

# STSM Scientific Report 2009

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

Reference code: COST-STSM-863-04557

Subject: Assessment of Bioactive constituents in Bulgarian raspberry genotypes

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**Objectives:** The main goal of this STSM was to assess fruit quality on more perspective Bulgarian raspberry varieties based on Total phenols, anthocyanins and antioxidant measurements in fruits; Characterization on bioactive berry constituents (phenolic compounds) by LC/MS analysis (fruit and leaves).

Differential metabolite profiling subjected on pathogenic attack against root rot disease /*Phytophthora fragariae* var *rubi* FVR 11/ for evaluation on compounds strongly associated with disease resistance.

The STSM was accomplished at Scottish Crop Research Institute (Dundee, Scotland), by supervision of Dr. Derek Stewart in the period from 20/04/09 to 18/05/09.

## Materials and Methods

### Plant material

In our study we include more important Bulgarian cultivars from Small Fruit Department, Kostinbrod: Bulgarian rubin (Preussen x Ll. George), Samodiva (Bulg. rubin x Sh. Alena), Shopska alena [(Preussen x Ll. George) x Newburgh], Lyulin [(Newburgh x Bulg. Rubin) x Heritage], Esenna pozlata [Sh. alena x (G. Prosen x Ll.George) x Neburgh] and one American cv. Marlboro (chance seedling).

The reference genotypes cv 'Glen Moy' (susceptible) and cv 'Latham' (resistant) to root rot disease are inoculated by *P. fragariae* var *rubi* /FVR 11 race 3/.

## **Extraction method**

For evaluation of Total phenols, Anthocyanins, Antioxidant activity (fruits) and LC/MS analyses (leaves, fruits, and roots) were used a basic acetonitrile method according to G. McDougall (2005).

## **Total phenols quantifications**

The total phenolic content of the 1% fruit extracts was determined using the Folin–Ciocalteu method (Slinkard and Singleton, 1997). Phenolic content were estimated from a standard curve of gallic acid. Total phenol determination was carry out spectrophotometrically at 750 nm (Ultraspec 2100 UV/Visible Spectrophotometer Amersham Bioscience).

Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (DW) of tissue, and reported as a mean value  $\pm$  standard deviation (SD) for three repetitions.

## **Anthocyanins quantifications**

Total anthocyanin content of the fruit extracts are measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid–potassium chloride, 0.2 M) and 4.5 (acetate acid–sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 12,100 (cyanidin–3–glucoside) and absorbance of  $A = [(A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}]$ . Results were expressed as mg cyanidin–3–glucoside equivalents  $100 \text{ g}^{-1} \text{ fw}$ .

## **FRAP Assay of Antioxidant capacity**

Antioxidant 'power' can be presented as ferric reducing ability. The assay follows the formation of a colored ferrous:

2,4,6–tripyridyl–s–triazine ( $\text{Fe}^{3+}$  –TPTZ) complex from ferric ion.

The FRAP assay was developed to measure the ferric reducing ability of plasma at low pH (Benzie & Strain, 1996). A sample containing 3 ml of freshly prepared FRAP solution (0.3 M acetate buffer (pH 3.6) containing 10 mM 2,4,6–tripyridyl–s–triazine (TPTZ) and 40 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) and 100  $\mu\text{l}$  of extract (prepared as for phenol determination) was incubated at 37°C for 4 min and the absorbance was measured at 593 nm (Ultraspec 2100 UV/Visible Spectrophotometer Amersham Bioscience).

An intense blue color is formed when the ferric–tripyrindyltriazine ( $\text{Fe}^{3+}$ –TPTZ) complex is reduced to the ferrous ( $\text{Fe}^{2+}$ ) form at 593 nm.

A standard solution of 1 mM L–ascorbic acid in distilled water was prepared. The absorbance change was converted into a FRAP value, by relating the change of absorbance at 593 nm of the test sample to that of the standard solution of L–ascorbic acid (AsA) and results were expressed as  $\mu\text{mol AsA g}^{-1}$  dw.

