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Guidelines for characterization of extracts for the recycling/upcycling of organic agrifood wastes

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COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This CEN Workshop Agreement (CWA 18149:2024) has been developed in accordance with the CEN-CENELEC Guide 29 "CEN/CENELEC Workshop Agreements – A rapid prototyping to standardization" and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by a Workshop of representatives of interested parties on 2024-05-31, the constitution of which was supported by CEN following the public call for participation made on 2024-03-11. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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Introduction

In recent years, the demand for natural antioxidants in various industries, including food, cosmetics, and nutraceuticals, has surged significantly. These antioxidants play a pivotal role in extending the shelf life of products and protecting them from oxidative degradation, thereby enhancing their overall quality and stability. In response to this growing demand, the exploration of natural sources rich in bioactive compounds has become a focal point of research and development.

Extracts derived from agro-industrial residues present a promising avenue for obtaining potent antioxidants due to their abundance in polyphenolic compounds and flavonoids. The agro-industry generates vast quantities of by-products during the processing of fruits and vegetables such as lemon, apple, artichoke, broccoli, and grape, among others. These by-products, if not efficiently managed, can pose environmental challenges. However, they also represent a valuable resource for the extraction of bioactive compounds with potential applications as antioxidants in various sectors.

The European Committee for Standardization (CEN) plays a crucial role in setting standards and guidelines to ensure the quality, safety, and efficacy of products and processes within the European Union (EU) and beyond. As part of this endeavour, this document aims to provide guidance for a comprehensive characterization of extracts obtained from agro-industrial residues for use as antioxidant additives in food, cosmetics, and nutraceutical formulations.

This document focuses on three key aspects of extract characterization: polyphenolic content analysis, flavonoid content analysis, and antioxidant activity assessment. The determination of polyphenolic content utilizes the Folin-Ciocalteu reagent, a widely recognized method for quantifying total phenolic compounds in various matrices. Additionally, the analysis of flavonoids employs a mixture of sodium borohydride and chloranil as a reducing agent, enabling the estimation of flavonoid content in the extracts.

Furthermore, the antioxidant activity of the extracts is evaluated using multiple assays, including the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power (FRAP) assays. These assays provide complementary information regarding the ability of the extracts to scavenge free radicals and reduce oxidative stress, essential parameters for assessing their potential as antioxidants.

The harmonization of methodologies for extract characterization is paramount to ensure consistency and comparability of results across different laboratories and stakeholders. By establishing standardized procedures, this document seeks to facilitate the development and commercialization of antioxidant-rich extracts from agro-industrial residues, thereby promoting sustainability, innovation, and competitiveness in the European market.

In conclusion, the utilization of agro-industrial residues as a source of antioxidant-rich extracts holds great promise for addressing the increasing demand for natural antioxidants in various industries. Through collaborative efforts guided by standardized methodologies, we can harness the potential of these extracts to enhance the quality and functionality of a wide range of products, contributing to the advancement of sustainable practices and the promotion of human health and well-being.

1 Scope

This document specifies a collection of methods for the characterization of extracts obtained from agro-industrial residues and by-products, with the purpose to be evaluated for being recycled/upcycled as antioxidant additives in food, cosmetics, and/or nutraceutical formulations.

The collection of methods applies to wastes and by-products from vegetables and fruits processing.

A method for the determination of total polyphenol content by a colorimetric assay using Folin-Ciocalteu phenol reagent [1] is provided. Along with polyphenols, this method quantifies flavonoids, ascorbic acid, reducing sugars, some amino acids, and some aromatic amines.

For the determination of total flavonoids content, a method based on the colorimetric assay using sodium borohydride/Chloranil based reagent [2] is provided. Amongst flavonoids are included: flavones, flavonols, flavonones, falvonols, isoflavonoids, flavanols, and anthocyanins.

For the determination of the antioxidant capacity of extracts, three different methods based on a colorimetric assay using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) [3], 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric-tripyridyltriazine (Fe^{3+} - TPTZ) [4], respectively, are specified.

2 Normative References

There are no normative references in this document.

3 Terms and definitions

No terms and definitions are listed in this document.

