EUBerry

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The sustainable improvement of European berry production, quality and nutritional value in a changing environment: Strawberries, Currants, Blackberries, Blueberries and Raspberries.

Work Package nr.3

FRUIT QUALITY CHARACTERIZATION AND DETERMINATION

METHODOLOGIES P1

For fruit sensorial and nutritional analysis as prescreening tools for genotype evaluation.

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FRUIT BIOACTIVE COMPOUNDS EXTRACTION METHOD

- 10g of fruit is weighed and used for the extraction.
- Strawberry: the extraction takes place in a solution of methanol and water (80% v/v) added to the pieces of strawberry in ratio of 1:5 (1 part of fruit:5 part of extraction phases, 10 g of fruits in 50 ml of extraction phase)
- Blueberry and Raspberry: the extraction takes place in a solution of acidified methanol (1% glacial acetic acid on extra volume methanol). The extraction phase is added to the pieces of strawberry in ratio of 1:5 (1 part of fruit:5 part of extraction phases, 10 g of fruits in 50 ml of extraction phase)
    - First extraction with 20ml of methanol, extraction phase
    - Homogenize the mixture, it has to be placed in continuous agitation (or ultrasound assisted) along 30 minutes. The extraction has to be in dark (cover the falcon tube with aluminum foil).
    - Separate the solid phase from the liquid phase by centrifugation at 4500g for 10 min.
    - Recover the supernatant and stock it in a new falcon tube by a glass Pasteur pipette.
    - Second extraction adding 20ml of methanol in the falcon where are placed the grinded fruit that have been extracted a first time yet.
    - Homogenize the mixture, it has to be placed in continuous agitation (or ultrasound assisted) along 30 minutes. The extraction has to be in dark (cover the falcon tube with aluminum foil).
    - Separate the solid phase from the liquid phase by centrifugation at 4500g for 10 min.
    - Recover the supernatant by a glass Pasteur pipette and stock it in the falcon tube where was placed the supernatant from the first extraction.
    - Transfer with a glass pipette the supernatant from the falcon tube to vials and store in freezer at -20°C.

For this type of extraction the determination of Anthocyanin content has to take place immediately after extraction.

TOTAL ANTIOXIDANT CAPACITY (CAT)

by Trolox Equivalent Antioxidant Capacity method (TEAC)

Intention

The pre-formed blue/green radical of $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate. The radical cation has an absorption maximum at 734 nm. It is reduced in the presence of such hydrogen-donating antioxidants. The decolorization of the $ABTS^{\bullet+}$ radical is determined as a function of concentration and calculated relative to the reactivity of Trolox, a water-soluble vitamin E analogue, as a standard under the same condition (Miller et al.,1993; Re et al., 1999) .

Material

Equipment

Photometer

Plastic Cuvette 1 cm

Stopwatch

Ultrasonic bath/ Shaker

Chemicals

- ABTS (2,2´-azinobis
- Trolox (6-hydroxy-2,5,7,8 tetramethychroman-2-carboxylic acid)
- Potassium persulfate (di-potassium peroxidisulfate)
- Dipotassium hydrogen phosphate
- Potassium dihydrogen phosphate
- Ethanol
- PhosphaTe buffered saline (**PBS**, 5mM, pH 7,2 - 7,4) 7,14 g (41 mmol/L) of dipotassium hydrogen phosphate (K_2HPO_4) and 1,23 g (9 mmol/L) Potassium dihydrogen phosphate (KH_2PO_4) filled up with water to 1 L.
- ABTS stock solution 77 mg ABTS are resolved in a 20 mL volumetric flask with a few ml PBS. 13 mg Potassium persulfate are weighed in a beaker and equally resolved with PBS (Ultrasound assisted!), before added to the ABTS. The flask is filled up with PBS to its mark. Before use it is necessary that the mixture stays in the dark (aluminium foil) at room temperature for 12 to 16 hours (at night). The solution is stable in the dark for five days.
- ABTS working solution. The ABTS stock solution has to be diluted with PBS, then filtered wit a paper filter, to an absorbance of 0,7 – 0,8. (1:50 to 1:70).