### Liquid chromatography–mass spectrometry (LC–MS) analysis

Berry samples were analysed on an LCQ–Deca system, comprising Surveyor autosampler, pump and photodiode array detector (PDAD) and a Thermo – Finnigan ion–trap mass spectrometer. The PDAD scanned discrete channels at 280 nm, 365 nm and 520 nm.

The samples were applied to a C18 column (Synergi Hydro C18 with polar endcapping, 4.6 mm X 150 mm, Phenomenex Ltd.) and eluted using a gradient of 5% acetonitrile (0.1% formic acid) to 40% acetonitrile (0.1% formic acid) over 45 min at a rate of 400  $\mu\text{l}/\text{min}$ . The LCQ–Deca LC–MS was fitted with an ESI (electrospray ionisation) interface and analysed the samples in positive and negative ion mode. There were two scan events; full scan analysis followed by data–dependent MS/MS of the most intense ions using collision energies (source voltage) of 45%. The capillary temp was set at 250°C, with sheath gas at 60 psi and auxiliary gas at 15 psi.

### Results and discussions

**Total phenols and anthocyanins quantification:** The Total phenols content was detected for seven raspberry genotypes (fruit samples). In our study we observed a wide range in total phenols content varied between **416.3** for cv ‘Bulgarian rubin’ and cv. ‘Samodiva’ **415.4** (mg/100g FW) to **156.3** (mg/100g FW) for cv ‘Iskra’ (table 1). Generally all genotypes could be divided in three groups according to their phenols content: 1<sup>st</sup> group including cv ‘Bulgarian rubin’ and cv ‘ Samodiva’ characterized with dark–colored fruits and higher level of total phenols content (416.3 and 415.4 mg/100 g FW) which is closer (equal) to some wild berry species and hybrids.

The second group containing three genotypes ('Marlboro', 'Lyulin' and 'Esenna pozlata' – white colored fruits) characterized with medium level of phenols content (260.4–186.2 mg/100 g FW). 3<sup>rd</sup> group consist lowest level of total phenols content (156.3 – 160.23 mg/100 g FW) for two genotypes 'Shopska alena' and 'Iskra'.

The total phenols content evaluated for all accessions fully corresponding with data obtaining from other authors (Moyer et al., 2002).

The Anthocyanins could be also successfully used as biotic and abiotic stress marker was undertaken in our preliminary study.

Total Anthocyanins content in analyzed set ranged between 175.79 for cv. 'Lyulin' to 6.68 mg/100 g FW for cv. 'Esenna pozlata' characterized with white – colored fruits (table 1). Relatively higher anthocyanins content were estimated for commercially important Bulgarian varieties 'Bulgarian rubin' and 'Samodiva' – 152.79 and 164.01 mg/100 g FW.