4 Content of total polyphenols — Colorimetric method using Folin-Ciocaulteu reagent

4.1 Generalities

This part of the document specifies a method for the determination of total polyphenol content in wastes and by-products from vegetables and fruits processing by a colorimetric assay using Folin-Ciocalteu phenol reagent [1].

Along with polyphenols, this method quantifies flavonoids, ascorbic acid, reducing sugars, some amino acids, and some aromatic amines.

4.2 Principle

Samples are dissolved/diluted with 70 % methanol. The polyphenols in the solution are determined colorimetrically using Folin-Ciocalteu phenol reagent. The reagent contains phospho-tungstic acids as oxidants, which on reduction by readily oxidized phenolic hydroxy groups yield a blue colour due to the formation of so-called tungsten and molybdenum blues, which have a broad maximum absorption at 765 nm. The Folin-Ciocalteu reagent reacts with a wide range of polyphenol compounds and, although the response can vary with the individual components, selection of gallic acid as a calibration standard enables useful total polyphenol data to be obtained.

4.3 Reagents

— Analytical balance. Capacity to weigh to an accuracy of ± 0,001 g.

— Methanol 70 % (v/v) (Methanol – distilled water, 70:30).

— Distilled water.

— Folin-Ciocalteu reagent 10 %. 20 ml of Folin-Ciocalteu phenol reagent to 200 ml with distilled water. Reagent solution freshly prepared, daily.

— Sodium carbonate solution, 7,5 % (m/v). Weight 7,5 g of anhydrous sodium carbonate and dissolve it with 100 ml of distilled water. This step could be assisted by heating a portion of the water, dissolve the solid, leaving it to cool at room temperature and adding the remaining water at room temperature up to the final 100 ml.

— Gallic acid stock standard solution 1 000 µg/ml. Weight 100 mg of gallic acid anhydrous (110 mg of gallic acid monohydrate) and dissolve in 100 ml of distilled water. Stock standard solution freshly prepared, daily.

4.4 Apparatus

— Analytical balance. Capacity to weigh to an accuracy of ± 0,001 g.

— Pipettes to cover the volume range for the solutions.

— Nylon filters (0,22 µm).

— One-mark volumetric flask of different capacities (50 ml, 100 ml, 250 ml, 500 ml, 1 L).

— Vortex mixer.

— Spectrophotometer set at 765 nm and able to accommodate 10 mm path length cells.

— 10 mm path length absorption cuvettes (quartz or plastic).

— 10 ml graduated tubes (glass or plastic).

4.5 Sampling

Store a representative sample in well-sealed containers protected from light, under inert atmosphere and freeze (- 20 °C). Thaw the samples analysis.

4.6 Procedure

4.6.1 Calibration curve

Starting from the stock standard solution of gallic acid (1 000 ppm), the following dilutions shall be prepared:

Table 1 — Standard levels for the calibration curve of gallic acid

Concentration (ppm)	Total volume (ml)	Stock solution (ml)	Solvent (ml)
5	10	0.125	9.875
25	10	0.250	9.75
50	10	0.500	9.5
100	10	1	9
200	10	2	8
300	10	3	7
400	10	4	6

4.6.2 Sample preparation

1. Dilute 100 µL of the sample extract to 10 ml using methanol (70 %). In case the sample is solid, weigh 100 mg and dissolve it with 10 ml of methanol (70 %).

2. Pour 1.0 ml of water in tubes for the blank. Do it twice to have two replicates.

3. Pour 2.0 ml of diluted samples, in duplicate, into separate tubes.

4. Add 5.0 ml of Folin-Ciocalteu phenol reagent (10 %) in each tube and mix it with vortex.

5. After 5 minutes, pipette 4,0 ml of sodium carbonate solution in each tube. Stopper and mix with vortex.

6. Allow to stand 60 minutes at room temperature and measure the absorbance at 765 nm using 10 mm path length cuvettes.

4.7 Calculation

$$T_{PhOH} = \frac{Abs_{765nm} \times V_{sample}}{S_{std} \times w}$$

Where

Abs_{765nm}	is the absorbance at 765 nm;
V_{sample}	is the volume of the sample;
S_{std}	is the slope obtained for the best-fit linear calibration;
w	is the mass of the sample.

4.8 Precision

4.8.1 Interlaboratory test

The preliminary validation of this method is recommended by an interlaboratory test to compare results between several laboratories.

