

# STSM Scientific Report 2006

**Short-term scientific mission (STSM) within COST project programme on Euroberry Research: FROM GENOMICS TO SUSTAINABLE PRODUCTION, QUALITY AND HEALTH.**

## *Phytochemical profiling of different genotypes of strawberries*

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**Objective:** The aim of this STSM was to perform metabolic profiling to 27 samples of strawberry belonging to 9 different genotypes, provided by the home institution.

Analyses performed include moisture content analysis, determination of total flavonoid content by spectrophotometry, vitamin C analysis by HPLC, separation and characterisation of the antioxidant compounds by C18-HPLC coupled to on-line postcolumn antioxidant detection, analysis of procyanidins by HPLC. In addition, 4 from the selected samples of strawberry were analysed by C18-HPLC coupled to QTOF/MS.

The STSM was accomplished at the Plant Research International (Wageningen, Netherlands) in the work group of Dr. Jules Beekwilder within the period stipulated (1/11/06 to 1/12/06).

### **Materials and Methods**

**Plant material and sample preparation.** Strawberry fruits from 9 selected genotypes (6 varieties and 3 selections) were previously harvested at the experimental field of the Faculty of Agriculture, Polytechnical University of Marche, Ancona, Italy (Table 1). Fruits from seven of these cultivars were sampled in two main harvest times; in addition, fruits from six of these cultivars were sampled and characterized both as fresh material and after short refrigeration (3 days at 4°C, followed by 24 h at room temperature), to check for the potential role of these pre- and post-harvest factors on the metabolic profiling and nutritional attributes of different cultivars.

Whole fruits were sent to the host institution on dry-ice, and stored at -80°C. Then berries

were snap-frozen in liquid nitrogen and ground to a fine powder using a precooled IKA grinder. For each sample, a small amount of the frozen powder was freeze-dried, to check for differences on preservation, and both the wet and freeze-dried (FD) powders were stored at –80°C until analysis.

**Determination of Total Flavonoid content.** Total flavonoid content was determined by using a colorimetric method described previously (Jia et al., 1999; Dewanto et al., 2002; Meyers et al., 2003). The absorbance was measured immediately against blank at 510 nm by using a spectrophotometer in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results were expressed as mean (micrograms of catechin equivalents per gram of freeze-dried strawberry)  $\pm$  SD; the analysis was performed at least in duplicate for each sample.

**Determination of vitamin C content.** Vitamin C (ascorbic acid) is an antioxidant and as such sensitive to oxidation. Therefore, working temperatures were always kept as low as possible and exposure to high pH prevented. We decided to choose wet frozen powder for HPLC analysis since the freeze-drying procedure could affect and underestimate vitamin C recovery. In addition, 4 samples were randomly selected and analysed both as wet and FD materials; the FD samples were added with the corresponding volume of water lost during the freeze-drying (450  $\mu$ l), before mixing with the extraction solution.

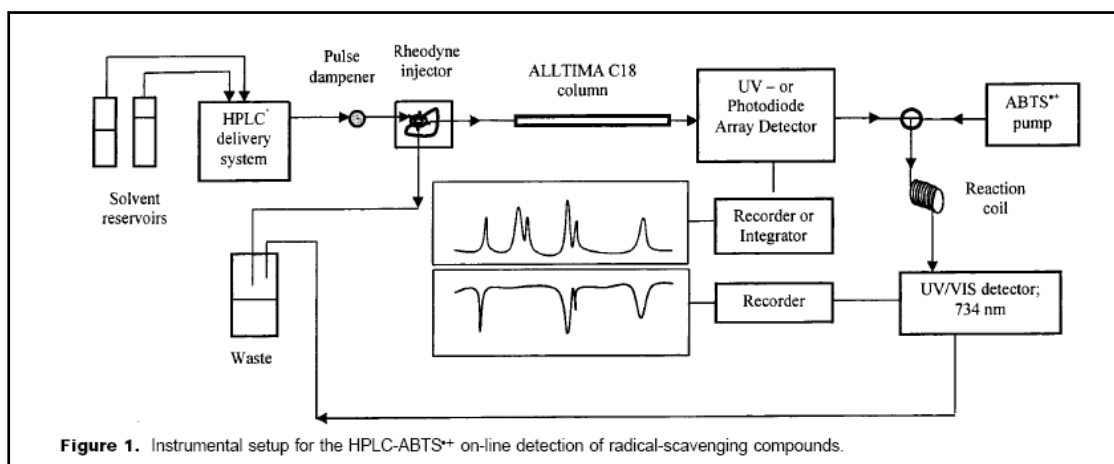
The HPLC system comprised a YMC Pack Pro 150x4.6 mm column, a Photodiode array (PDA) detector set at an absorbance of 262 nm, and a column incubator at 30 °C. The elution was isocratic using K-phosphate buffer pH 4.4, and analysis consisted in a 10 minutes run, after which the column was cleaned with 50% acetonitrile.

