

## STSM REPORT 2006

Short Term Scientific Mission, (STSM) within COST 863 project programme on Euroberry Research: from Genomics to sustainable production, Quality and Health.

### **Methods to determine the Total Antioxidant Capacity of different *F. x ananassa* genotypes.**

Dr AMPARO MONFORT, Institut de Recerca i Tecnologia Agroalimentàries, Cabrils,(Barcelona), Spain.

Host: Battino Maurizio, Institute of Biochemistry, Faculty of Medicine, Università Politecnica delle Marche. 60100 Ancona (IT)

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The aim of this STSM was to learn about different methods to determine the total antioxidant capacity and phenolic compounds of different cultivars of *F. x ananassa*. The STSM was accomplished at the laboratory of Maurizio Battino at the Università Politecnica delle Marche (Ancona, Italy), that is included in WG4 of COST Action 863, has a lot of experience on antioxidant capacity determination in strawberry. The STSM was accomplished within the period stipulated 21/01/07 to 26/01/07. At the same time I have started collaboration with Bruno Mezzetti around the possibility to apply together to FP7 project.

### **Methods experimented**

#### *Sample preparation procedure*

The first method experimented was a simplified method to prepare extracts from strawberry fruits to **Hydrophilic and Lipophilic extracts from strawberry fruits**. This method permits to work with high number of frozen fruits, and

prepare an extract that can be freeze a new and it is valuable to asses the antioxidant capacity of fruits. 10 g from seven fresh undamaged ripe fruits of average size were cut in 100ml of extraction solution (methanol: water 80:20 + 0.5% formic acid and checked for the pH  $5.0 \pm 0.2$ .) Fruit suspension was homogenized using one ultra-Turrax T-25 homogenizer during 15 seconds. The juice was kept in the dark at room temperature with agitation during two hours. After this time solution was centrifuged on a table centrifuge for 15 min at 3500 rpm. The supernatant was recovered and centrifuged a new at the same conditions. After the second centrifugation recover and filter 15 ml of supernatant in dark cryovials at  $-20\text{ }^{\circ}\text{C}$  until analysis. and freeze. Lipophilic extract was recovered on a pellet by adding acetone (1:5 weight/volume) keeping in the dark at room temperature with agitation during two hours and it was centrifuged for 15 min at 3500 rpm. The supernatant was recovered and centrifuged a new at the same conditions. After the second centrifugation the supernatant was recover and filter in dark cryovials at  $-20\text{ }^{\circ}\text{C}$  until analysis. This method was experimented in four different genotypes of strawberry.

#### *Ferric reducing antioxidant power (FRAP) to determine Antioxidant capacity*

The antioxidant capacity of the sample solution is determined by its ability to reduce ferric to ferrous ion. When iron is complexed with TPTZ (2,4,6-trypiridyl-s-trizine) in sodium acetate solution at an acidic pH, its reduction results in a solution colour change. The variation of absorbance at 593 nm reflects the extent of reduction. The analogue to vitamin E, Trolox was used as a standard for antioxidant power determination

-The standard Trolox 5mM stock solution were prepared in ethanol, and dilute at different concentrations between 50 to 400  $\mu\text{M}$  in  $\text{H}_2\text{O}$  and conserved in dark.

-Different dilutions of extracts in  $\text{H}_2\text{O}$  (1:2, 1:5, 1:10, 1:15, 1:20) were prepared.

-FRAP reagent combine 10 volume of sodium acetate trihydrate solution (300mM ph=3.6 with glacial acetic acid) with 1 volume of TPTZ solution (10mM in 40mM HCl) and 1 volume of ferric chloride solution (20 mM in  $\text{H}_2\text{O}$ ) prepared fresh prior the procedure.

- the samples were prepared adding to 0.9 ml of FRAP solution + 0.1 ml of sample or standard and vortex quickly for 20 seconds. Samples were readed at the sepctrofotmeter at 593 nm exactly 4 minutes after the addition of the samples to the FRAP reagent.

-The final values on a  $\mu\text{mol TE}$  (Trolox equivalent) /100 gr fruit FW(fresh weight)=

$$\frac{\text{Raw FRAP value} \times \text{DF (dilution factor)}}{10 \times \text{weight (g) of strawberry extracted in 100ml extraction}}$$

The Trolox standard values were used to design the standard basal line.

Samples with Absorbance of 0.4 o 0.5 detect an antioxidant capacity of 8 to 12  $\mu\text{M}$  Trolox/g FW strawberry.