- Trolox stock solution. 32 mg Trolox is weighed in a 50 ml volumetric flask and resolved with a few ml ethanol and filled up with PBS to its mark (2,5mM).

- **Procedure**
  - Sample preparation

See extraction methodology. Supernatant is diluted 1:20 (100µL sample: 2000µL).

  - Measuring

At first transfer by pipette 1900 µL of ABTS working solution into the cuvette. The reaction starts after addition of the sample solution respectively blank or standard (100 µL) and should be mixed immediately. The absorbance of the sample is measured after 6 minutes at 734 nm.

	Sample	Blank
ABTS Working Solution	1900 µL	1900 µlL
PBS	-	100 µL
Sample solution	100 µL	-
Total	2000 µL	2000 µL

  - Calibration

The Trolox stock solution is diluted with PBS so that the final concentration of the dilution series ranges from 0,025 to 0,450 mmol/L. The Trolox solutions are measured like sample 3.2.

Calculation

To obtain the percentage of inhibition:

$$\%inhibition = \frac{Abs_{blank} - Abs_{sample/standard}}{Abs_{blank}} \times 100\%$$

The calibration is calculated by linear regression (Δ A = ac + b, c = concentration trolox mmol/l, Δ A = %inhibition, a = %slope, b = %intercept).

$$TEAC - Value\ (mg\ Trolox\ eq/kg\ Fruit) = \frac{(\Delta A - b) \times F}{a \times E}$$

Δ A = %inhibition

a = slope

b = intercept

F = Dilution factor (20)

E = sample weight [kg/L extracting agent]

- **Results**

TEAC-Value is expressed as [mmol Trolox equivalent/ kg] fruit with one decimal accuracy.

Attention: The TEAC-Value comprehends the antioxidative capacity of ascorbic acid.

TOTAL ANTIOXIDANT CAPACITY (CAT)

by Ferric reducing antioxidant power (FRAP).)

Intention

Antioxidant capacity of the sample solution is determined by its ability to reduce ferric to ferrous iron. When iron is complexed with 2,4,6-tripyridyl-s-triazine (TPTZ) in sodium acetate solution at an acidic pH, its reduction results in a colour change of the solution, from pale rust to blue. The absorbance of the solution at 593 nm reflects the extent of reduction. The reducing power is usually compared to that of ferrous sulphate, but other aqueous antioxidants, such as the vitamin E analogue Trolox are used as alternative standards.

Material

Chemicals

- Sodium acetate trihydrate 300mM Solution: dissolve 3.1 g sodium acetate in 950 ml H_2O, adding 16 ml glacial acetic acid. Bring total volume to 1000ml. Store in glass bottle at room temperature.
- Ferric chloride hexahydrate 20mM solution: dissolve 0.270 g ferric chloride hexahydrate in H_2O, total volume 50 ml. This reagent must be prepared fresh immediately prior to procedure.
- 2,4,6-tripyridyl-s-triazine (TPTZ) 10mM solution: add 340 µl concentrated HCl to H_2O, total volume 100ml. Dissolve 0.312 g TPTZ in HCl solution with stirring. Store in dark glass bottle at room temperature.
- Hydrochloric acid
- Ferrous ammonium sulphate or Ferrous sulphate heptehydrate: dissolve 0.0098 g ferrous ammonium sulphate hexahydrate (mol wt 392.1) in 25 ml H_2O (1000 µM stock). (Alternatively can use ferrous sulphate heptahydrate (mol. Wt 278.0), 0.0278 g in 100ml H_2O) to make the 1000 µM stock. The stock and dilutions should be prepared fresh prior to performing procedure

  Dilute appropriately to prepare remaining standards:

  500 µM std = 500 µl of 1000 µM stock + 500 µl H_2O
  200 µM std = 400 µl of 500 µM std + 600 µl H_2O
  150 µM std = 150 µl of 500 µM std + 350 µl H_2O
  100 µM std = 500 µl of 200 µM std + 500 µl H_2O
  50 µM std = 500 µl of 100 µM std + 500 µl H_2O
  0 µM std = H_2O

- Trolox Standards: dissolve 0.0624 g Trolox in 25 ml methanol (10 mM stock; this may be frozen for up to 6 months). These should be prepared fresh from the frozen stock prior to performing procedur

Additional dilutions are made as follow:

500 μM std = 50 μl of 10 mM stock + 950 μl H_2O
250 μM std = 500 μl of 500 μM std + 500 μl H_2O
100 μM std = 500 μl of 250 μM std + 750 μl H_2O
75 μM std = 300 μl of 100 μM stock + 100 μl H_2O
50 μM std = 500 μl of 100 μM stock + 500 μl H_2O
25 μM std = 500 μl of 50 μM stock + 500 μl H_2O
0 μM std = H_2O

- Milli-Q water for all reagent preparation
- FRAP reagent**: c**ombine 10X volume of sodium acetate solution with 1X volume TPTZ and 1X volume of ferric chloride solution.

This reagent must be prepared fresh immediately prior to procedure.

Equipment

- Spectrophotometer
- Plastic Cuvette 1 cm (plastic)
- Volumetric flasks
- Falcon tube

Procedure

Prepare appropriate dilutions of extracts to be analyzed in H_2O. 100 μl of the appropriate dilution is needed for each sample tested in singlet

Prepare ferrous sulphate and Trolox standards, and hold at room temperature.

Prepare ferric chloride solution; then use the sodium acetate solution, TPTZ solution and ferric chloride to prepare the FRAP reagent, as introduced above. This reagent is stable for at least 2 hours at room temperature.

For reading in non automated spectrophotometer, pipette 900 μl FRAP reagent into cuvette (acrylic disposable are OK), followed by 100 μl EXTRACT SAMPLE or STANDARD and mix quickly by pipetting up and down (start timer immediately after addiction). The most complete and efficient mixing occurs when the tip is held just under the surface of the mixture in the cuvette, with rapid aspiration and expulsion. Blanks for zeroing the spec are prepared by adding 100 μl H_2O to 900 μl FRAP reagent, unless interference from the extraction solvent is suspected (doesn't occur with methanol or ethanol/water when diluted as above). Note thet the blank can be used as the zero standard, unless an adjustment for solvent is necessary.

Zero the spec with the blank at 593 nm and then EXACTLY 4 minutes following the addition of the samples to the FRAP reagent, read the absorbance.

Raw values of the FRAP are determined from the respective standard curves. The Trolox curve provides values in mmoles Trolox equivalent (TE), while the ferrous sulphate provides them in ferric reducing equivalents.

Results

For final values on a mmoles (TE or ferric reducing equivalents)/Kilogram of Fresh Weight

$$\frac{\text{raw FRAP value X dilution factor (e.g. 100) X volume of solvent extract (e.g. 100ml)}}{\text{wt (g) of fruit sample (e.g. 20 g berries) X } 10^3}$$

The ratio of ferrous sulphate/Trolox values on a molar basis is usually about 2:1. It's requires twice as many moles ferrous sulphate to achieve the same reducing power as Trolox.

TOTAL PHENOL CONTENT

by Folin Ciocaltou's reagent method

Intention

The total phenolics assay does not only determine phenolics but also reducing agents like ascorbic acid, because the basic mechanism is an oxidation/reduction reaction. The exact chemical nature is not known, but it is believed to contain heteropolphospho-tunstates molybdates. Molybdenum seems to be easily reduced in the complex. An electron-transfer reaction occurs between reductants and Mo(VI) under alkaline conditions, which results in blue color with an absorbance maxima about 720 nm.