Quantification of the vitamin C content was conducted through a standard calibration curve prepared by running standard concentrations of vitamin C similarly prepared, and measured in duplicate at the beginning and the end of the analysis.

**HPLC Antioxidant Detection System.** The HPLC antioxidant detection system was used as previously adapted by the work group (Beekwilder et al., 2005).

The scheme of the instrumental setup of the system is presented in Figure 1, taken from Koleva et al. 2001. The HPLC system used comprised an HPLC eluent pump, a programmable photodiode array detector, a column incubator set at 40°C, a dual-wavelength

UV-VIS absorbance detector connected to a recorder, and a syringe pump for delivery of ABTS<sup>+</sup> solution.



Separation of compounds in the extracts was carried out by using a C18 10 mm x 4,6 mm pre-column followed by a C18 250 mm x 4,6 mm column. A step gradient with MQ water acidified with 0,1 % Trifluoroacetic acid (TFA) (Eluent A) and acetonitrile acidified with 0,1% TFA (Eluent B) was used for separation.

Compounds eluting passed first through the PDA detector set at an absorbance range of 240 to 700 nm. Then they passed through a postcolumn reaction loop consisting in a stainless steel tube (internal diameter 0,508 mm) at 40°C, where they reacted for 30 seconds with a buffered solution of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) cation radicals online pumped into the loop. The solution finally passed through the second detector.

The ABTS radical solution prepared at least 16 h before analysis and was diluted in 0,1 M Na-phosphate buffer.

The amount of ABTS cation radicals quenched by reaction with antioxidants expressed as a decrease in absorption and was subsequently measured when the mixture passed through the dual-wavelength UV-VIS detector set at 412, one of the maximum absorption of the ABTS cation, and 650 nm to check for interfering compounds in the reaction.

In addition, the use of 0,1% TFA as acidifying agent in the mobile phase was compared to the use of 0,05% FA, to check for the best setting giving both a good chromatographic resolution and acceptable pH for ABTS reactivity.

**C18-HPLC coupled to QTOF/MS.** To identify the main polyphenolic compounds in the extracts, four FD samples were subjected to C18-HPLC coupled to QTOF/MS, in

combination with chromatographic and spectral informations previously obtained. Separation of compounds was obtained by the same chromatographic conditions used for the HPLC antioxidant analysis; the acidifying agent of both Eluent A (MQ water) and Eluent B (acetonitrile) was 0,05% FA.

**HPLC analysis of Procyanidins.** The analysis of the procyanidin content and the assessment of the degree of polymerization was conducted on 11 samples of FD material (9 cultivars + 2 samples for the assessment of the biological variability within cultivar E), corresponding to fruits of the first harvest time. The samples were subjected to two differential extractions and preparative treatments (hydrolysis and not-hydrolysis), and runned separately in the same chromatographic conditions.

We used a RP-HPLC method to separate the procyanidin monomers, by using the same extraction procedure and chromatographic conditions used for the HPLC antioxidant analysis and previously described (mobile phase acidified with 0,1% TFA). The excitation wavelength was set at 275 nm, and fluorescence detection at 310 nm.

The proportion of the constituent units (as total catechin and total epi-catechin content) after hydrolysis and the average degree of polymerization (DP) of procyanifidins were calculated.

**Biological variability.** To check for the biological variability within cultivar, different fruits (n=9) from a selected sample (E1-F) were divided in three subgroups (E1-Fa, E1-Fb, E1-Fb) and separately subjected to each one of the analyses.

### **Statistical analysis**

Average concentrations, standard deviations (SD) and coefficients of variation (CV %) were calculated for all samples, generally analysed in triplicate, using the computer program Excel 97 from Microsoft.

To check significant variations, a Student's *t*-test was used to analyze the values and a  $p < 0.05$  was taken as significant.

