

STSM Scientific Report 2009

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

***In vitro* digestion of fruits from Portuguese endemic *Rubus* species**

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Objective

The aim of this STSM is to perform *in vitro* digestion of fruits from Portuguese endemic *Rubus* spp. to assess the gastrointestinal stability of key polyphenol components.

The STSM was accomplished at Scottish Crop Research Institute (Dundee, Scotland), by supervision of Drs. Gordon McDougall and Derek Stewart in the period from 20th September to 10th October

Materials and Methods

i) **Plant material and sample preparation:** Fruits from 5 Portuguese endemic *Rubus* sp. were harvested in the north-east of Portugal and were stored at -80°C. Based on their high phenolic content and high *in vitro* antioxidant activity, two (*R. vagabundus* and *R. brigantinus*) were screened for *in vitro* digestion study. The standard was a commercial Blackberry (cv. Apache) grown at Herdade Experimental da Fataca (Odemira, Portugal). The fruits were collected and stored in the same condition as the others. Whole samples were freeze-dried and ground. Phenolic extraction was done using hydro-ethanolic extraction method. Briefly, lyophilized plant material was mixed with 50% (v/v) ethanol and kept on orbital shaker for 30 min, followed by centrifugation at 2350 g for 30 min. The supernatant was then filtered.

After phenolic quantification, ~100mgGAE (Gallic acid equivalent) of polyphenols were taken and aliquoted into 4 falcons having 25 mgGAE each and freeze-dried. The extracts were sent to the host institute in dry-ice, and stored at -20°C until analysis.

ii) Total polyphenol quantification: The total phenolic content was determined using a modified Folin-Ciocalteu method (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 ml.

iii) Anthocyanin quantifications: The total anthocyanin content of the extracts was determined using a pH differential absorbance method (Deighton et al. 2000). Absorbance readings were related to anthocyanin content using molar extinction coefficient of 12100 L/mol cm calculated for cyanidin-3-O-glucoside. Results were expressed as micrograms of cyanidin 3-glucoside equivalents per ml.

iv) *In vitro* digestion: The procedure was done as described in previous work by Coates *et al* (2007). The method consists of two sequential steps: an initial pepsin/HCl digestion for 2 h at 37 °C to simulate gastric conditions followed by a digestion with bile salts/pancreatin for 2 h at 37 °C to simulate small intestine conditions. The dried fruit extracts (containing 25mg GAE) were suspended in 20 mL distilled water and adjusted to pH 1.7 with 5 M HCl and 315 units/mL pepsin was added and then incubated at 37 °C in a heated water bath for 2 h with shaking at 100 rpm. After 2 hr, 2 mL aliquots of the post-gastric digestion were removed and frozen to be used for phenol and anthocyanin quantification when required. The remainder was placed in a 250 mL glass beaker, and 4.5 mL of 4 mg/mL pancreatin and 25 mg/mL bile salts mixture was added. A segment of cellulose dialysis tubing (molecular mass cutoff, 12 kDa) containing sufficient NaHCO₃ to neutralize the sample's titratable acidity was added, and the beaker was sealed with Parafilm. After 2 h of incubation at 37 °C, the solution outside the dialysis tubing was taken as the OUT sample representing material that remained in the gastrointestinal tract called "Colon available fraction" and the solution that entered the dialysis tubing was taken as the IN sample representing the material that entered the serum called "Serum available fraction". The NaHCO₃ diffused out of the dialysis tubing, and the pH of the OUT sample reached neutrality within 30-45 min. The amount of 1 M NaHCO₃ required to

neutralize an 18 mL aliquot of the postgastric digest plus 4.5 mL of bile salts/pancreatin was defined as the titratable acidity. The postgastric IN and OUT samples were thawed when required and centrifuged at 13200 g in a microfuge, and the supernatants were assayed for anthocyanin and phenol contents.

v) Liquid Chromatography-Mass Spectrometry (LC-MSⁿ): After polyphenol quantification, samples containing 250 µg GAE were aliquoted and the solvent was removed by rotary evaporation. The dry material was resuspended in 125 µL 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, Phenomenex 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 µL/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 quercetinglucoside (negative mode).

