

Short term scientific mission, COST 863: From genomic to sustainable production, quality and health

***RUBUS* ELLAGITANNINS – COMPARISON OF DIFFERENT ANALYTICAL METHODS**

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STSM Scientific report.

Objective: This STSM represented a natural, timely and scientifically relevant collaboration between two well placed groups. The aim of the STSM was to compare four different methods for analysis of *Rubus* ellagitannins on the same sample set of blackberry and raspberry samples grown in Trentino (Italy). Two methods were developed at IASMA and the other two at SCRI.

Introduction

Ellagitannins are a major class of phenolics largely responsible for the astringent, antioxidant and potential health benefits of the *Rubus* species (particularly raspberries and blackberries). The *Rubus* ellagitannins constitute a complex mixture of monomeric and oligomeric tannins which contain the sanguisorboyl linking ester group as the well known ellagic acid and gallic acid moieties. Different methods for ellagitannin analyses were reported in the last 20 years. The wide range of different ellagitannins and ellagic acid derivatives make analytical approaches difficult, owing both difficulties in separation and lack of commercial standards. In order to simplify the identification process as a consequence most of the studies were done after acid hydrolysis of ellagitannins. The positive aspect of this methods is that they are very robust, easy applicable, while the main drawback is that the essential information on the authentic structure of ellagitannins are lost in this way. Rapid methods are fundamentally important for the analysis of large data sets (e.g very fast screening of breeding programs). On the other hand, in the postgenomic area where gene-function

studies are required, metabolomic approaches with precise, accurate methods which identify and quantify the highest number of the individual metabolites become evermore important.

Materials and methods

Plant material. 34 blackberry and 59 raspberry samples were grown under standardised conditions in the experimental field at Vigolo Vattaro (Trentino, Italy) over four different years (2004, 2005, 2006, 2007). Berries were harvested manually, every 2-3 days from plant that were deemed mature, placed immediately in ice packs and transported on the same day to the laboratory for extraction.

Extraction of polyphenols. Polyphenols were extracted following the method of Mattivi et al (2002) in which 60 g of fresh fruit was homogenized in a blender in 250 mL of mixture acetone/water (70/30v/v). The centrifuged extracts were stored at -20 C until analysis.

Sample purification. For the application of **Methods 1, 2, and 4** the samples were prepared according to the method published by Hager et al., 2008.

Methods.

Method 1: Short Column: Synergy, (20 x 2 mm, 2.5µm) MAX-RP 100A Mercury (Phenomenex). LC: Finigan LTQ system, controlled by the XCALIBUR software (2.07, ThermoFinnigan).

Method 2: DIMS method (McDougall et al., 2008)

Method 3: Acid hydrolysis (Vrhovsek et al., 2006, 2008).

Method 4: Direct method for analysis of ellagitannins and ellagic acid derivatives, column Luna (Phenomenex) UPLC-Synapt (Waters), controlled by MassLynx software; (manuscript in preparation)

Method 1 was developed during this STSM and **Method 4** was applied to a unique and detailed set of 93 blackberries and raspberries samples. The two methods are used for different purposes.

Method 1 is a quick method aimed to screen large populations, while **Method 4** is suitable to study ellagitannin profiles and for the fine quantification of individual ellagitannin components.

To the selected data set of 8 blackberry and 16 raspberry samples (year 2005) two additional already published methods were applied. **Method 3** is a new procedure for acid hydrolysis of *Rubus* ellagitannins in methanol, which allows quantification of all the major reaction products (ellagic acid, methyl-sanguisorboate, methyl gallate and an unknown ellagic acid derivative) and estimation of the mean degree of polymerization (mDP) of *Rubus* ellagitannins (Vrhovsek et al, 2006, 2008) and the **Method 2** involves direct infusion mass spectrometry (DIMS) and was published by (McDougall et al., 2008).

Description of the work carried out during the visit

- 1) Development and fine-tuning of **Method 1** and its application to the data set of 93 blackberry and raspberry samples. Analyses were done in triplicates.
- 2) Application of the **Method 2** to a representative sub-set of 24 samples chosen from the data set described in point 1. Analyses were done in triplicates.
- 3) Statistical analysis and comparisons of the results of the data set of 93 samples obtained with the **Methods 1 and 4**.
- 4) Comparison of the data of 24 samples obtained with the **Methods 1, 2, 3 and 4**.

Description of the main results obtained

The main aim was the comparison of the result of the sample sets of 93 samples obtain with the **Method 1** (“short method”), developed at SCRI during this STSM and **Method 4** (“long method”), previously developed at IASMA and applied to the data set. **Method 1** is a fast method aimed for rapid screening of the large sample sets while the purpose to use **Method 4** is the possibility to identify the profiles of ellagitannins and ellagic acid derivatives, and their quantification. With the newly developed **Method 1** it was possible to obtain a partial separation (Figure 1, Method 1) between anthocyanins, early eluting ellagitannins, sanguin H6 and lambertianin C, ellagic acid derivatives, flavonols and some unknown compounds having UV-VIS spectra similar to hydroxycinnamic acid (Figure 2). By combining the two different methods, it is possible to quantify the main ellagitannin structures in these sample sets and assess the year to year natural environmental and genetic contributions to the content.