4.8.2 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 short interval of time, should not exceed the repeatability limit values obtained in an interlaboratory test in more than 5 % of cases. The result can be expressed like standard deviation (s_r), coefficient of variation (*%*) or repeatability limit ($r = 2{,}8\ s_r$).

4.8.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed the reproducibility limit values, *R*, in more than 5 % of cases. The result can be expressed like standard deviation (s_R), coefficient of variation (*%*) or repeatability limit ($R = 2{,}8\ s_R$).

5 Content of total flavonoids - Colorimetric method using Sodium Borohydride / Chloranil reagent.

5.1 Generalities

This part of the document specifies a method for the determination of total flavonoids content in wastes and by-products from vegetables and fruits processing by a colorimetric assay using sodium borohydride/Chloranil based reagent [2]. Amongst flavonoids are included: flavones, flavonols, flavonones, falvonols, isoflavonoids, flavanols, and anthocyanins.

5.2 Principle

Samples are dissolved/diluted with Tetrahydrofuran/Ethanol (1:1). The flavonoids in the solution are determined colorimetrically after treating the sample with a reductive reagent (mixture of sodium borohydride and aluminium trichloride), acid and chloranil at 100 °C. This procedure (Figure 1) yields a mixture with a peak of maximum absorption at 490 nm.

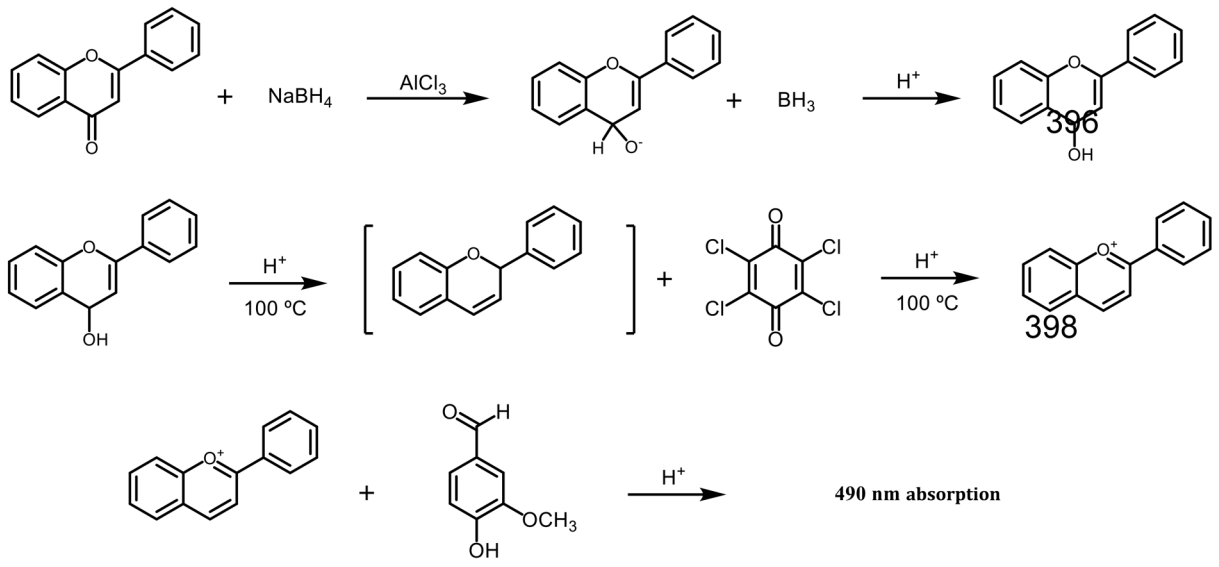

Figure 1 — Reaction mechanism involved in the flavonoid's quantification method

5.3 Reagents

— Sodium borohydride (NaBH4) in ethanol, 50 mM (0,19 % (p/v)).

— Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) in ethanol. 74,56 mM (1,8 % (p/v)).

— Chloranile in THF, 20 mM (0,5 % (p/v)).

— Acetic acid aqueous solution, 0,8 M.

— Vainillin in methanol, 1 M (0,16 % (p/v)).

— Hydrochloric acid concentrated (37 %).

— THF/EtOH (1:1, v/v).

— Methanol.

