclause 5.3) is added.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 7 min and then absorbance is measured at 748 nm.

5.6.2 Sample preparation

The samples (in liquid form) are homogenized at room temperature in a vortex and filtered through 0,22 µm PTFE filters. Then, different dilutions (1:2, v/v; 1:5, v/v; 1:10, v/v;1:20, v/v; 1:50, v/v; 1:100, v/v and 1:200, v/v) are carried out in Milli-Q® water as follows in Table 4.

Table 4 — Data to carry out the sample dilutions

Code	Dilution factor (v/v)	Sample volume (µL)	Milli-Q® water volume (µL)	Total volume (µL)
A	2	500	500	1 000
B	5	200	800	1 000
C	10	100	900	1 000
D	20	50	950	1 000
E	50	20	980	1 000
F	100	10	990	1 000
G	200	5	995	1 000

Then, 50 µL of each dilution (A, B, C, D, E, F, G) are placed in the microplate, as follows in Figure 6.

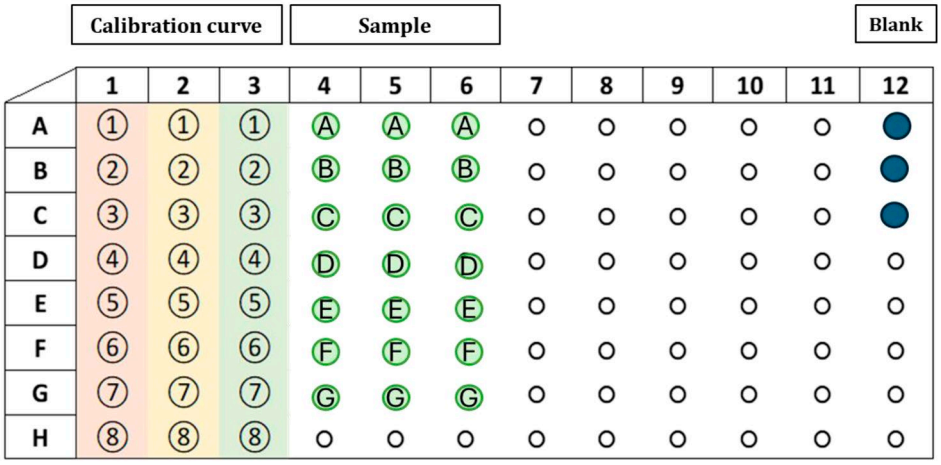

Figure 6 — Representation of the 96-well microplate for the sample and blank preparation

Then, 200 µL of the ABTS•+ radical solution (see subclause 5.3) is added.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 7 min and then absorbance is measured at 748 nm.

5.6.3 Blank preparation

To avoid overestimating the results, a blank sample is performed with each batch of samples as follows: 50 µL of Milli-Q® water is placed (by triplicate) on the 96-well microplate as it is also depicted in Figure 6. Afterwards, 200 µL of the $ABTS^{\bullet+}$ radical solution (see subclause 5.3) is added. Its absorbance value (Abs_{ABTS}) will be used as the blank (see subclause 5.7).

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 7 min and then absorbance is measured at 748 nm.

5.7 Quantification

The Trolox calibration curve (see subclause 5.6.1) is:

Calibration curve: $y = a + bx$

where y: absorbance (blank – sample); a: intersection; b: slope; x: Trolox concentration

AA results are expressed in milimol of Trolox equivalents per Liter of sample (mmol TRE L^{-1})

$$AA\left(\text{mmol TRE } L^{-1}\right) = \frac{\left(\left(Abs_{ABTS} - Abs_{SAMP}\right) - a\right) * V_{TOT}}{b * V_{SAMP}}$$

where Abs_{ABTS}: blank absorbance; Abs_{SAMP}: sample absorbance; V_{TOT}: final volume of the dilution; V_{SAMP}: sample volume (see Table 4).

5.8 Precision

5.8.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a day (24 h), should not exceed 5 % (expressed as coefficient of variation).

5.8.2 Intralaboratory reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within different days (>24 h), should not exceed 8 % (expressed as coefficient of variation).

6 Determination of Antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method for UV-Vis microplate reader

6.1 Introduction

Clause 6 describes a methodology for the determination of Antioxidant Activity (AA) in white grape marc extracts in a UV-Vis 96-well microplate through the DPPH method.