- **Material**

Equipment

- Spectrophotometer
- Plastic Cuvette 1 cm (glass, plastic, quartz??)
- Stopwatch

Chemicals

- Folin-Ciocalteu-Reagent
- Sodium carbonate
- Gallic Acid
- Sodium carbonate solution 20% 200 g sodium carbonate is filled up with water to 1 L.
- Stock solution: 1000 Gallic Acid mg /L 200mg. Gallic Acid is solubilized in a few drops of Methanol to avoid the formation of agglomerate hard to resolve, and filled up with water to 200 mL.
- Standard: Gallic Acid. The Gallic Acid stock solution is diluted with water so that the final concentration of the dilution series ranges from 10 to 50 mg Gallic Acid/L (0,1ml; 0,2ml; 0,3ml; 0,4ml; 0,5ml in 10ml volumetric flask)

Procedure

- Sample preparation.

See extraction methodology. Supernatant is diluted 1:20 (100µL sample: 2000µL).

- Measuring

A test tube (glass) is filled with 7.0 ml water. Afterwards 1 mL of the diluted sample (only water is used for the blank measurement) is added which is followed by 500 µL Folin-Ciocalteu-Reagent an

vortexed. After 3 minutes 1,5 mL sodium carbonate is added and the tube is mixed one more time. The absorbance of the sample is measured after exactly 60 minutes at 760 nm.

- Calibration

The Gallic Acid standards are measured like sample 3.2. The calibration has to be repeated when a new Folin-Cioclateu reagent is used.

Calculation

The calibration is calculated by linear regression (Δ A = ac + b, c = concentration Gallic Acid mg/l, Δ A = absorbance, a = slope, b = intercept).

$$TP(mg\ GallicAcid\ \ eq/kg\ Fruit) = \frac{(\Delta A - b) \times F}{a \times E}$$

Δ A = $A_{sample/standard}$

a = slope

b = intercept

F = Dilution factor (20)

E = sample weight [kg/L extracting agent]

- **Results**

TP is expressed as [mg Gallic Acid equivalent/ kg] fruit without decimals.

TOTAL ANTHOCYANIN CONTENT

by pH Differential Shift Method

Intention

Anthocyanin pigments change hue and intensity according to pH. At pH 1.0, anthocyanins exist in the colored oxonium or flavylium form and at pH 4.5 predominantly in the colorless carbinol form. One aliquot of an aqueous anthocyanin solution is adjusted to pH 1.0 and another aliquot to pH 4.5. The difference in absorbance is proportional to the anthocyanin content. Determination of anthocyanin content is based on Lambert-Beer's Law. Published Molar absorbance values for purified pigments are used, making determination unnecessary . Pelargonidin-3-glucoside is the major anthocyanin in Strawberry, so the total anthocyanin content is calculated as pelargonidin-3-glucoside.

Material

Equipment

- Spectrophotometer
- Plastic Cuvette 1 cm (glass, plastic, quartz??)
- Volumetric flasks

Chemicals

- Potassium chloride (KCl)
- Sodium acetate (NaAc)
- Hydrochloric acid (HCL)
- Acetic acid
- Buffer pH 1 (potassium chloride (M= 74,55 g/mol) solution)

A solution of 0,025 mol/L potassium chloride is produced. (1,86 KCl g/L) and adjusted to pH 1 with hydrochloric acid.

- Buffer pH 4.5 (sodium acetate (M= 82,03 g/mol) solution)

A solution of 0,4 mol/L sodium acetate is produced (32,81 NaAc g/L) and adjusted to pH 4.5 with acetic acid

Procedure

Sample preparation

See Extraction methodologies as TEAC and TPC.

- **Measurement**

The supernatant is diluted 1:10 with each buffer solution. The absorbance maximum is determined (about 500 nm, It depends on fruits variety). Each dilution is measured at the absorbance maximum and 700 nm. The spectrophotometer is zeroed with distilled water.

Notice: Dilute the sample further if absorbance is greater than 1.0 AU.

