

STSM scientific report 2008.

A short term scientific mission (STSM) within the COST 863 Euroberry Research: FROM GENOMICS TO SUSTAINABLE PRODUCTION, QUALITY AND HEALTH.

Comparison of analytical methodologies for the evaluation of nutraceutical parameters in strawberries fruits.

Jacopo Diamanti, Marche Polytechnic University, Ancona, Italy

Objective: the aim of this STSM was to compare the analytical methodologies involved in the evaluation of nutraceutical parameters of the strawberries cultivated within the project “*Climatic influences on health beneficial components of strawberry*”.

The need to compare the methodologies used to analyze fruit nutritional quality at Geisenheim Research Centre and at Polytechnic University of Marche emerged after the comparison of the results of the harvesting year 2008. In fact, in this first experience were encountered difficulties to obtain vegetable materials from the different experimental fields involved in the project. First of all, because of the nature of the samples coming from trials started in Croatia, Switzerland and Norway were hold to the barrier customs and never reached the laboratories for the analyses so data were lost. Furthermore, when the results from Geisenheim and Ancona were compared emerged some differences in the methodologies of fruit sampling and analyses, and for these difference it was quite difficult to compare the results.

From this experience the possibility to have STSM specifically addressed to discuss and prepare a common protocol for the analytical methods, resulted as an important opportunity for better developing this research.

During the week spent in Geisenheim, Melanie Josuttis (PhD. student from geisenheim) and I, developed a sampling preparation method that could be used by scientists working in the different experimental sites, including also a method for storage and transport of the samples to the experimental laboratories. This sampling preparation method will solve the problem linked with the custom rules (particularly for non EU countries). In addition, the analytical methods used in Ancona and Geisenheim, were compared, by including the different steps, i.e. sampling - and analysis connected to the following analysis within the project:

- Total Antioxidant Capacity (TAC) by Trolox Equivalent Antioxidant Capacity method (TEAC);

- Total Phenols Content with Folin Ciocalteu's method (TPC);
- Anthocyanins Content (ACY) by the pH Differential Shift method;
- Ascorbic Acid (Vit.C) by titrimetric determination;
- Total Acidity (TA) by titrimetric determination.

About the sampling preparation was decided to prepare a standard protocol describing the different steps (from fruit collections to shipment). This protocol was delivered to all the experts involved in the research work on berry nutritional quality. The analytical protocols for the different main components of fruit nutritional values were also harmonized.

Following are reported all the protocols developed during the STSM.

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 tetramethylchroman-2-carboxylic acid)
- Potassium persulfate (di-potassium peroxodisulfate)
- 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 (K₂HPO₄) and 1,23 g (9 mmol/L) Potassium dihydrogen phosphate (KH₂PO₄) 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.

	Sample	Blank
ABTS Working Solution	1900 µL	1900 µL
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 ($\Delta A = ac + b$, $c =$ concentration trolox mmol/l, $\Delta A =$ %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?

- Have you mixed the solution?
- Have you wait six minutes?

TOTAL PHENOL CONTENT

by Folin Ciocalteu'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 (100 μ L sample: 2000 μ L).

- 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 and 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-Ciocalteu 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)

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

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.

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?

ASCORBIC ACID CONTENT

by titrimetric evaluation

Intention

Ascorbic acid is stable in oxalic acid. The amount of ascorbic acid is determined in the oxalic acid extract of the fruits by titration with iodide - iodate - solution. It described the titrimetric determination of ascorbic acid in strawberries.

Material

Equipment

- 100 mL beaker
- 250 mL beaker
- 10 mL burette
- 50 mL Erlenmeyer flask without grinding (Beuta)
- 50 mL graduated cylinder
- 100 mL volumetric flask
- Hand blender
- 1 mL single volume pipette
- balance
- centrifuge
- centrifuge tips

Chemicals

- oxalic acid
- L (+) – ascorbic acid p.a.
- iodide – iodate – solution, $c(I_2) = 1/128$ Molarity

- Oxalic acid solution, 2%ig. 40g of oxalic acid are weighed in a 250 mL beaker, and transfered with water into a 2 L volumetric flask and filled up with water to its mark.
- Ascorbic acid standard. About 100 mg ascorbic acid are weighed accurately to 0,1 mg in a 100 mL volumetric flask and filled up with oxalic acid – solution to its mark.