Furthermore, we wanted to compare the results obtained with two new developed methods (**Method 1** and **Method 4**) with two already established and published methods (**Method 2** and **Method 3**). This would allow the ranking of samples by the longer, more time-consuming but data-rich method against the shorter but necessarily less data-rich methods. Through this comparison, we may be able to identify commonalities which would allow us to infer structural data on (say) ellagitannin structures through the mass spectral data obtained from the abbreviated methods.

The advantages and disadvantages of the two techniques are given in Table 1.

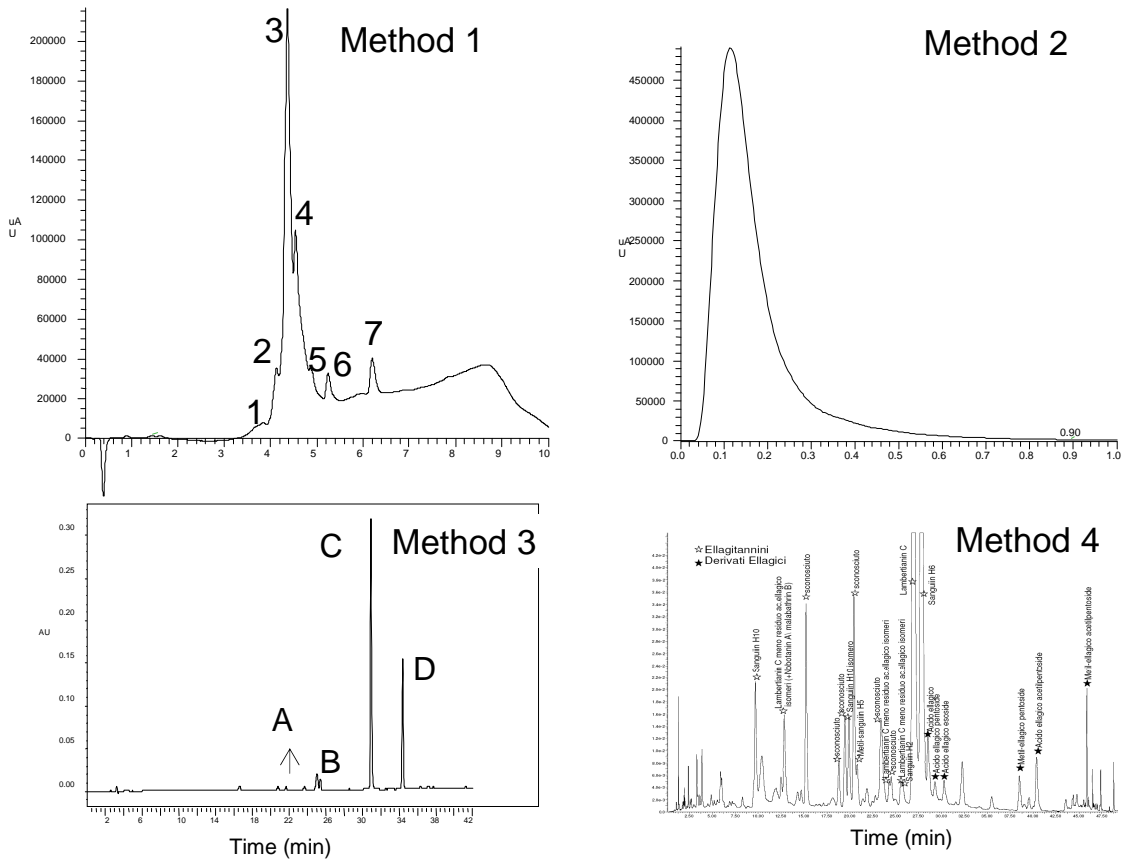


Figure 1: Examples of chromatograms obtained with different method

Legend: **A**, methyl gallate; **B**, derivative 1; **C**, ellagic acid; **D**, methyl sanguisorboate.