- **Calculation**
- Calculation of anthocyanins as Pg-3-glu/kg fresh weight (FW)

$$mg\,\text{Pel-3-glu}\,/\,kg\,FW = \frac{[(A_{\lambda\max} - A_{700})_{pH1} - (A_{\lambda\max} - A_{700})_{pH4,5}] \times MW \times F \times 1000}{\varepsilon \times d \times E}$$

A = absorbance [-]

MW = molecular weight of pelargonidin-3-glucosid = 433.2 [g/mol]

F = dilution factor [-] = 10

d = cell pathlengths [cm]

ε = molar absorbance of Pel-3-glu = 15600 $[\frac{L}{mol \times cm}]$

E = sample weight [kg/L extracting agent]

1000 = Factor for mg

Results

Anthocyanins are expressed as Pel-3-gl [mg/kg FW] fruit.

FLAVONOID CONTENT

Intention

Total flavonoid content was determined by using a colorimetric method described by Dewanto and coworkers 2002.

Material

Equipment

- Spectrophotometer
- Plastic Cuvette 1 cm (plastic)
- Volumetric flasks
- Falcon tube

Chemicals

- Sodium nitrite ($NaNO_2$) solution 5% (w/v) is produced dissolving 5 grams of sodium nitrite in 100ml distilled water.
- Alluminium Chloride ($AlCl_3*6H_2O$) solution 10% (w/v) is produced dissolving 10 grams of sodium nitrite in 100ml distilled water.
- Sodium hydroxide (NaOH) 1M solution is produced dissolving 4 g of NaOH in 100ml distilled water.
- Catechin standard is produced dissolving 0,01g of catechin in 10ml of methanol, so to obtain a batch solution with concetration 1ml/mg then dilute to obtain a standard gradient that goes from 0,0125g/L to 1g/L.

Procedure

The supernatant is diluted 1:5 with distilled water, 250 µL of sample extract or (+)-catechin standard solution was mixed with 1.25 mL of distilled water in a test tube followed by addition of 75 µL of a 5% NaNO2 solution.

After 6 min, 150 µL of a 10% AlCl3*6H2O solution was added and allowed to stand for another 5 min. Successively add 500 µL of 1 M NaOH was added. The mixture was brought to final volume of 2.5 mL with distilled water and mixed with a mechanical agitator.

The absorbance was measured immediately against the blank at 510 nm using a spectrophotometer and calculate the catechin concentration comparing the absorbance value with the calibration curve made with the standards prepared similarly with known (+)-catechin concentrations.

The results are expressed as mean (mg of Catechin Equivalents /Kg of Fresh Weight).

HPLC DETERMINATION OF VITAMIN C CONTENT.

Intention

Ascorbic acid is measured as described by Helsper *et al.* 2003.

Material

Equipment

- High Pressure Liquid Chromatography System
- mono use syringe filter sterile (size pore 0.45μm)
- C18 250 mm × 4.6 mm column
- Syringe for HPLC sample and standard injection
- Electrochemical Detector Coulochem®/Photo Diode Array (PDA) Detector
- Electronic blender
- Refrigerated centrifuge
- Ultrasonic bath

Chemicals

- Mobile phase of 10 mM phosphate buffered solution, pH 3, 5% metaphosphoric acid, isocratic gradient. Mobile phase is prepared by weighing 14,28 g (82 mmol/L) of dipotassium hydrogen phosphate (K_2HPO_4) and 2,46 g (18 mmol/L) Potassium dihydrogen phosphate (KH_2PO_4) filled up with water to 1 L and add 5grams of EDTA. Adjusted to pH 3 with hydrochloric acid.
- Solution of metaphosphoric acid 5% and 1 mM EDTA, solution of 1mM EDTA is produced diluted 0,29g of EDTA in 1L of ultrapure water for HPLC analysis, and adding 5g of metaphosphoric acid..
- Ascorbic acid Standard, diluted in ice cold solution of metaphosphoric acid 5% and 1mM EDTA. Range values that goes to 1mg/ml to 0,00125 mg/ml

Procedure

Pooled frozen strawberries are powdered with an electric blender. Vitamin C is extracted by sonication of 0.5 g of wet frozen strawberry powder in 2 mL of ice-cold water with 5% metaphosphoric acid and 1 mM EDTA during 5 minutes.