— Quercetin stock standard solutions (20 mM). Weigh 67,7 mg of quercetin dihydrate in a 10 ml one-mark volumetric flask, dilute to the mark with THF/EtOH (1:1) and mix. Stock standard solution freshly prepared, daily.

To avoid the undesirable hydrolysis of sodium borohydride, use absolute ethanol instead of ethanol at 96 %.

All the reagents shall be of recognized analytical grade.

5.4 Apparatus

— Analytical balance. Capacity to weigh to an accuracy of ± 0,001 g.

— Pipettes to cover the volume range for the solutions.

— Nylon filters (0,22 µm).

— One-mark volumetric flask of different capacities (50 ml, 100 ml).

— Vortex mixer.

— Heater capable to maintain the temperature at 100 °C (± 1 °C) while stirring.

— Spectrophotometer set at 490 nm and able to accommodate 10 mm path length cells.

— 10 mm path length absorption cuvettes (quartz or plastic).

— 10 ml graduated tubes (glass or plastic).

5.5 Sampling

A representative sample shall be stored in well-sealed containers protected from light, under inert atmosphere and freeze (- 20 °C). Thaw the samples before analysis.

5.6 Procedure

5.6.1 Calibration curve

Starting from the stock standard solution of quercetin (20 mM), the following dilutions shall be prepared.

Table 2 — Standard levels for the calibration curve of quercetin

Concentration (mM)	Total volume (ml)	Stock solution (µl)	THF/EtOH (µl)
0,1	1	5	995
0,5	1	25	975
1	1	50	950
2,5	1	125	885
5	1	250	750
7,5	1	375	625
10	1	500	500

Alternative, catechin as standard could be used.

5.6.2 Sample preparation

1. In a 10 ml test tube, dilute 100 µL of the sample extract/standard solution in 1 ml of THF/EtOH (1:1) solution. In case the sample is solid, weigh 100 mg and dissolve it with 1 ml of THF/EtOH (1:1). For the blank, instead of sample add 100 µL of EtOH.

2. Pour 0,5 ml of the sodium borohydride solution and 0,5 ml of the aluminium chloride solution in the test tube with the sample/standard solution. Shake during 30 minutes in an orbital shaker.

3. Pour another 0,5 ml of sodium borohydride solution and continuing shaking for another 30 minutes.

4. Add 2,0 ml of cold (4 °C) acetic acid solution. Mix with vortex and keep it in the dark for 15 minutes.

5. Add 1 ml of chloranil solution and heat at 100 °C with shaking for 60 minutes.

6. Let the mixture to cool to room temperature and bring the final volume to 4 ml with methanol.

7. Add 1 ml of vanillin solution and mix.

8. Add 2 ml of HCl concentrated, mix and keep the reaction mixture in the dark for 15 minutes.

9. Measure the absorbance at 765 nm using 10 mm path length cuvettes.

5.7 Calculation

Expressed in mg of quercetin equivalent per g/ml of extract.

5.8 Precision

5.8.1 Interlaboratory test

The preliminary validation of this method is recommended by an interlaboratory test to compare results between several laboratories.

5.8.2 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 short interval of time, should not exceed the repeatability limit values obtained in an interlaboratory test in more than 5 % of cases. The result can be expressed like standard deviation (s_r), coefficient of variation (*%*) or repeatability limit ($r = 2{,}8\ s_r$).

5.8.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed the reproducibility limit values, *R*, in more than 5 % of cases. The result can be expressed like standard deviation (s_R), coefficient of variation (*%*) or repeatability limit ($R = 2{,}8\ s_R$).

6 Antioxidant activity – Trolox Equivalent Antioxidant Capacity (TEAC)

6.1 Generalities

This part of the document specifies a method for the determination of the antioxidant capacity of extracts obtained from wastes and by-products after the vegetables and fruits processing by a colorimetric assay using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) [3].

6.2 Principle

TEAC assay is based in the reaction between a 7 mM solution of ABTS with 2,45 mM potassium persulfate during 16 h produced the $ABTS^{\cdot+}$ cation which can be detected at a λ = 734 nm due to its intense blue-green colour (Figure 2), In presence of an antioxidant substance (ROH), $ABTS^{\cdot+}$ cation is reduced to its original uncoloured form ($ABTS^{+}$).