Sample extraction

- extraction takes place in a solution of oxalic acid 2% 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):
 - Double extraction (twice with 20 mL of oxalic acid)
 - First extraction with 10 mL of oxalic acid solution 2%.
 - Successively the homogenized mixture have to rest for 5 min in dark for both extraction.
 - Then separate the solid phase from the liquid phase by centrifugation at 4500g for 10 min.
 - At this point we have to recover the supernatant with help of a sieve and stock it in falcon tube.
 - Second extraction with 10 mL of oxalic acid solution 2%.
 - Successively homogenization the mixture have to rest for 5 min in dark for both extraction.
 - Then it needs to separate the solid phase from the liquid phase by centrifugation at 4500g for 10 min.
 - At this point we have to recover the supernatant with help of a sieve and stock it in the same falcon tube where was placed the first extraction phase.

The ascorbic acid standard has to be prepared freshly.

- Iodide – iodate – solution, $c(I_2) = 1/128$

Using Titrisol® solution, it has to be filled up with water to **2 L**.

If using Iodide – iodate – solution it has to be diluted with water in a 2 L volumetric flask at a ratio of 1:1.

The iodide – iodate – solution has to be stored in the dark.

• Procedure

- Determination of the Iodide – iodate – solution titre

1,0 mL of the ascorbic acid solution will be pipetted into a 50 mL Erlenmeyer flask and mixed with 20 mL oxalic acid solution. After addition of some drops starch indicator, it has to be titrated with Iodide – iodate – solution to a permanent blue colour.

The titration has to be done quickly because of the susceptibility of oxidation of the ascorbic acid.

The determination of the titre has to be repeated daily with a freshly prepared ascorbic acid solution.

For the blind testing 1,0 mL water will be used.

- Sample preparation

About 250 g strawberries will be weighed exactly into a 2 L beaker and have to be completed with oxalic acid – solution to 1 kg. Then the fruits are crushed with a hand blender, transfer the mash into centrifuge tubes and centrifuge for 5 minutes at 10 000 U / min.

- Titration

20 mL supernatant are titrated with iodide-iodate-solution after adding the starch indicator.

• Calculation

$$mg \text{ ascorbic acid} / kg \text{ FW} = \frac{c \times MW \times V}{v \times E}$$

c = concentration of Iodide-iodate-solution [mol/L]

MW = molecular weight ascorbic acid 176,13 = [g/mol]

V = volume used Iodide – iodate [mL]

v = volume extract

E = sample weight [kg/L extract]

• Results

Ascorbic acid is expressed as [g / kg] fruit.

• Check list

- Have you pipetted ascorbic acid solution into a 50 mL Erlenmeyer flask?
- Have you added 20 mL oxalic acid solution?
- Have you added the starch indicator?
- Have you done the blind test?

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

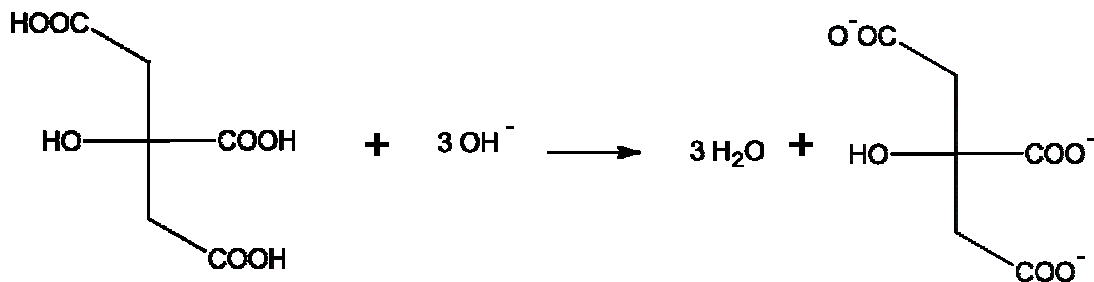
One aliquot of about 10 g strawberry mash, produced as described in 3.2., is given into a 100 mL beaker (high size), weighed exactly and filled up to 10 mL with distilled water. After immersion

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:



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

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 pHmeter 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

- Miller NJ, Rice-Evans C, Davis MJ (1993): A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates, *clin. Sci.* 84, 407-412
- Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999): Antioxidant activity applying an improved ABTS Radical Cation decolorization assay, *Free Radical Biology & Medicine*, vol. 26, No. 9/10, 1231-1237
- Giusti M. and Wrolstad R. E., Characterization and Measurement of anthocyanins by UV-visible Spectroscopy, *Current Protocols in Food Analytical Chemistry*, F1.2.1-F1.2.13 (2001)
- R. Matissek, F. Schnepel, G. Steiner; *Lebensmittelanalytik*; Springer – Verlag; Berlin Heidelberg; 1989.