### *TEAC method (Trolox Equivalent Antioxidant Capacity)*

This method so-called ABTS assay detects the Total antioxidant capacity of biological samples.

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, a chromogen and a colourless substance, is changed into its coloured monocationic radical form (ABTS<sup>+</sup>) by an antioxidant molecules of strawberry juice.

Addition of antioxidants reduces ABTS into its colourless form.

-The ABTS radicalized solution (ABTS<sup>+</sup>) was prepared by reacting ABTS (7 mM in water) with K<sub>2</sub>S<sub>2</sub>O<sub>8</sub> (140 mM in water) to obtain a molar ratio of 1:0.35. This solution was maintained in the dark 12 hours before use.

-The ABTS<sup>+</sup> working solution was prepared by dissolving ABTS<sup>+</sup> radicalized solution in ethanol (1:100).

-A dose-response curve was derived for Trolox (0, 0.625, 1.25, 1.75, and 2.5 mM) diluted in 1 mL of ABTS working solution. The Trolox stock solution was prepared as FRAP method at 5mM.

- 20 ul of samples were diluted in 1 ml of ABTS<sup>+</sup> reagent, vortexed during 30 sec., incubated a room temperature 20 sec and the absorption peak of ABTS is measured at 734 nm in a spectrophotometer (Kontron Uvikon 941 Plus).

-The antioxidant abilities of fruits were expressed as Trolox equivalent antioxidant capacity (TEAC) by using the calibration curve plotted against different amounts of Trolox. TEAC values were calculated and expressed using Trolox equivalents (TE) per gram of fresh weight (FW). Data are expressed as antioxidant capacity induced by hydrophilic and lipophilic components, and total antioxidant capacity as the sum of the two phases. But in strawberry the antioxidant capacity of lipophilic fraction is minimal.

The ABTS assay was also analyzed on a FIA system (Bompadre, Leone et al. 2004). Flow injection system was composed by a Beckman 126 HPLC pump, a Beckman model 126 UV/Vis detector, a Rheodyne Model 7125 manual injection valve equipped with a 5ul sample loop, and a reaction coil 100 cm long folded and included in a oven for temperature controlled reaction. The pump flow rate was 1.2 ml/min, the oven temperature was 35°. The absorbance of ABTS reagent will be between 1.0 and 1.5 to detect colourless on standard Trolox dilutions and samples.

### *Total Polyphenol determination FOLIN-CIOCALTEU method*

Phenolic content of strawberry fruits was determined by the Folin-Ciocalteu method by using Gallic acid (GA) as a standard for the calibration curve.

- Samples (fruit juice) or standards (0, 0.6, 1.2, 1.8, 2.4, and 3 mM of GA) were diluted 1:10.

- The reagent Folin-Ciocalteu was diluted 1:10.

- 400ul of samples were diluted in 2 ml of Folin diluted reagent. The final dilution ratio is 1:5 (v/v). After 6 min incubation at room temperature we add 1.6 ml Sodium Carbonate solution (700 mM in water) and maintained in the dark 2 hours.

- Absorbance was read on spectrophotometer at 760 nm after 2 h.
- Results were calculated and are expressed as milli-grams of Gallic Acid equivalent (GAE) per litre of juice.

## Conclusion

The Short Term Scientific Mission was successfully accomplished and the objective proposed to learn and compare different methods to determine the total antioxidant capacity on strawberry fresh fruits was completely assumed. I have acquired knowledge of methods to apply to large and quickly screening of our populations of *Fragaria* plants, and will be very important for enlarge our work on QTL determination analysis of strawberry genotypes.

I will thank here the collaboration of Pr. Maurizio Battino for scientific discussion and especially to Sara Tulipani, and Stefania that they show me all the techniques described before and Stefano Bompadre for help on HPLC methods.

On other way in this STSM I have started collaboration with Pr. Bruno Mezzetti around the possibility to apply together to FP7 project. In several meetings during the STSM we have prepared a first draft of new proposal to EU- FP7 COOP2-1-1-01: Development of new tools and processes to support R&D in crop plants: molecular breeding.

The draft concern to "Development of molecular tools in breeding strawberry", and we appoint to use *F.vesca* as a model plant of breeding *Fragaria*, genotyping several populations of *F. vesca* and *F x ananassa* in nutritional quality of fruit aspects.

## References

(Bompadre, Leone et al. 2004; Scalzo, Battino et al. 2005; Scalzo, Mezzetti et al. 2005; Scalzo, Politi et al. 2005)

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