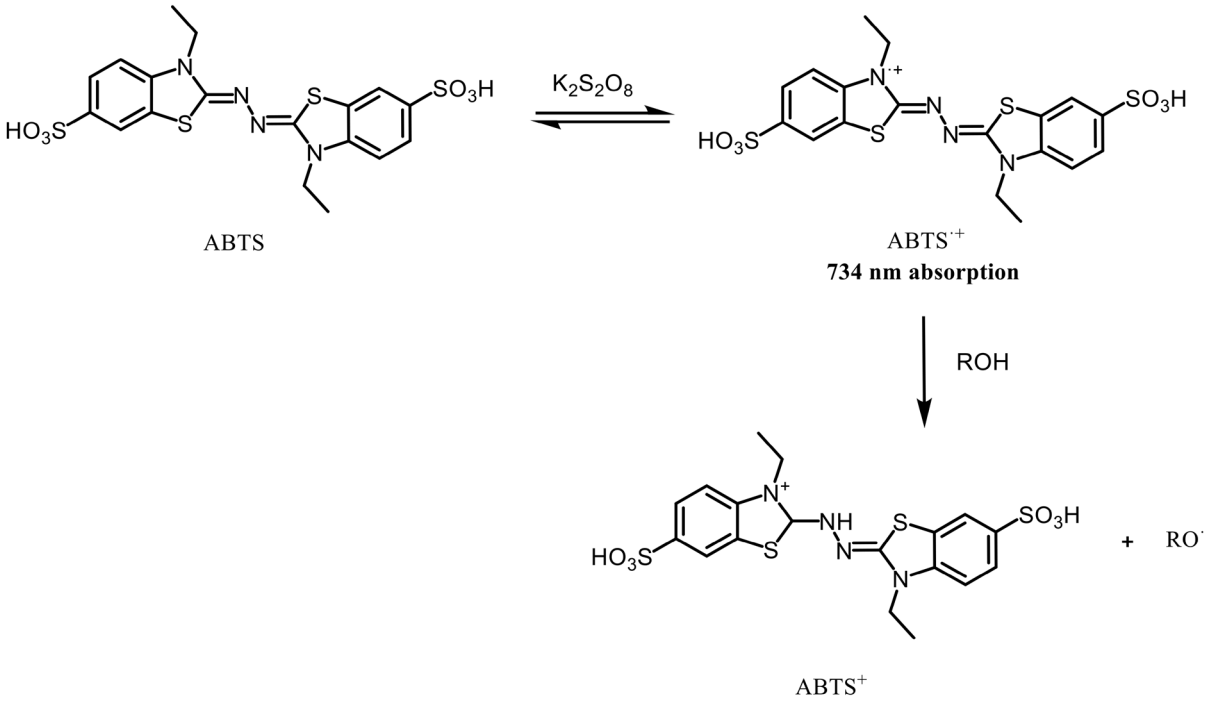

Figure 2 — Mechanism involved in the TEAC assay

The mechanism which describes this method is based on electrons transferences.

The method could be applied to hydrophilic and lipophilic antioxidant. For the latest, dichloromethane shall be used instead of methanol at 80 %.

The reference used is Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) a water soluble analogous of vitamin E.

6.3 Reagents

— Methanol 80 % ($MeOH/H_2O$, 80:20).

— Ethanol.

— ABTS: 2,2'-azino-di-(3-ethyl benzthiazoline-5-sulphonic acid).

— Potassium persulfate: $K_2S_2O_8$.

— Reagent A (78,4 mg ABTS dissolved in 10 ml of distilled water) (stable refrigerated a maximum of 5 days).

— Regent B (13,2 mg of potassium persulfate dissolved in 10 ml of distilled water) (stable refrigerated a maximum of 5 days).

— Trolox stock standard solution of 600 ppm in MeOH (80 %). Weigh 26,00 mg of Trolox in a 50 ml one-mark volumetric flask and dilute to the mark with MeOH (80 %).

All the reagents shall be of recognized analytical grade.

6.4 Apparatus

— Analytical balance. Capacity to weigh to an accuracy of ± 0,001 g.

— Pipettes to cover the volume range for the solutions.

— Nylon filters (0,22 µm).

— One-mark volumetric flask of different capacities (10 ml, 50 ml, 100 ml).

