

# STSM Scientific Report 2008

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

## Phytochemical profiling of different tissues from Portuguese endemic *Rubus* species

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**Objective:** The main goal of this STSM was to apply the techniques of liquid-chromatography-mass spectrometry (HPLC-PDA and LC-MS) for identification of the main phenolic compounds in extracts prepared from Portuguese endemic and uncharacterized *Rubus* species tissues (fruits and leaves).

The STSM was accomplished at Scottish Crop Research Institute (Dundee, Scotland), by supervision of Dr. Derek Stewart in the period from 10<sup>th</sup> to 23<sup>rd</sup> August.

### Materials and Methods

**Plant material and sample preparation:** Fruits and leaves from Portuguese endemic *Rubus* sp. were harvested in the Northeast of Portugal (Serra da Nogueira and Parque Natural da Serra de Montesinho, Portugal) and was stored at -80°C. The standard was a commercial Blackberry (cv. Apache) grown at Herdade Experimental da Fataca (Odemira, Portugal) which leaves and fruits were collected and stored in the same condition as the others. Whole samples were freeze-dried, ground and sent to the host institution on dry-ice, and stored at -80°C until analysis. 200 mg of each sample were extracted with 2 mL of a mixture of water and a solution of 2% (v/v) acetic acid in acetonitrile (1:1). The mixture was shaken overnight in an orbital shaker and centrifuged for 5 minutes at 16000 *g*. The supernatant was stored at 4°C until analysis. The samples were extracted in duplicate.

**Total polyphenol quantification:** The total phenolic content of the 1% (v/v) fruit extracts and 0.25% (v/v) leaf extracts was determined using the Folin–Ciocalteu method modified (Singleton and Rossi 1965). Phenolic content were estimated from a standard curve of gallic acid. 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 six measurements.

**Anthocyanins quantifications:** The total anthocyanin content of the fruit extracts was determined using a pH differential absorbance method (Deighton, Brennan *et al.* 2000). Absorbance readings were related to anthocyanin content using the molar extinction coefficient of 12100 calculated for cyanidin–3–O–glucoside. Results were expressed as milligrams of cyaniding 3–glucoside equivalents per gram of dry weight, and reported as a mean value  $\pm$  standard deviation (SD) for four measurements.

**Liquid Chromatography–Mass Spectrometry (LC–MS<sup>n</sup>):** After polyphenol quantification, samples containing 500  $\mu$ g GAE were aliquoted and the solvent was removed by rotary evaporation. The dry material was resuspended in 500  $\mu$ L 5% (v/v) acetonitrile in water and was analyzed on a LCQ–DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan). The LCQ–Deca system comprised a Surveyor autosampler, pump and photo diode array detector (PDAD) and a Thermo Finnigan mass spectrometer iontrap. The PDA collected data from 200–600 nm and scanned three discrete channels (at 280, 365 and 510 nm). The samples were applied to a C–18 column (Synergi Hydro C18 column with polar end capping, 4.6 mm x 150 mm, Phenomonex Ltd.) and eluted over a gradient of 95:5 solvent A:B at time=0 minutes to 60:40 A:B at time=60 minutes at a flow rate of 400  $\mu$ L/min. Solvent A was 0.1% (v/v) formic acid in ultra pure water and solvent B 0.1% (v/v) formic acid in acetonitrile. The LCQ–Deca LC–MS was fitted with an ESI (electrospray ionization) interface and analyzed the samples in positive and negative–ion mode. Two scan events, full scan analysis in mass range 80–2000 m/z followed by data dependent MS/MS of the most intense ions, were used for compounds detection and identification. The data–dependent

MS/MS used collision energies (source voltage) of 45%. The capillary temperature was set at 275 °C with sheath gas at 60 psi and auxiliary gas at 10 psi. Before the analysis, the system was tuned by using known concentrations of cyanidin-3-glucoside (positive mode) and quercetin-glucoside (negative mode) in ultrapure water.

## Results and Discussion

**Total polyphenols and anthocyanins quantification:** The total polyphenol content was determined for fruit and leaf samples. As can be seen in Table I for fruits, the phenolic content of the commercial blackberry was lower than the majority of wild blackberries species fruits. The phenolic content of the leaves is not significantly different between commercial and *R. brigitinus* and *R. vigo* leaves. *R. genevieri* leaves have the highest amount of polyphenols.

In relation to anthocyanins content, the commercial blackberry, *R. vigo* and *R. brigitinus* seem to have high content (not significantly different) while the *Rubus genevieri*, one of the wild *Rubus* species has the lowest.

