Methodologies for the evaluation of nutraceutical parameters in strawberries fruits.

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STRAWBERRY FRUIT EXTRACTION METHOD

- 10g of fruit is weighed and used for the extraction.
- 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):
  - 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\(^{++}\) 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\(^{++}\) 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 tetramethylichroman-2-carboxylic acid)
- Potassium persulfate (di-potassium peroxidosulfate)
- 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\(_2\)HPO\(_4\)) and 1,23 g (9 mmol/L) Potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)) 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 with 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.

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Blank</th>
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</thead>
<tbody>
<tr>
<td>ABTS Working Solution</td>
<td>1900 μL</td>
<td>1900 μL</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>100 μL</td>
</tr>
<tr>
<td>Sample solution</td>
<td>100 μL</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>2000 μL</td>
<td>2000 μL</td>
</tr>
</tbody>
</table>

• 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 (ΔA = ac + b, c = concentration trolox mmol/l, ΔA = %inhibition, a = %slope, b = %intercept).

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

ΔA  = %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.

• Check list
  • Have you put the potassium persulfate?
  • Have you covered the ABTS radicalized with aluminium foil?
  • Have you filtered the abts solution?
  • Have you resolved the standard?
  • Have you diluted the supernatant?
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 ($\Delta A = ac + b, c =$ concentration Gallic Acid mg/l, $\Delta A =$ absorbance, $a =$ slope, $b =$ intercept).

\[
TP(\text{mg Gallic Acid eq/kg Fruit}) = \frac{(\Delta A - b) \times F}{a \times E}
\]

$\Delta A =$ $A_{\text{sample/standard}}$
$a =$ 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.

• Check list
  • Have you diluted the sodium carbonate?
  • Have you prepared the Gallic acid standards?
  • Have you put the water in the tube glass?
  • Have you add the sample/standard?
  • Have you add the Folin reagent?
  • Have you wait one minute?
  • Have you add the sodium carbonate?
  • Have you put the tube in dark for one hour?
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)

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

A = absorbance [-]
MW = molecular weight of pelargonidin-3-glucosid = 433.2 [g/mol]
F = dilution factor [-] = 10
d = cell pathlengths [cm]
\(\varepsilon\) = 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.

Check list
• Have you prepared the pH 1 buffer solution?
• Have you prepared the pH 4.5 buffer solution?
• Have you diluted the sample with both buffer solution?
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 water. 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:

\[ w(\text{total acid}) = \frac{V \times c \times M}{3 \times E} \]

with:
- \( w(\text{total acid}) \) = 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 pH meter on?

CONCLUSIONS
The STSM in Geisenheim was helpful to compare and harmonized the different methodologies used by the different laboratories performing analyses on berry nutritional quality within the Euroberry COST863 network. The protocols developed and described above will help in increasing the accuracy in the analyses of factors affecting berry nutritional quality, such as the climatic conditions, and in general to be able to compare the results from analyses performed in different laboratories.
Beside these outputs, that we expect quite beneficial for the COST863 network, the short period spent in Geisenheim resulted as an important experience to exchange and gain experiences specifically related to fruit nutritional values, for sure useful for my future research program.

**Literature**

  & Medicine, vol. 26, No. 9/10, 1231-1237
- R. Matissek, F. Schnepel, G. Steiner; Lebensmittelanalysik; Springer – Verlag; Berlin Heidelberg; 1989.
Sample preparation

Instruments:

- Liquid Nitrogen
- Dewar Jar to transport Liquid Nitrogen or Polystyrene Box
- Termic Gloves
- Aluminium Foil
- Mortar and Pistil
- Analytical Balance
- Falcon tube 50 ml
- Knife, Spoon and Forceps
Put Nitrogen in the deware jar to transport it.

To manage the Liquid Nitrogen (-196°C) it is necessary wearing termic gloves and glasses.

If you don´t have a deware jar, it is possible use a polystyrene box.
Sample size have to be of 500g of fruits selected from marketable fruits in the middle of the harvest, in a way to discard first and last fruits.

The fruits have to be similar in colour and dimension.

Fruits don’t have to show physical and pathological damage on their surface and beneath the calyx.

Cut the calyx and split the fruit
Cut two slices from the splitted fruit in a way to obtain two specular section.

The sections have to collect from opposite side of fruits in a way to eliminate eventually bias.

The fruit section have to show a triangular shape and have to be represented for all the length of fruit.
Cut the slices in small pieces to facilitate the homogenization with liquid nitrogen.

The pieces have to be of the dimension of the picture aside. But it is better cut it straight in the aluminium foil bags (see next slide).
Cut the fruit sections inside the bag in small pieces as explained before.
Close the bag and put it in the liquid nitrogen. It is better manage it with forceps and gloves.

The bag have to be totally covered with liquid nitrogen and have to stay in it few seconds (minimum 10sec).
After freezing it is possible to store the samples in freezer at -20°C, and go on later.

Frozen fruits have to look like picture aside.
Before to begin the homogenization it is necessary to cool the mortar and even the pistill.

The pistill has to be totally submerged with liquid nitrogen.
Fruit pieces have to be crushed strongly with the pistill.

The result is to obtain a powder consistence of strawberries. As you can see on the picture aside.
Fill up four falcon tubes with the homogenized powder of strawberries obtained. All in all 100 g sample.

Two falcons have to be sent to the analytical lab for the analysis. The other two should be stored to keep a safe sample in the eventuality of transport mistakes.