— Vortex mixer.

— Heater capable to maintain the temperature at 60 °C (± 1 °C).

— Spectrophotometer set at 734 nm and able to accommodate 10 mm path length cells.

— 10 mm path length absorption cuvettes (quartz or plastic).

— 10 ml graduated tubes (glass or plastic).

— 4 ml glass vials.

6.5 Sampling

A representative sample shall be stored in well-sealed containers protected from light, under inert atmosphere and freeze (-20 °C). Thaw the samples before analysis.

6.6 Procedure

6.6.1 Calibration curve

Starting from the stock standard solution of Trolox (600 ppm), the following dilutions shall be prepared.

Table 3 — Standard levels for the calibration curve of Trolox

Concentration (ppm)	Toral volume (ml)	Stock standard solution vol. (µl)	MeOH (80 %) vol (µl)
40	10	667	9333
80	10	1333	8667
100	10	1667	8333
120	10	2000	8000
140	10	2333	7667
160	10	2667	7333
180	10	3000	7000
200	10	3333	6667

6.6.2 Sample preparation

1. Preparation of the ABTS stock solution. Mix 1,5 ml of reagent A and 1,5 ml of reagent B, mix and store in the dark at room temperature for 16 - 20 hours.

2. Preparation of the diluted ABTS solution. Take 1 ml of the previously prepared solution, after the 16 - 20 hours incubation and dilute it with 60 ml of Ethanol.

3. Adjusting of the diluted ABTS solution. Measure the absorbance at 734 nm of the diluted ABTS solution (step 2). The value shall be 1,1 ± 0,02. If the absorbance is higher than 1,12 the solution shall be diluted with ethanol. If the absorbance is lower than 1,08, it shall be adjusted with the stock solution (step 1).

4. In a 50 ml one-mark volumetric flask, weigh 25 mg (or 25 µL) of the extract and dissolve/dilute it to the mark with MeOH (80 %) and mix.

5. Add 150 µl of the sample (extract diluted, standard solutions or Methanol for the blank) into a 4 ml glass vial. Then add 2,85 ml of the ABTS adjusted diluted solution from step 3. Mix with vortex and keep the reaction mixture in the dark for 30 minutes.

6. Measure the absorbance at 734 nm using 10 mm path length cuvettes.

6.7 Calculation

Expressed in mg equivalents of trolox per “g” or “ml” of extract.

mg Eq trolox/g sample = ppm trolox * solvent volume (L)/ (sample mass (g))

6.8 Precision

6.8.1 Interlaboratory test

The preliminary validation of this method is recommended by an interlaboratory test to compare results between several laboratories.

6.8.2 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 short interval of time, should not exceed the repeatability limit values obtained in an interlaboratory test in more than 5 % of cases. The result can be expressed like standard deviation (s_r), coefficient of variation (*%*) or repeatability limit ($r = 2{,}8\ s_r$).

6.8.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed the reproducibility limit values, *R*, in more than 5 % of cases. The result can be expressed like standard deviation (s_R), coefficient of variation (*%*) or repeatability limit ($R = 2{,}8\ s_R$).

7 Antioxidant activity — DPPH Antioxidant Assay

7.1 Generalities

This part of the document specifies a method for the determination of the antioxidant capacity of extracts obtained from wastes and by-products after the vegetables and fruits processing by a colorimetric assay using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) [4].

7.2 Principle

DPPH antioxidant assay is based in the reaction between a stable free radical (DPPH) and an antioxidant compound with scavenging capacity towards it. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine.