Cultivar (ID letter)	Harvest	Storage	Code
<b>A</b>	1	Immediately frozen	<b>A1-F</b>
		Short refrigeration	<b>A1-C</b>
	2	Immediately frozen	<b>A2-F</b>
		Short refrigeration	<b>A2-C</b>
<b>B</b>	1	Immediately frozen	<b>B1-F</b>
		Short refrigeration	<b>B1-C</b>
	2	Immediately frozen	<b>B2-F</b>
		Short refrigeration	<b>B2-C</b>
<b>C</b>	1	Immediately frozen	<b>C1-F</b>
		Short refrigeration	<b>C1-C</b>
	2	Immediately frozen	<b>C2-F</b>
		Short refrigeration	<b>C2-C</b>
<b>E</b>	1	Immediately frozen	<b>E1-F</b>
		Short refrigeration	<b>E1-C</b>
	2	Immediately frozen	<b>E2-F</b>
		Short refrigeration	<b>E2-C</b>
<b>M</b>	1	Immediately frozen	<b>M1-F</b>
		Short refrigeration	<b>M1-C</b>
	2	Immediately frozen	<b>M2-F</b>
		Short refrigeration	<b>M2-C</b>
<b>O</b>	1	Immediately frozen	<b>O1-F</b>
		Short refrigeration	<b>O1-C</b>
	2	Immediately frozen	<b>O2-F</b>
<b>D</b>	1	Immediately frozen	<b>D1-F</b>
	2	Immediately frozen	<b>D2-F</b>
<b>H</b>	1	Immediately frozen	<b>H1-F</b>
<b>G</b>	1	Immediately frozen	<b>G1-F</b>

**Table 1. Cultivars ID letters and codes with the corresponding harvest time and storage method, used in the presented report.**

## Results and Discussion

**Moisture content.** We found interesting intercultural differences in the moisture content which ranged between 87,36 % (cultivar D) and 94,17% (cultivar C).

**Total Flavonoid content.** Total Flavonoid content was expressed as mg catechin equivalents/g of FDW. Cultivars G, O and M had higher flavonoid content than all the other cultivars. In particular, strawberries from cultivar G showed almost twice the flavonoid content of cultivar B (averaging 5,33 mg/g FDW and 2,7 mg/g FDW, respectively). No relevant cultivar-to-cultivar differences were found among the other samples, and storage at short refrigeration conditions didn't seem to affect the total amount of flavonoid.

**Vitamin C content.** Vitamin C (ascorbic acid) concentration was expressed as mg of vitamin C/g FW (Figure 1). Fruits from G cultivar had the highest vitamin C content which represented the double of amount present in fruits from cultivar H. Compared to the corresponding wet samples, lower concentrations in vitamin C were found in all FD samples analysed. Storage at short refrigeration conditions didn't seem to affect the vitamin C content.