Results and Discussion

i) **Total polyphenols and anthocyanins quantification:** Total polyphenol and anthocyanin content was determined for original, gastric, IN and OUT fruit samples and the control (Table 1 and 2 respectively). As depicted in Table 3 (& Figs. 1 & 2), both phenols and anthocyanins were fairly stable during gastric digestion (~70-100%) for all three fruits. However, % recovery of phenols is higher than that of anthocyanins in OUT fractions.

Only 4% of anthocyanins were recovered in OUT fraction for both fruits and 9% for blackberry, as compared to 56.02%, 43.37% and 24.72% of phenol recovery for *R. vagabundus*, *R. brigantinus* and blackberry respectively. A significantly lower amount of phenols and anthocyanins were recovered in the IN fractions (as seen more clearly in figures 1 and 2).

Table 1: Polyphenol content of original, gastric, IN and OUT samples of *R. vagabundus*, *R. brigantinus* and blackberry.

Sample	Polyphenol content ($\mu\text{gGAE/mL}$)			
	Original	Gastric	IN	OUT
<i>R. vagabundus</i>	1192.87	940.25	58.70	675.05
<i>R. brigantinus</i>	1057.65	971.70	12.58	463.31
Blackberry	835.43	776.73	76.52	208.60

Table 2: Anthocyanin content of original, gastric, IN and OUT samples of *R. vagabundus*, *R. brigantinus* and blackberry.

Sample	Anthocyanin content ($\mu\text{g cy-3-glucoside /mL}$)			
	Original	Gastric	IN	OUT
<i>R. vagabundus</i>	738.43	724.51	11.13	32.47
<i>R. brigantinus</i>	718.02	792.23	5.57	28.76
Blackberry	1333.07	1428.62	92.77	115.00

Table 3: % recovery of polyphenols and anthocyanins during different phase of digestion

Samples	Polyphenol (% recovery)	Anthocyanin (% recovery)
<i>R. vagabundus</i>		
Gastric	70.94	88
OUT	56.02	4
IN	1.55	0.40
<i>R. brigantinus</i>		
Gastric	82.69	99
OUT	43.37	4
IN	0.43	0.20
Blackberry		
Gastric	83.68	96
OUT	24.72	9
IN	4.95	4

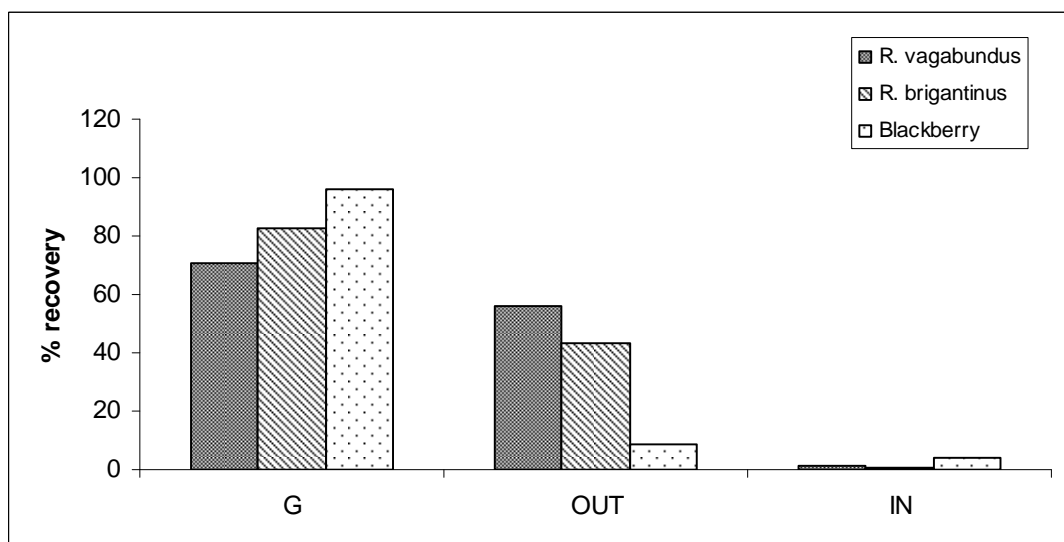


Fig 1: Recovery of polyphenols during different phase of digestion: G- post gastric fraction; OUT- colon available fraction; IN- serum available fraction.