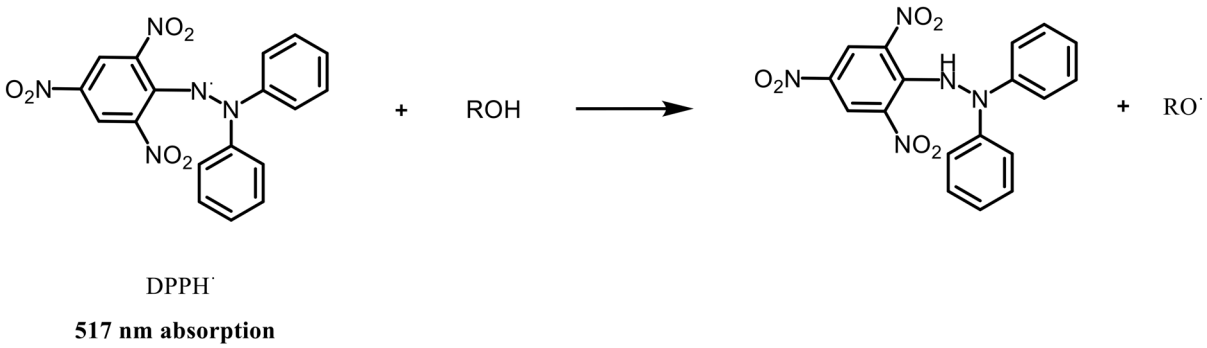

Figure 3 — Mechanism involved in the DPPH assay

The radical DPPH in ethanolic solution has a peak of absorption at 517 nm (it is a deep violet colour solution). In the presence of the antioxidant and after the DPPH accept an electron or hydrogen radical to pair off its odd electron, the absorbance at 520 nm vanishes.

The method works even with weak antioxidants and may be used in aqueous and nonpolar organic solvents (with hydrophilic and lipophilic antioxidants).

7.3 Reagents

— Methanol 80 % ($MeOH/H_2O$, 80:20).

— Ethanol.

— DPPH: 2,2-Diphenyl-1-picrylhydrazyl.

All the reagents shall be of recognized analytical grade.

7.4 Apparatus

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

— Pipettes to cover the volume range for the solutions.

— Nylon filters (0,22 μm).

— One-mark volumetric flask of different capacities (10 ml, 50 ml, 100 ml).

— Vortex mixer.

— Spectrophotometer set at 734 nm and able to accommodate 10 mm path length cells.

— 10 mm path length absorption cuvettes (quartz or plastic).

— 10 ml graduated tubes (glass or plastic).

7.5 Sampling

A representative sample shall be stored in well-sealed containers protected from light, under inert atmosphere and freeze (-20 °C). Thaw the samples before analysis.

7.6 Procedure

7.6.1 Sample preparation

1. Prepare a DPPH stock solution of 500 ppm. Weigh 12,5 mg (± 0,0001 g) and dissolve it with 25 ml of MeOH 80 %.

2. Add 11,8 ml of the stock solution to a 250 ml one-mark volumetric flask, dilute it to the mark with MeOH (80 %) and mix to prepare the DPPH working solution.

3. Prepare, at least, 8 sample solutions of the extract at different concentrations.

4. Mix 3,9 ml of the DPPH working solution and 0,1 ml of sample solution.

5. Measure the absorbance at 734 nm after 0 min, 1 min, 15 minutes and so on until reach stability in the absorbance (3 reads with the same value). Then, the reaction is considered finished. The reaction could be prolonged for 6 hours.

7.7 Calculation

Results can be expressed in two ways:

1. Antiradical activity

$$I\ (\%) = \{[(A_{DPPH} - A_{Blank}) - (A_{s\text{-}\ DPPH} - A_{s\ blank})]/(A_{DPPH} - A_{blank})\} \times 100$$

Where

A_{DPPH} is the absorbance of the DPPH solution;

A_{Blank} is the absorbance of MeOH (80 %);

$A_{s\text{-}DPPH}$ is the absorbance of the DPPH with the sample after reaction;

$A_{s\text{-}Blank}$ is the absorbance of the DPPH in presence of the sample.

2. EC50 (antioxidant concentration required to obtain a radical inhibition of 50 %)

$$\%\ \text{DPPH residuary} = (A_f / A_0) \times 100$$

Where

A_f is the absorbance of the samples after the reaction

A_0 is the absorbance of the blank (without antioxidants)

A graph with data of the % DPPH residuary (ordinate) and the concentration of the antioxidant solution (abscissa) is plotted. The EC50 corresponds with the antioxidant concentration at which % DPPH residuary is equal to 50.

It could be expressed as well as the antiradical power (ARP):

$ARP = 1/EC_{50}$

7.8 Precision

7.8.1 Interlaboratory test

The preliminary validation of this method is recommended by an interlaboratory test to compare results between several laboratories.