6.2 Principle

The DPPH• radical is a molecule with an intense purple colour, which has a characteristic absorption band around 517 nm. When an antioxidant compound is added, it donates an electron or a hydrogen atom to the DPPH• radical and, as a result, the radical is reduced and loses its purple colour, changing to a pale yellow hue (non-radical form) [3] (see Figure 7).

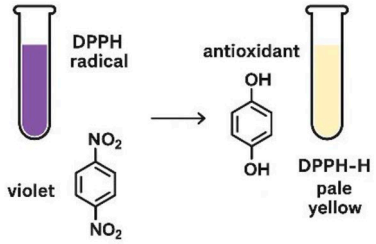

Figure 7 — DPPH methodology principle

6.3 Reagents

— Milli-Q® water (resistivity of 18,2 MΩ·cm).

— Methanol (purity > 99,9 %).

— DPPH reagent: 2,2-diphenyl-1-picrylhydrazyl free radical (purity > 97 %). 0,25 mM solution in methanol (weight 0,0080 g of DPPH reagent and dilute it to 50 mL in a 50 mL volumetric flask). Keep the solution at 4 °C and protected from light. This solution must be prepared weekly.

— Trolox reagent: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (purity > 99 %). 4 mM solution in methanol (weight 10 mg of Trolox reagent and dilute it to 10 mL in a 10 mL volumetric flask).

6.4 Apparatus

— Spatula.

— Analytical balance. Capacity to weigh to an accuracy of ± 0,0001 g.

— Pipettes to cover the volume range for the solutions.

— Volumetric flasks (10 mL, 50 mL).

— Polytetrafluoroethylene (PTFE filters (0,22 μm).

— Glass vials (or Eppendorf).

— Vortex mixer.

— 96-well microplate (polystyrene with optical transparency).

— Spectrophotometer set at 515 nm and able to accommodate 96-well microplates.

6.5 Samples

At least 5 mL of sample (liquid grape marc extract) will be kept at −20 °C in a glass vial (or Eppendorf) and protected from light until their analysis.

6.6 Experimental procedure

6.6.1 Calibration curve

Trolox calibration curve is prepared in methanol from the initial stock solution (see subclause 6.3), following the dilutions in Table 5.

Table 5 — Standard solutions to carry out the calibration curve of Trolox

Concentration level	Trolox concentration (mmol L^{-1})[a]	Trolox stock solution volume (µL)	Methanol volume (µL)	Total volume (µL)
1	0,008	10	4 990	5 000
2	0,016	20	4 980	5 000
3	0,032	40	4 960	5 000
4	0,064	80	4 920	5 000
5	0,096	120	4 880	5 000
6	0,128	160	4 840	5 000
7	0,144	180	4 820	5 000
8	0,160	200	4 800	5 000
[a] Linear range.				

Then, 100 µL of each concentration level are placed (by triplicate) on the 96-well microplate as shown in Figure 8.

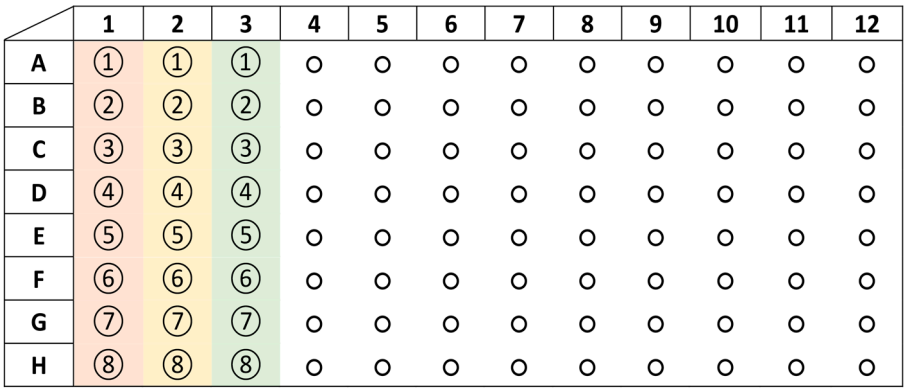

Figure 8 — Representation of the 96-well microplate for the Trolox calibration curve preparation

Afterwards, 100 µL of the DPPH 0.25 mM solution (see subclause 6.3) are added.