Table I – Total polyphenols content and total anthocyanin quantification of *Rubus* samples (fruits and leaves).

Samples		Total polyphenols content (mg GAE / g DW)	Anthocyanins content (mg cy-3-glucoside / g DW)
Fruit	Blackberry cv. Apache	26.9 ± 6.3	3.037 ± 0.966
	<i>Rubus brigitinus</i>	44.6 ± 1.1	2.503 ± 0.041
	<i>Rubus genevieri</i>	31.5 ± 1.1	0.666 ± 0.034
	<i>Rubus vigo</i>	45.2 ± 1.0	2.549 ± 0.056
Leaf	Blackberry cv. Apache	118.0 ± 9.7	
	<i>Rubus brigitinus</i>	106.2 ± 6.4	
	<i>Rubus genevieri</i>	141.7 ± 5.1	
	<i>Rubus vigo</i>	138.7 ± 2.4	

**Liquid Chromatography–Mass Spectrometry (LC–MS<sup>n</sup>):** Although the fruit samples analyzed are from different species their chromatograms are similar, but presenting some quantitative differences in the relative amount of some compounds (Figure 1).

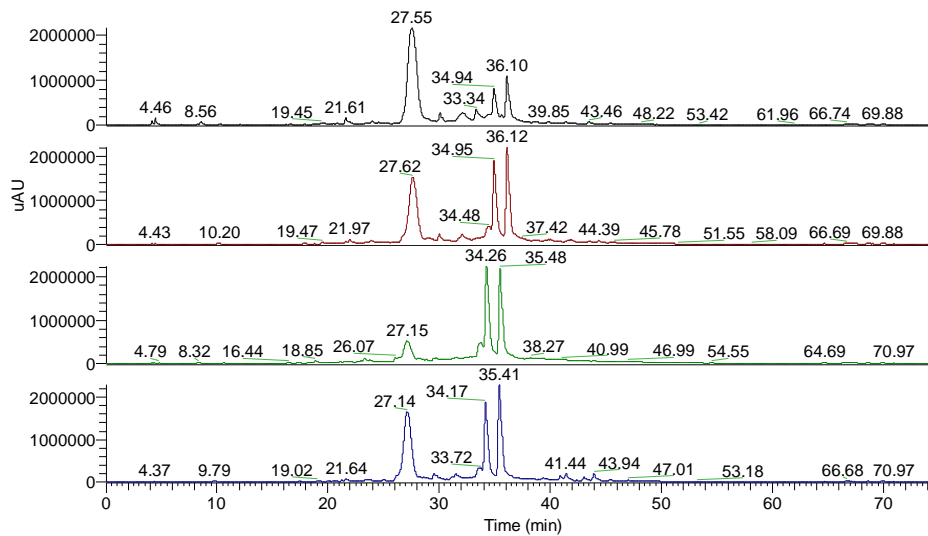


Figure 1 – HPLC profile obtained with PDA of the fruit samples, recorded at 280 nm. The first chromatogram refers to commercial blackberry, the second to *R. brigantinus*, the third to *R. genevieri* and the fourth to *R. vigoii*.

For the same amount of phenolics (500 µg GAE), commercial blackberry presents a higher amount of anthocyanins (Figure 2). It has almost five fold the anthocyanin content of *R. genevieri*. This result corroborates the values obtained previously for anthocyanins quantification.