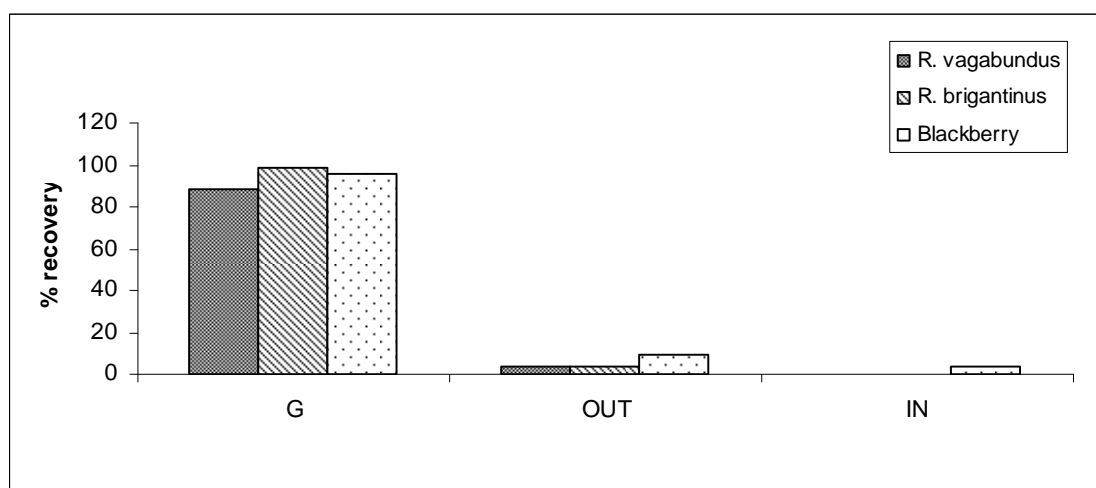


Fig. 2: Recovery anthocyanins during different phase of digestion: G- post gastric fraction; OUT- colon available fraction; IN- serum available fraction.

ii) **Liquid Chromatography-Mass Spectrometry (LC-MSⁿ):** The LC-MS profiles of both the fruits and control show a similar metabolite profile, only differing quantitatively for some metabolites (Fig. 3). As seen in Fig. 4, the LC-MS profiles of *R. vagabundus* for original and post gastric samples appear similar with some exceptions like an increase in the peak at Retention Time 14.2min. This shows the stability of most metabolites during gastric digestion. However, the diminishing peaks in IN and OUT fractions suggests low survival of metabolites. This result also

corroborates the previous phenol and anthocyanin quantification results. Similar results were obtained for *R. brigantinus* and blackberry (Fig. 5 & 6)