After that the strawberries are centrifuged at 2500 rpm for 10 min at 4°C, filtering with a mono use syringe filter sterile (size pore 0.45μm), and immediate analysis on an HPLC system.

The HPLC system is coupled with a C18 250 mm × 4.6 mm column. Mobile phase of 10 mM phosphate buffered solution, pH 3, with 5% of metaphosphoric acid is at isocratic gradient.

The HPLC system is coupled to an Electrochemical Detector Coulochem®. Quantification was made through a standard calibration curve prepared by running increasing standard concentrations of vitamin C (ascorbic acid) prepared similarly and measured in duplicate at the beginning and end of the analysis.

The intracultivar biological variability is assessed by separate samplings of strawberries genotypes.

Results

Results are expressed as milligrams of vitamin C (vit C) per gram of FW (SD for biological variability).

TOTAL ACIDITY

by Titrimetric Evaluation

Intention

This method is used for the determination of titratable total acid in strawberries.

The sample has to be titrated potentiometrically with 0.1 N NaOH (sodium hydroxide) to pH 8.1

Material

Equipment

- 100 mL beaker (high size)
- 2 L beaker
- 1 L volumetric flask
- balance
- hand blender
- pH - measuring instrument
- single-rod measuring cell (storage in 3 mol/L potassium chloride – solution)
- magnetic stirrer
- 50 mL burette

Chemicals

- Water (aqua dest)
- 3 mol/L potassium chloride (KCl)
- buffer solutions for calibration the pH measuring instrument at pH 4,00 and 7,00
- 0,1 n sodium hydroxide (NaOH)

- **Procedure**
  - Calibration

Calibration of the pH measuring instrument with two buffer solutions with different but exact pH-values (two-point-calibration). The buffers have to be stirred during calibration.

  - Sample Preparation

Approx. 10 g of mash strawberries are weighed exactly into a beaker and are supplemented with 10 ml of water.

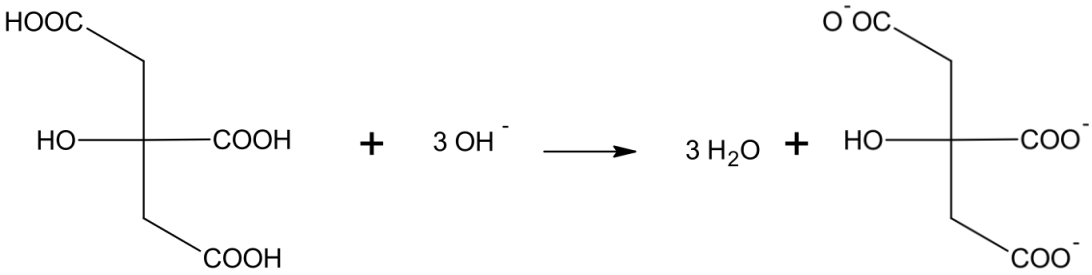
- Measuring

One aliquot of about 10 g strawberry mash, produced as described in 3.2., is given into a 100 mL beaker (high size), weighed exactly and filled up to 10 mL with distilled watrer. After immersion the single-rod measuring cell the sample has to be titrated with 0.1 N NaOH to pH 8.1 during constant stirring.

The addition of the volumetric standard solution has to be slow.

- **Calculation**

The content of total acidity will be calculated as citric acid at pH 8.1 as follows:



$$w(total\ acid) = \frac{V * c * M}{3 * E}$$

with: *w(total acid)* = content of total acid calculated as citric acid [g / kg]

V = volume of NaOH – solution [mL]

c = concentration of NaOH-solution [mol/L]

M= molecular weight of citric acid [g / mol] 192,12

E = initial weight of the mash [kg]

- **Results**

Total Acidity at pH 8,1 is expressed as [g citric acid / kg].

- **Check list**
  - Have you added 10 mL of water?
  - Is the pHmeter on?

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