### HPLC Antioxidant Detection System.

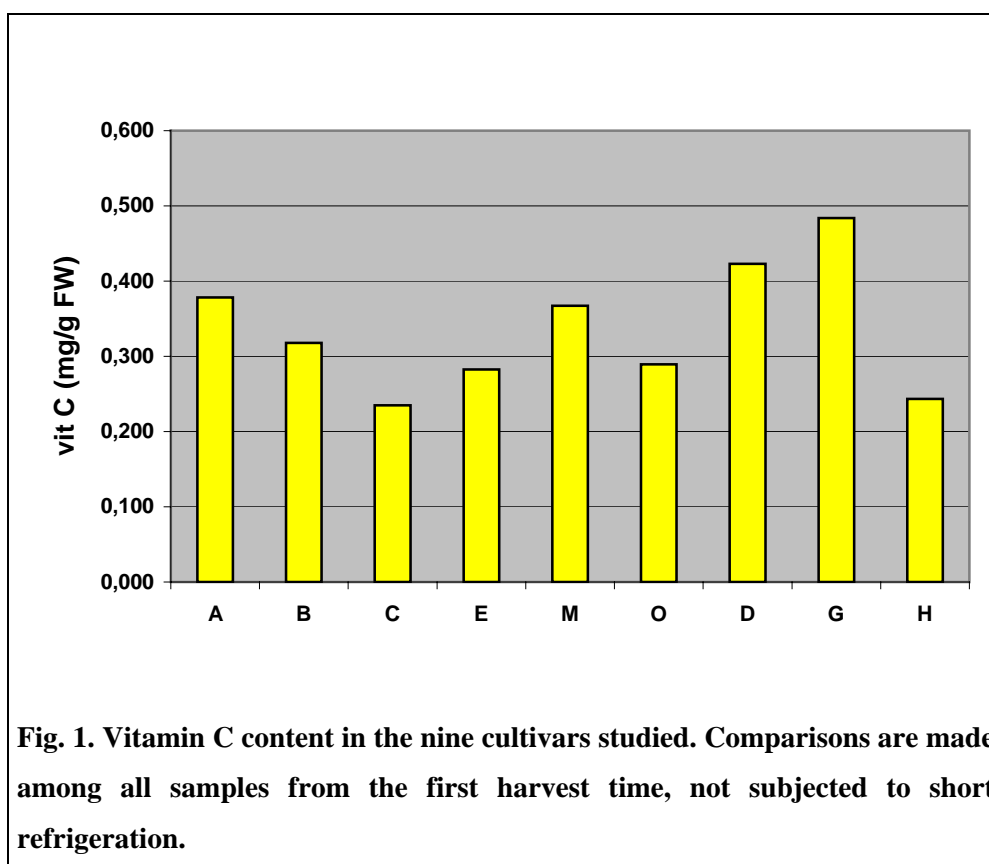
The on-line antioxidant system allowed to obtain the chromatographic separation and detection of compounds in the extracts, their identification through PDA spectrum and elution pattern, and simultaneously the detection of individual antioxidant capacity of each eluting compounds.

TFA was elected as the acidifier of choice of the eluents. In fact, when comparing the chromatographic and antioxidant analysis obtained by using the two different mobile phases (acidified with 0,1% TFA vs 0,05% FA) it was evident the the use of TFA resulted in better chromatographic resolution and also good ABTS reactivity. The reason is probably related to the buffering action of the Na-phosphate buffer, which allowed to compensate the pH and keep it not so dramatically lower than in physiological conditions.

The compounds of interest were quantified by using external standard calibration curves and reading their absorbance at the specific maximum wavelength. For quantification of derivatives compounds we used the calibration curve of the native compound showing closely similar spectral characteristics and molar extinction coefficients. We concentrated our attention on ellagic acid derivatives ( $\lambda_{\max}$ = 254 nm), p-coumaric acid derivatives ( $\lambda_{\max}$ = 312

nm), flavonols ( $\lambda_{\max}$ = 360 nm) and anthocyanidins ( $\lambda_{\max}$ = 512nm).

The capacity of each antioxidant compound expressed as negative peaks in the postcolumn chromatogram (Fig 2); the identification of the compounds responsible for each peak was possible since the delay in retention time observed between the corresponding PDA and ABTS peaks (64 seconds) is fixed, corresponds to the eluent travel time from the first to the second detector, and includes the antioxidant reaction time within the postcolumn coil (30 seconds).



**Contribution of single compounds to the total antioxidant capacity of strawberry extracts.** By integrating the main antioxidant peaks on the ABTS chromatogram the contribution % of each antioxidant compound to the total antioxidant capacity (TAC) of the extracts was calculated (Fig. 2. Numbers refer to the main antioxidants taken into account).

In all the strawberry extracts analysed, a first sharp peak corresponding to the early eluting

highly polar compounds (vitamin C) represented the main contribution to the TAC of extracts, and the system reached saturation during detection of the peak.

A second region (retention times RT=2.5-12 minutes) showed the present of high polar antioxidants, probably belonging to the class of phenolic acids. In particular, compound corresponding to peak 4 (RT=8.0, Fig.2) was evident only in some cultivars and showed an increase during short refrigeration. In some samples the compound represented alone >6% of the TAC.

A relevant contribution to the TAC of the extracts was due to the anthocyanins, observed in the central region of the chromatogram (RT=19-30 minutes).

Other compounds later eluting were identified mainly as ellagic acid derivatives, and flavonols. But some interesting antioxidant compounds are still unidentified only by the interpretation of their spectral characteristics and pattern of elution.