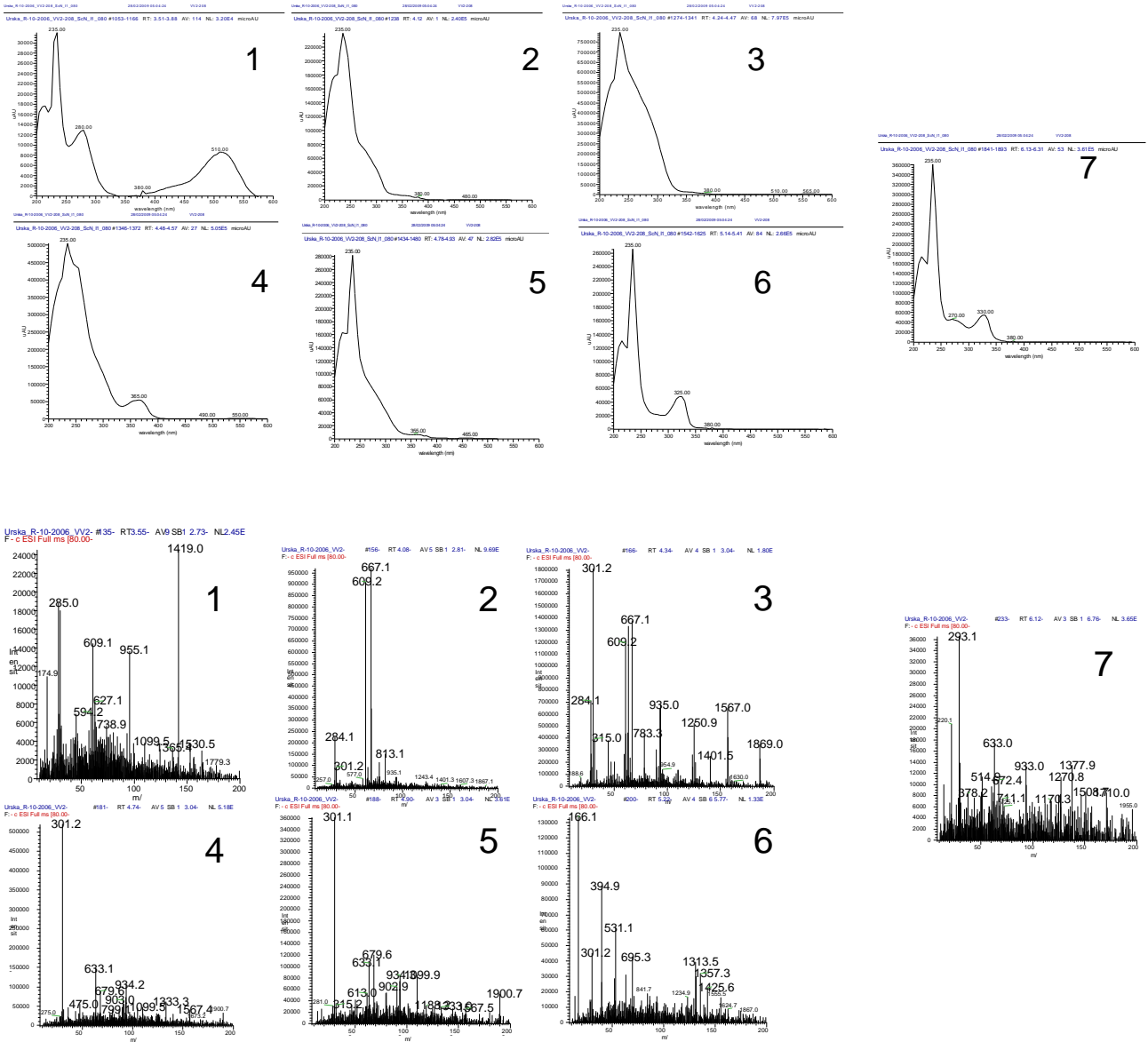


Figure 2: UV-VIS and MS spectra of the partially separated chromatographic peaks obtained with **Method 1** (for the legend see Figure 1, chromatogram Method 1).

Table 1: The comparison of four different methods included in the study

Method	Time of analysis (min)*	Positive aspects	Drawbacks	Suitable for
Method 1	10	Fast, partial separation and gives limited information on many components	Semi-quantitative	Fast screening for breeding programs Identification of outlying samples, and possible quality control application (e.g. minimum anthocyanin levels)
Method 2	1.5	Very fast and gives limited information on many components	No separation and semi-quantitative. Possible problems identified with in-source fragmentation and ion suppression	Very fast screening for breeding programs Identification of outlying samples, and possible quality control (QC) applications (e.g. minimum anthocyanin levels)
Method 3	47	Gives some structural information on ellagitannins, e.g. estimation of the mean degree polymerisation of ellagitannins. Chromatograms are easily processable. Robust and easy applicable Results comparable with already published data.	Indirect method, much structural information and profile of ellagitannins are lost. Only reports on ellagitannin structure Long sample preparation. Isolation of the methyl sanguisorboate is required.	Screening for breeding programs and comparison of ellagitannin profiles in berry products
Method 4	61	Analysis of the ellagitannins and ellagic acid derivatives on molecular level – possibility to identify the profiles Reports on other components	Slow, isolation of native compounds requested in order to do quantification	Detailed study of the ellagitannins on the molecular level. Important for gene function studies and detailed studies on nutrition, bioavailability and bioactivity.

Legend: * run time+equilibration time

CONCLUSIONS

The abbreviated methods developed in this STSM proved to be robust, reproducible and potentially amenable to other samples and wider applications. The comparative analysis of the longer and abbreviated methods is not yet completed but initial results suggest that the abbreviated methods may give a rapid assessment of ellagitannin diversity and be suitable as first pass methods to identify and partly quantify structural diversity in ellagitannin profiles before further analysis of the difference by the more time-consuming data-rich methods.

Future collaboration with host institution

We intend to write a joint paper on the comparison of the methods developed during this STSM and SCRI staff plan to visit IASMA in the coming year to discuss future collaborative efforts, especially with regard to obtaining EU funds.

Projected publication/articles resulting or to result from STSM

We intend to write at least one joint paper on this work and we hope to extend our collaboration with other studies and projects.

Literature

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