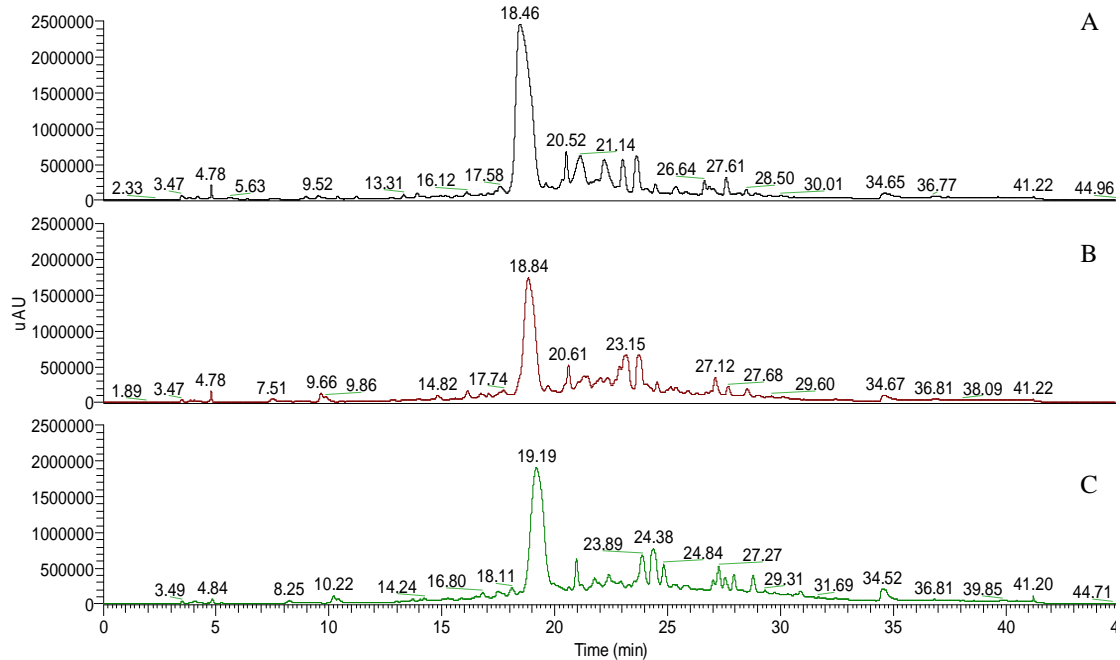


Fig. 3. HPLC profile obtained by PDA collected from 200-600 nm of blackberry (A), *R. vagabundus* (B) and *R. brigantinus* (C) original sample

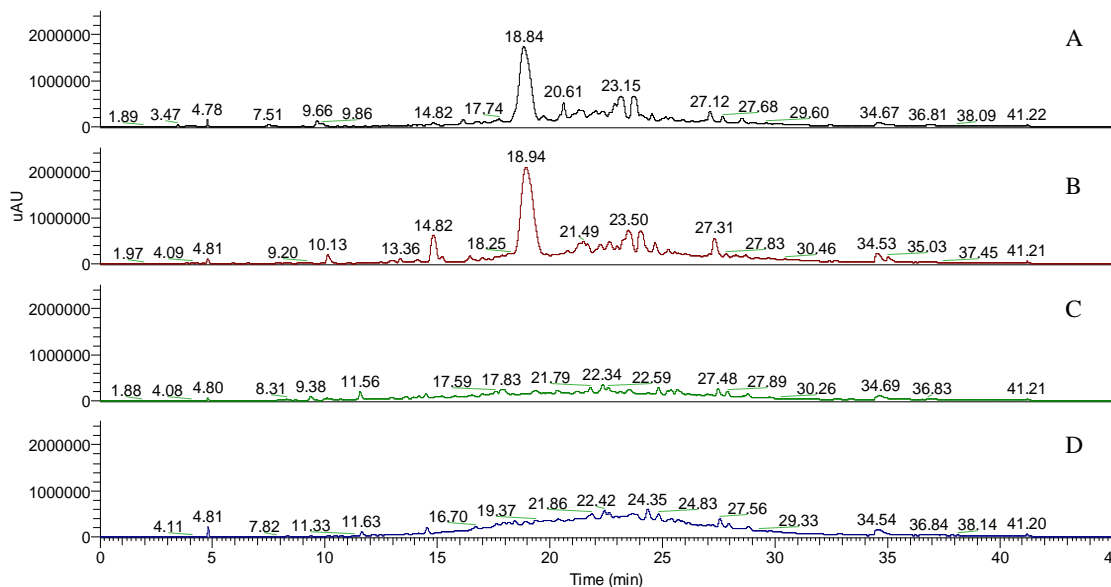


Fig. 4. HPLC profile obtained by PDA collected from 200-600 nm of *R. vagabundus* fruit sample, where chromatogram A is original sample; B is post-gastric; C is IN and D is OUT sample.