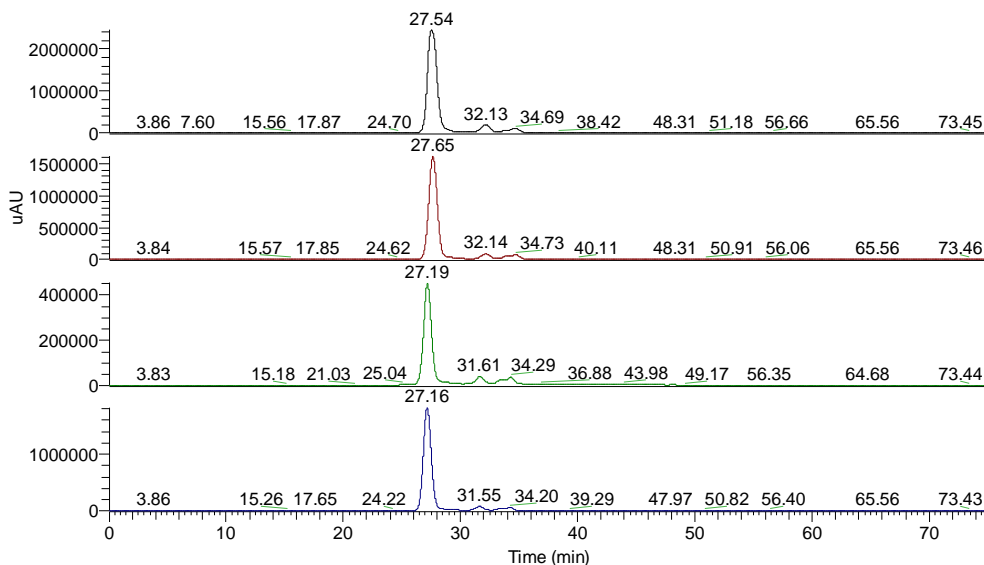


Figure 2 – HPLC profile obtained with PDA of the fruit samples, recorded at 510 nm. The first chromatogram refers to commercial blackberry, the second to *R. brigantinus*, the third to *R. genevieri* and the fourth to *R. vigoii*.

The difference between the different species could be compensated by the amount of others phenolics scanned at 280 nm and 365 and eluted between 30 and 50 minutes (Figure 1 and Figure 3). Further work will identify and quantify these compounds.

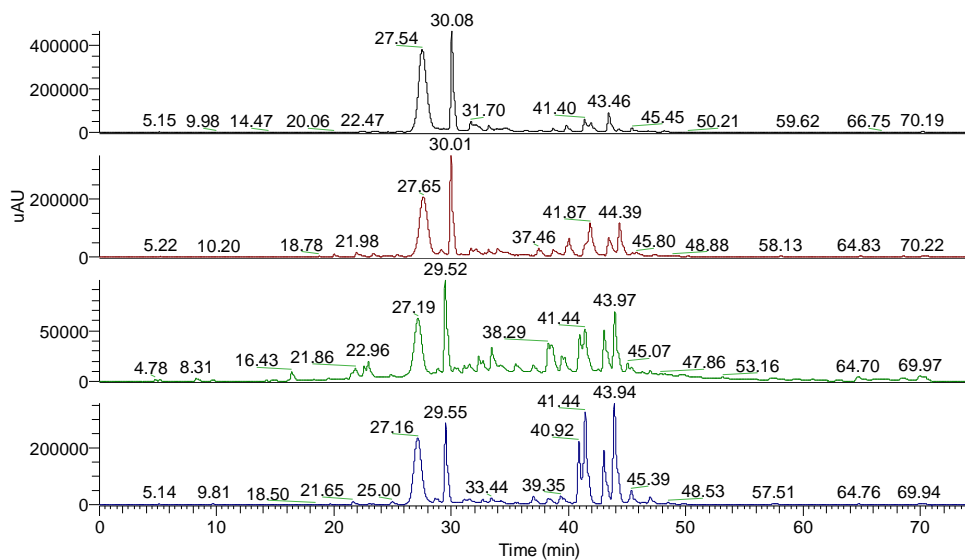


Figure 3 – HPLC profile obtained with PDA of the fruit samples, recorded at 365 nm. The first chromatogram refers to commercial blackberry, the second to *R. brigantinus*, the third to *R. genevieri* and the fourth to *R. vigoii*.

Regarding the leaf samples, the phenolic profile is as in fruit samples similar between the species analyzed. The differences are not qualitatively but quantitatively between the different compounds eluted (Figure 4).

For the same amount of GAE in all samples *R. vigoii* has a higher amount of compounds recorded at 365 nm than the other samples, mainly the compounds eluted between 40 and 50 minutes as can be seen in Figure 5.

No significant amounts of anthocyanins were detected in the leaf samples.