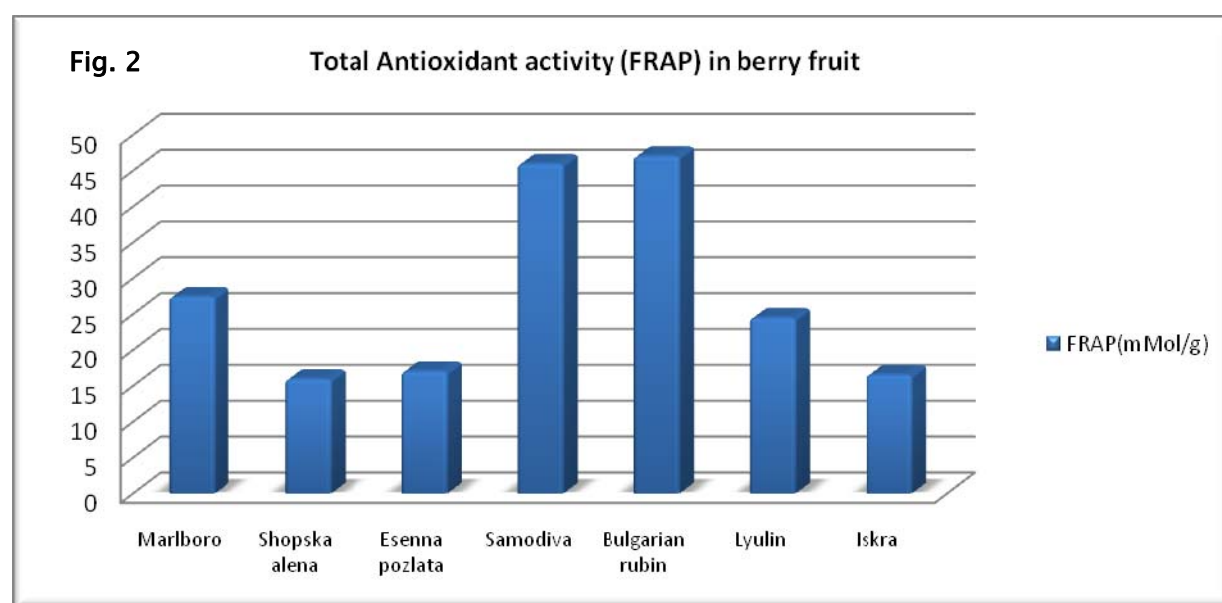
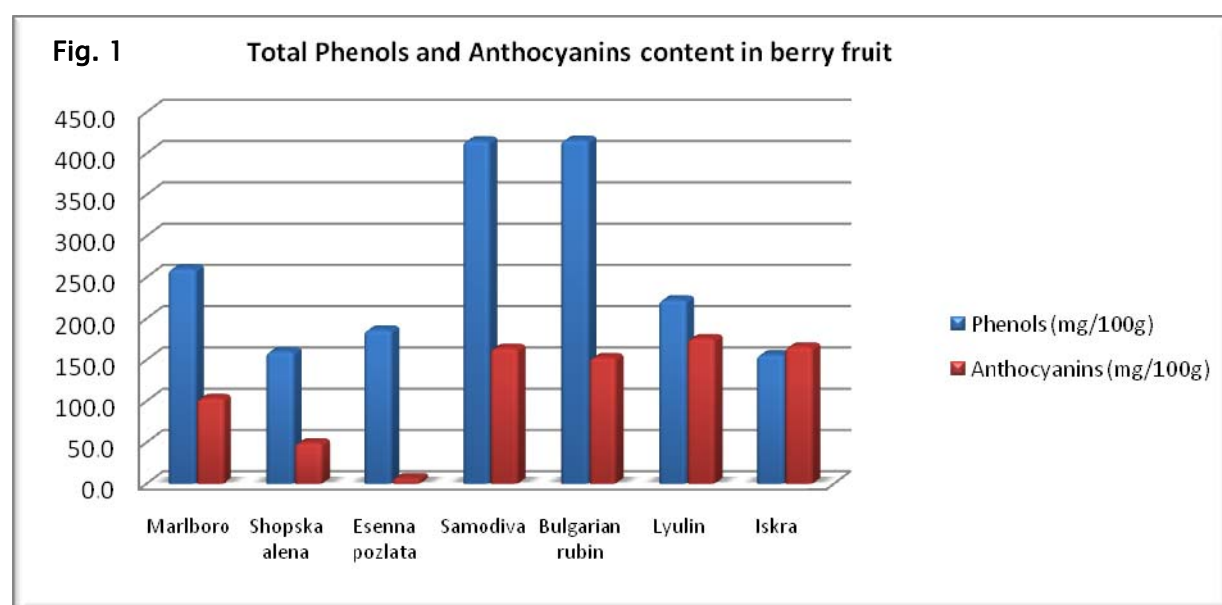
**Antioxidant activity:** Assessment of antioxidant activity (in berry fruits samples) for all seven accessions was carry out by FRAP method. FRAP values, derived from triplicate analyses, are expressed as mMol of ferric iron reduced per g of fresh–frozen fruit.