7.8.2 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 short interval of time, should not exceed the repeatability limit values obtained in an interlaboratory test in more than 5 % of cases. The result can be expressed like standard deviation (s_r), coefficient of variation (*%*) or repeatability limit ($r = 2{,}8\ s_r$).

7.8.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed the reproducibility limit values, *R*, in more than 5 % of cases. The result can be expressed like standard deviation (s_R), coefficient of variation (*%*) or repeatability limit ($R = 2{,}8\ s_R$).

8 Antioxidant activity — FRAP Antioxidant Assay

8.1 Generalities

This part of the document specifies a method for the determination of the antioxidant capacity of extracts obtained from wastes and by-product after vegetables and fruits processing by a colorimetric assay using ferric-tripyridyltriazine (Fe^{3+} - TPTZ) [4].

8.2 Principle

The FRAP (Ferric Reducing Antioxidant Power) assay is used to measure the antioxidant power based on the reduction at low pH of ferric-tripyridyltriazine (Fe3 +-TPTZ) to an intense blue colour ferrous-tripyridyltriazine complex (Fe2 +-TPTZ) with an absorption maximum at 593 nm. Antioxidants present in the sample donate electrons to the Fe^{3+}-TPTZ complex, resulting in the formation of a coloured ferrous complex.

This method is limited by the presence of certain substances, such a fluorides, phosphates, citrates, and tartrates which can interfere with the determination of polyphenol antioxidant.

8.3 Reagents

— Acetate buffer (300 mM, pH 3.6).

— 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM HCl.

— 20 mM $FeCl_3 \cdot 6H_2O$ solution.

— Trolox stock standard solution of 600 ppm in methanol (80%).

All the reagents shall be of recognized analytical grade.

8.4 Apparatus

— Analytical balance. Capacity to weigh to an accuracy of ± 0,001 g.

— Pipettes covering the volume range for the solutions.

— Vortex mixer.

— Spectrophotometer set at 593 nm and able to accommodate 10 mm path length cells.

— 10 mm path length absorption cuvettes (quartz or plastic).

— Glass vials (4 – 10 ml).

— Water bath capable of maintaining the temperature at 37 °C (± 1 °C).

8.5 Sampling

A representative sample shall be stored in well-sealed containers protected from light, under inert atmosphere and freeze (-20 °C). Thaw the samples before analysis.

8.6 Procedure

8.6.1 Calibration curve

Starting from the stock standard solution of Trolox (600 ppm), the following dilutions shall be prepared.

Table 4 — Standard levels for the calibration curve of Trolox

Concentration (ppm)	Total volume (ml)	Stock standard solution vol. (µl)	MeOH (80 %) vol (µl)
40	10	667	9333
80	10	1333	8667
100	10	1667	8333
120	10	2000	8000
140	10	2333	7667
160	10	2667	7333
180	10	3000	7000
200	10	3333	6667

8.6.2 Sample preparation

1. Prepare 50 ml of the FRAP reagent by mixing acetate buffer, TPTZ solution, and $FeCl_3 \cdot 6H_2O$ solution in a ratio of 10:1:1, respectively. Warm the mixture to 37 °C.

2. Add 900 µL of the warm FRAP reagent to a glass vial.

3. Add 30 µL of the sample (extract diluted, standard solutions, or methanol for the blank) to the glass vial and mix.

4. Incubate the mixture at 37 °C for 30 minutes.

5. Measure the absorbance at 593 nm using a spectrophotometer.

8.7 Calculation

Calculate the antioxidant capacity of the sample using the Trolox calibration curve.

Expressed in mg equivalents of trolox per “g” or “ml” of extract.

mg Eq trolox/g sample = ppm trolox * solvent volume (L)/ (sample mass (g))

8.8 Precision

8.8.1 Interlaboratory test

The preliminary validation of this method is recommended by an interlaboratory test to compare results between several laboratories.

8.8.2 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 short interval of time, should not exceed the repeatability limit values obtained in an interlaboratory test in more than 5 % of cases. The result can be expressed like standard deviation (s_r), coefficient of variation (*%*) or repeatability limit ($r = 2{,}8\ s_r$).

8.8.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed the reproducibility limit values, *R*, in more than 5 % of cases. The result can be expressed like standard deviation (s_R), coefficient of variation (*%*) or repeatability limit ($R = 2{,}8\ s_R$).

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