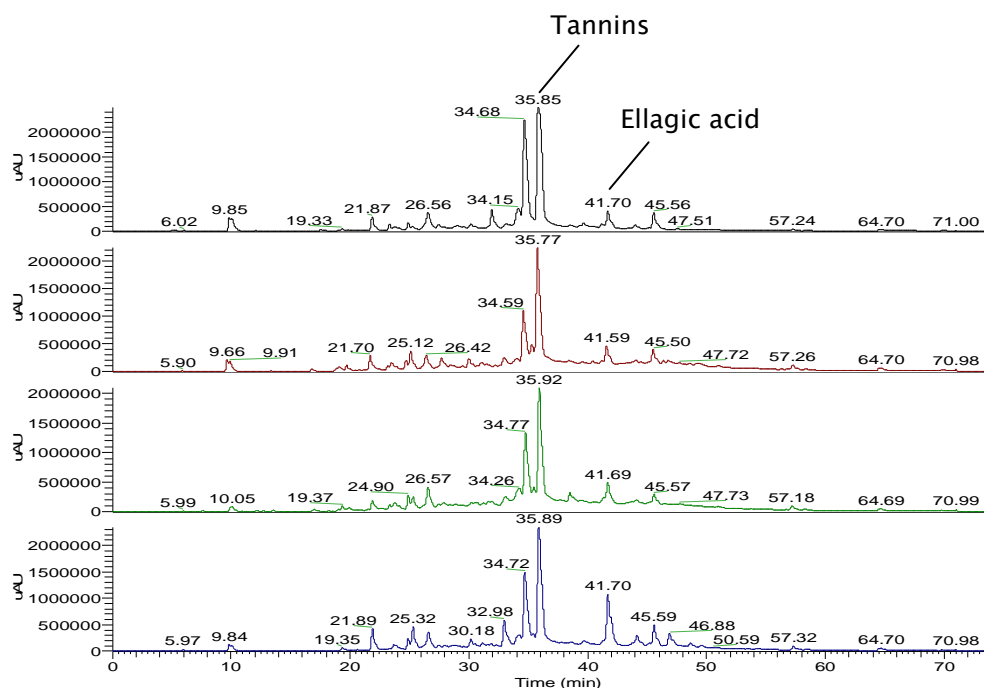


Figure 4 – HPLC profile obtained with PDA of the leaf samples, recorded at 280 nm. The first chromatogram refers to commercial blackberry, the second to *R. brigantinus*, the third to *R. genevieri* and the fourth to *R. vigo*.

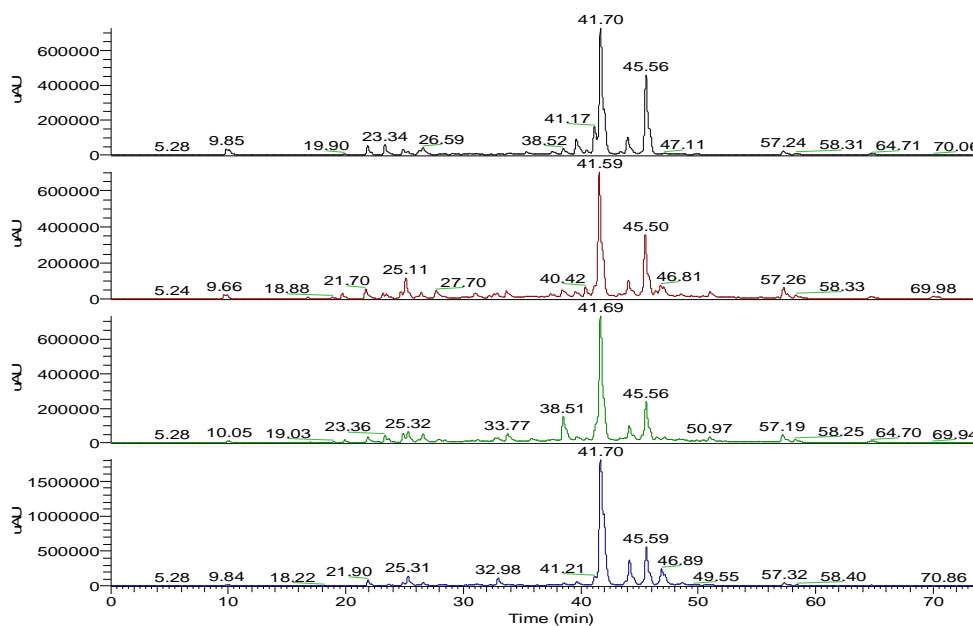


Figure 5 – HPLC profile obtained with PDA of the leaf samples, recorded at 365 nm. The first chromatogram refers to commercial blackberry, the second to *R. brigantinus*, the third to *R. genevieri* and the fourth to *R. vigo*.

**Conclusions:** Although there are some differences in phenolic content between the commercial and wild blackberries and between different wild blackberry species, more work is required, namely comparing all the species growing in the same conditions. The fact that two different species collected in similar locations presenting two different profiles for fruit and leaves seems to be indicative that the differences could be maintained when species growing on the same environment. If these differences will be confirmed, *R. brigantinus* and *R. vigoii* could be good applicants to blackberry breeding since they are species well adapted to hardiness conditions of the northeast of Portugal and they have a higher content in polyphenols nowadays related with health benefits.

## References

- Deighton, N., R. Brennan, et al. (2000). "Antioxidant properties of domesticated and wild *Rubus* species." *Journal of the Science of Food and Agriculture* 80(9): 1307–1313.
- Singleton, V. L. and J. A. Rossi (1965). "Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents." *American Journal of Enology and Viticulture* 16(3): 144–158.