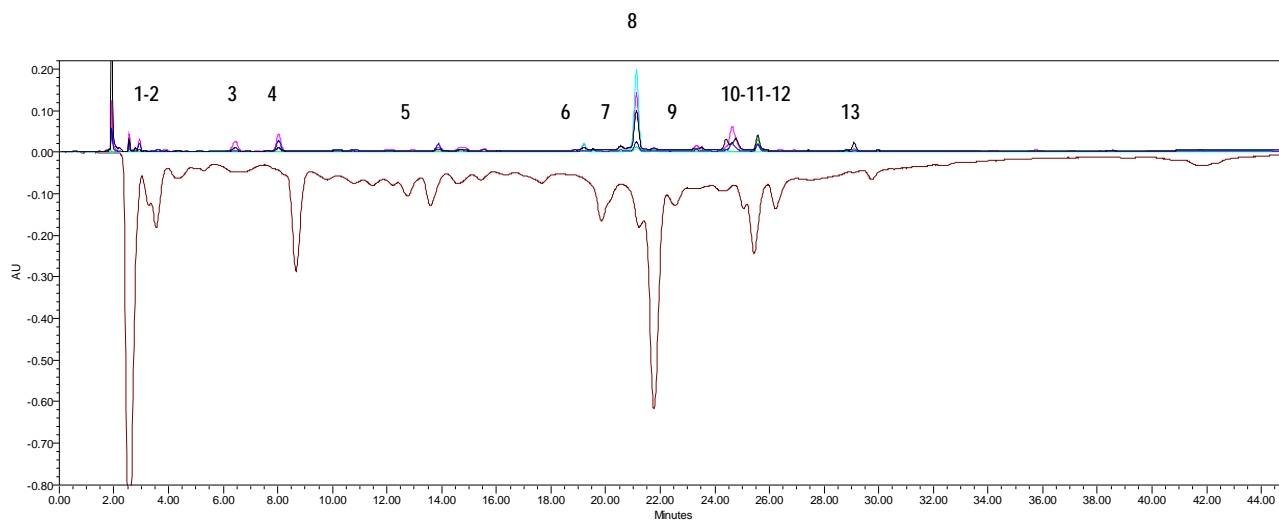
**C18-HPLC coupled to QTOF/MS.** The main anthocyanins and flavonols contributing to the TAC of strawberry extracts were further identified by running 4 selected samples in the same C18-HPLC system (mobile phase acidified with 0,05% FA) connected to QTOF-MS. Positive ions (parent ions and fragments obtained) were detected by analysing samples in the ESI-positive mode.

**Proanthocyanidins.** The increasing interest on proanthocyanidins arises from recent studies suggesting that the low proanthocyanidin oligomers (DP<4) could be absorbed in the gastrointestinal tract and so bioavailable (Santos-Buelga et al., 2001). Dimers have been detected in blood after human subjects consumed a proanthocyanidins-rich diet (Holt et al., 2002), and trimers have been shown to be absorbed through the human intestinal cell line Caco-2. For this reason the identification and quantification of proanthocyanidins should be emphasized, especially low oligomers.

In this study we analysed the procyanidins, which are the most representative proanthocyanidins. In ten samples analysed we found a DP ranging between 5,26 and 7,31.

**Biological variability.** No significant fruit-to-fruit differences were found in vitamin C and total flavonoid content. Small variations in the amount of single compounds were found in the strawberry extracts, but anyway they should be taken into account since differences showed always the same trend (E1-Fa with lowest amounts, E1-Fb and E1-Fc with similar

concentrations). Also the determination of procyanidins and total catechin and epicatechin content after hydrolysis gave different results in the 3 samples of cultivar E, with coefficient of variations of 18,32 and 15,68 respectively.



**Figure 2. HPLC analysis of antioxidant compounds of H1-F strawberries (sample D1-F). The upper part represents the overlappinf of the HPLC chromatograms recorded by PDA at all the wavelenght of interest (254, 280, 312, 360, 512 nm). The lower part corresponds to the HPLC chromatogram simultaneously recorded at 412 nm after reaction with ABTS.**

## Concluding Remarks

The data obtained from the one-month long STSM suggested us that differences exist in the phytochemical composition among different varieties and selections of italian strawberries.

Further studies are needed to confirm the results and go more into details. For these reasons, I am aiming at a future collaboration with the host institute and a joint publication.

I would like to take this opportunity to thank COST 863 for making this fruitful visit possible. I also thank Dr. Jules Beekewilder and colleagues for hosting me at the PRI Institute, and Dr. Esra Capanoglu for her help and friendship.

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