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 10 min and then absorbance is measured at 515 nm.

6.6.2 Sample preparation

The samples (in liquid form) are homogenized at room temperature in a vortex and filtered through 0,22 µm PTFE filters. Then, different dilutions (1:2, v/v; 1:5, v/v; 1:10, v/v; 1:20, v/v; 1:50, v/v; 1:100, v/v and 1:200, v/v) are carried out in Milli-Q® water as follows in Table 6.

Table 6 — Data to carry out the sample dilutions

Code	Dilution factor (v/v)	Sample volume (µL)	Milli-Q® water volume (µL)	Total volume (µL)
A	2	500	500	1 000
B	5	200	800	1 000
C	10	100	900	1 000
D	20	50	950	1 000
E	50	20	980	1 000
F	100	10	990	1 000
G	200	5	995	1 000

Then, 100 µL of each dilution (A. B, C, D, E, F, G) are placed in the microplate, as follows in Figure 9.

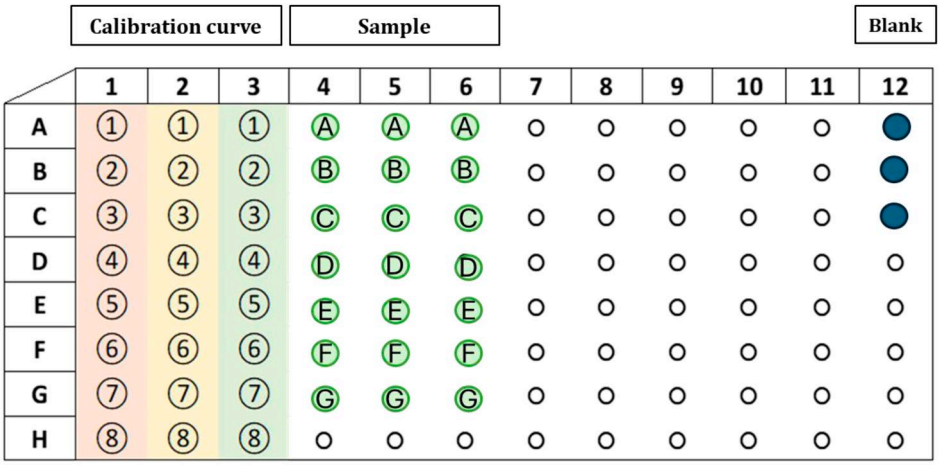

Figure 9 — Representation of the 96-well microplate for the sample and blank preparation

Then, 100 µL of the DPPH 0.25 mM solution (see subclause 6.3) is added. The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 10 min and then absorbance is measured at 515 nm.

6.6.3 Blank preparation

To avoid overestimating the results, a blank sample is performed with each batch of samples as follows: 100 µL of Milli-Q® water is placed (by triplicate) on the 96-well microplate as it is also depicted in Figure 9. Afterwards, 100 µL of the DPPH 0,25 mM solution (see subclause 6.3) are added. Its absorbance value (Abs_{DPPH}) will be used as the blank (see subclause 6.7).

The 96-well microplate is inserted into the spectrophotometer, where it is shaken and kept in darkness for 10 min and then absorbance is measured at 515 nm.

6.7 Quantification

The Trolox calibration curve (see subclause 6.6.1) is:

Calibration curve: $y = a + bx$ $\mathrm{AA}\left(\mathrm{mmol\ TRE\ L^{-1}}\right) = \frac{\left(\left(\mathrm{Abs_{DPPH}} - \mathrm{Abs_{SAMP}}\right) - \mathrm{a}\right) * \mathrm{V_{TOT}}}{\mathrm{b} * \mathrm{V_{SAMP}}}$

where *y*: absorbance*; a*: intersection; *b*: slope; *x*: Trolox concentration.

AA results are expressed in milimol of Trolox equivalents per Liter of sample (mmol TRE L^{-1})

where Abs_{DPPH}: blank absorbance; Abs_{SAMP}: sample absorbance; V_{TOT}: final volume of the dilution; V_{SAMP}: sample volume (see Table 6).

6.8 Precision

6.8.1 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a day (24 h), should not exceed 5 % (expressed as coefficient of variation).

6.8.2 Intralaboratory reproducibility

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within different days (>24 h), should not exceed 8 % (expressed as coefficient of variation).

Bibliography

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