The values ranged from **47.06** for cv. 'Bulgarian rubin' and **45.98** for cv. 'Samodiva' to **15.98** for cv. 'Shopska alena' (table 1).

In comparison with other authors (Pantelidis et al., 2006) we also observed good correlation ( $r=0.98$ ) between Total phenols content and Antioxidant activity (FRAP).

**Table 1. Total Phenols, Total Anthocyanins content and Antioxidant activity (FRAP)**

Genotype	Total Phenols (mg/100g FW)	Total Anthocyanins (mg/100g FW)	FRAP (mMol/g FW)
Marlboro	260.41 ± 0.49	103.30 ± 1.93	27.45 ± 0.29
Shopska alena	160.20 ± 4.83	49.54 ± 0.28	15.98 ± 0.33
Esenna Pozlata	186.22 ± 2.06	06.68 ± 0.59	17.06 ± 0.10
Samodiva	415.43 ± 1.98	164.01 ± 0.46	45.98 ± 1.34
Bulgarian Rubin	416.34 ± 0.37	152.79 ± 0.42	47.06 ± 0.77
Lyulin	222.65 ± 0.99	175.79 ± 0.38	24.51 ± 0.38
Iskra	156.31 ± 3.26	165.87 ± 0.37	16.57 ± 0.27
<i>mean</i>	<i>259.65 ± 2.01</i>	<i>116.85 ± 0.63</i>	<i>27.80 ± 0.50</i>



**Liquid chromatography–mass spectrometry (LC–MS) analysis:** In order to metabolite profiling of seven raspberry genotypes (leave and fruit extractions) LC/MS analysis was carry out. A total 14 bioactive phenolic compound was identified for negative and positive ion mode detection (Table 2).

**Table 2. Molecular ions, fragmentation pattern, and tentative identification of phenolic compounds in raspberry (fruits and leaves) by LC/MS**

Peak	Compound	M <sup>+/-</sup> (m/z)	Fragment ions (m/z)
1	Cyanidin-3,5-O-diglucoside	611 <sup>+</sup>	287 [Cyanidin] (M <sup>+</sup> - Glc <sup>a</sup> - Glc <sup>a</sup> )
2	Cyanidin-3-O-sophroside	611 <sup>+</sup>	287 [Cyanidin] (M <sup>+</sup> - Soph <sup>b</sup> )
3	Cyanidin-3-O-rutinoside	595 <sup>+</sup>	287 [Cyanidin] (M <sup>+</sup> - Rhamnose <sup>c</sup> - Glucose <sup>a</sup> )
4	Pelargonidin-3-O-rutinoside	579 <sup>+</sup>	271 [Pelargonidin] (M <sup>+</sup> - Rhamnose <sup>c</sup> - Glucose <sup>a</sup> )
5	Pelargonidin-3-O-glucoside	433 <sup>+</sup>	271 [Pelargonidin] (M <sup>+</sup> - Glucose <sup>a</sup> )
6	Delphinidin-3-O-rutinoside	611 <sup>+</sup>	303 [Delphinidin] (M <sup>+</sup> - Rhamnose <sup>c</sup> - Glucose <sup>a</sup> )
7	Delphinidin-3-O-glucoside	465 <sup>+</sup>	303 [Delphinidin] (M <sup>+</sup> - Gluc <sup>a</sup> )
8	Kaempherol-3-O-glucoside	447 <sup>-</sup>	285 [Kaempherol] (M <sup>-</sup> - Glucose <sup>a</sup> )
9	Kaempherol-3-O-rutinoside	593 <sup>-</sup>	285 [Kaempherol] (M <sup>-</sup> - Rhamnose <sup>c</sup> - Glucose <sup>a</sup> )
10	Ellagic acid - pentose conugate	433 <sup>-</sup>	301 [HHDP*] (M <sup>-</sup> - Pentose <sup>d</sup> )
11	Quercetin-3-O-rutinoside	609 <sup>-</sup>	301 [Quercetin] (M <sup>-</sup> - Rhamnose <sup>c</sup> - Glucose <sup>a</sup> )
12	Quercetin-3-O-glucoside	463 <sup>-</sup>	301 [Quercetin] (M <sup>-</sup> - Glucose <sup>a</sup> )
13	Ellagic acid - acetylxlyoside	475 <sup>-</sup>	301 [HHDP <sup>e</sup> ] (M <sup>-</sup> - acetylxlyoside <sup>f</sup> )
14	Kaempherol -3-O-glucoronide	461 <sup>-</sup>	285 [Kaempherol] (M <sup>-</sup> - glucoronosyl <sup>g</sup> )