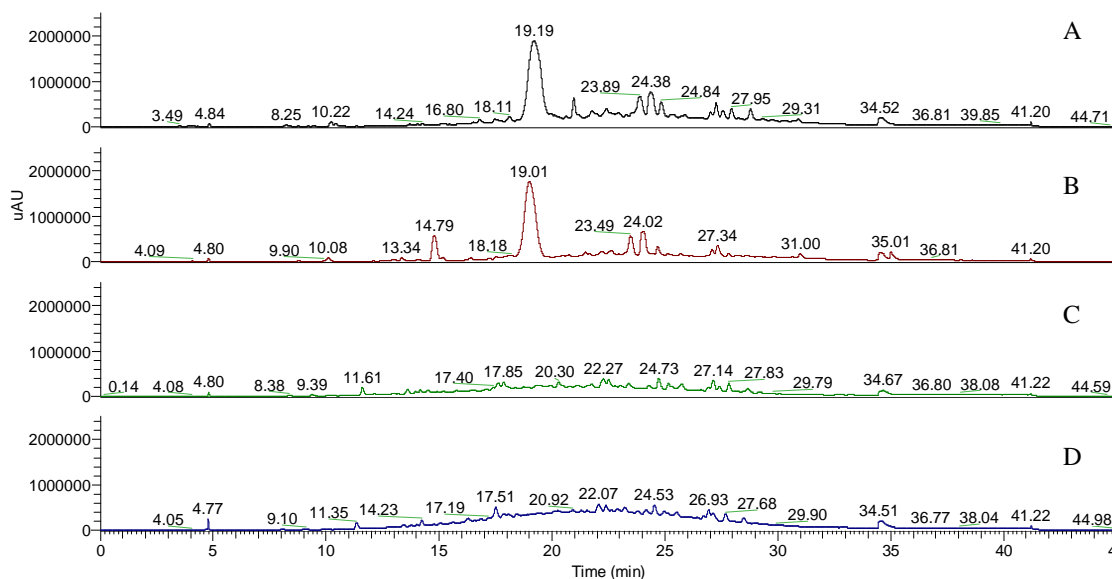


Fig. 5. HPLC profile obtained by PDA collected from 200-600 nm of *R. brigantinus* fruit sample, where chromatogram A is original sample, B is post-gastric, C is IN and D is OUT sample.

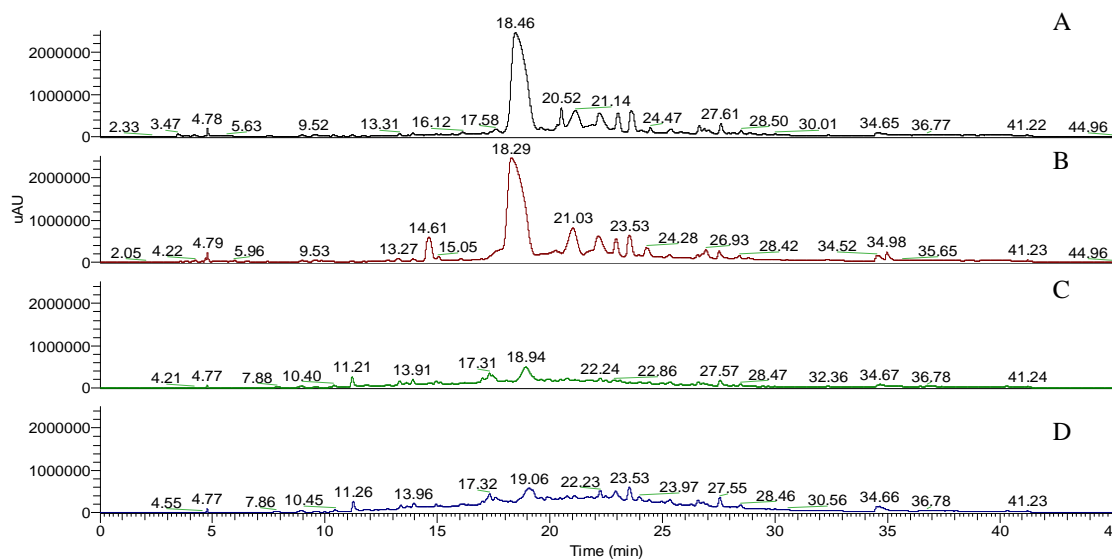


Fig. 6. HPLC profile obtained by PDA collected from 200-600 nm of blackberry fruit sample, where chromatogram A is original sample, B is post-gastric, C is IN and D is OUT sample.

Conclusion:

The endemic species under study (*R. vagabundus* and *R. brigantinus*) possess considerable polyphenol and anthocyanin content as compared with

commercial blackberry. *In vitro* digestion results suggest that both polyphenols and anthocyanins are stable under gastric digestion. Although, the stability of anthocyanins compounds decreases considerably under pancreatic digestion. This could be attributed to changing pH, increasing temperature and/or oxidation. Apparently, phenolic compounds survived better than anthocyanins during pancreatic digestion possibly as degradation products will be detected by the phenol method.

Future work:

1. Identify major compounds (phenols and anthocyanins) from LC-MS spectra.
2. To evaluate the effects of the serum and colon available metabolites from the digested fruits in the metabolism of a neurodegeneration cell model.

References

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