a – 162 units

b – 324 (2 x 162) units

c – 146 units

d – 132 units

e – hexahydroxyphenoyl units

f – 174 units

g – 176 units

Fig. 3. Total LC/MS spectrum (negative ion mode detection) of berry fruit

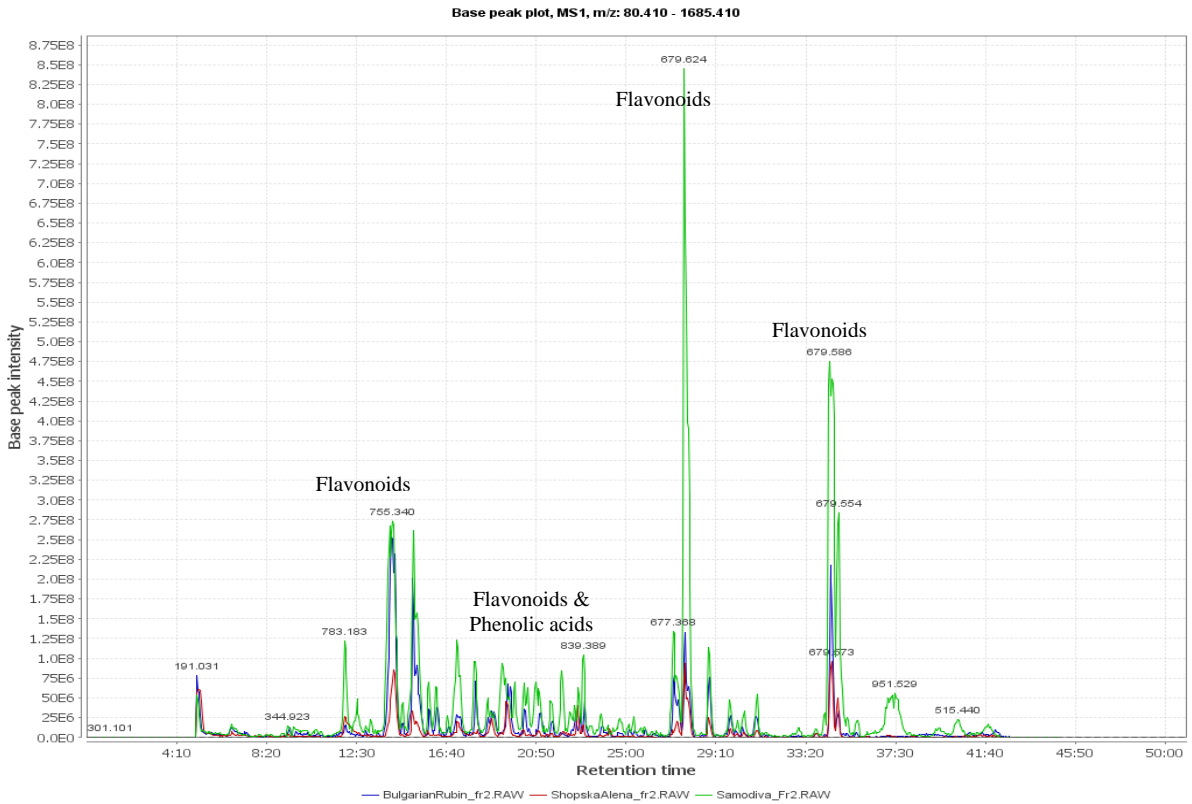
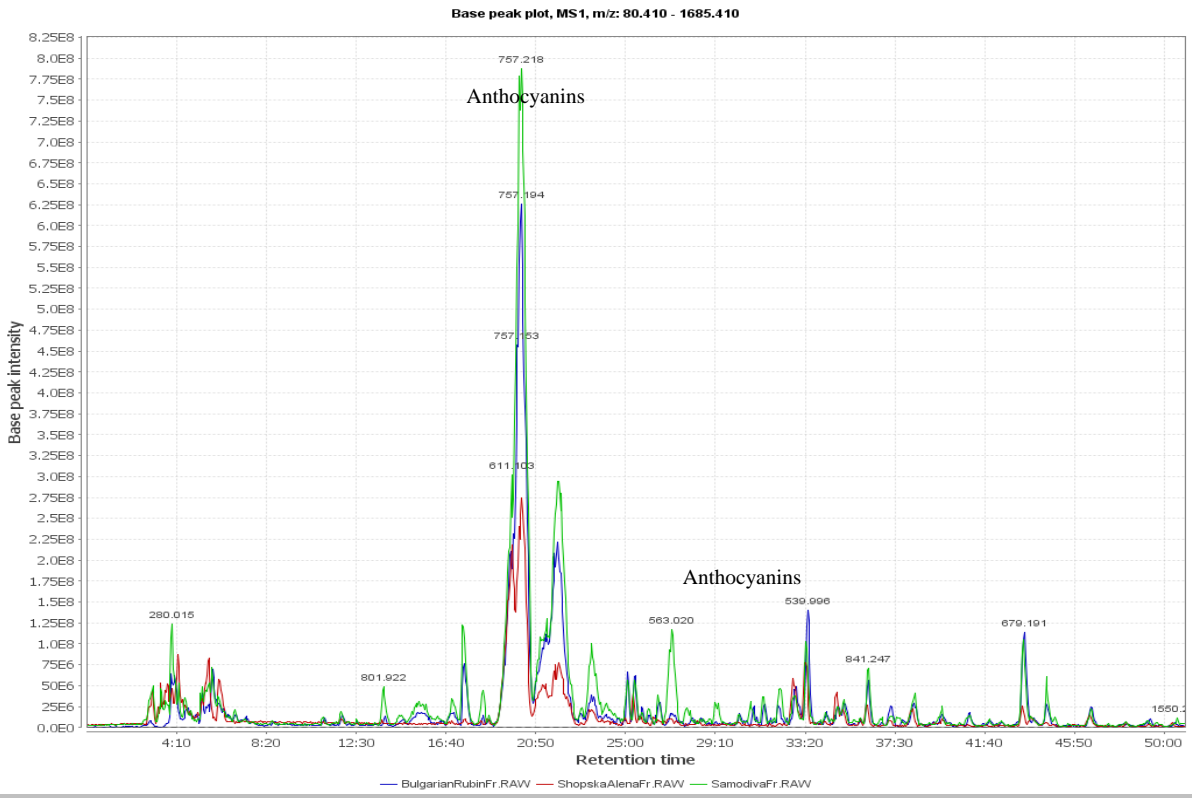


Fig. 4. Total LC/MS spectrum (positive ion mode detection) of berry fruit



LC/MS data analyses in our study show a higher similarity of phenolic profiles for cv 'Samodiva' and her parental form cv 'Bulgarian rubin'. This confirms previously data for phenols and anthocyanins assessing between these two genotypes.

A higher content of analyzed bioactive compounds in cv 'Bulgarian rubin' and cv 'Samodiva' could be suggested their using as fruit quality standards.

The work is a part of long term scientific programme directed to fruit quality improvement.

### **Acknowledgements**

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