

STSM Scientific Report 2007

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

Phenolic metabolites from strawberries in human plasma during a medium-term consumption study, and metabolic profiling of ripe fruits from the same selected cultivar

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Objective: The aim of this STSM was to use the liquid-chromatography- mass spectrometric (LC-MS) facilities to examine the uptake of phenolic compounds from strawberries in human serum of 12 healthy volunteers involved in a medium-term behavioral counseling study. The main challenge was to investigate the putative extent of the human absorption and metabolism of the ingested phytochemicals, after at least 12-14 hours from the last intake of strawberries.

Analyses performed also included the LC-MS metabolic profile of the strawberry selected cultivar grown in Italy at different locations, to check for the potential role of the environmental factor on the phenolic composition of the fruits.

The STSM was accomplished at the Scottish Crop Research Institute (Dundee, Scotland) in the work group of Dr. Gordon McDougall within the period stipulated (18/11/07 to 19/12/07).

Materials and Methods

Study design and serum preparation. Among the strawberry genotypes previously investigated, Sveva strawberry variety, cultivated at the Azienda Acciarri in Ripatransone (Ascoli Piceno, Italy), was selected for this behavioral counseling study organized in the home Institute. During may 2007, twelve healthy volunteers previously recruited and trained were invited to consume 500 grams of strawberries per day for 16 days, preferably at mid-morning and mid-afternoon between meals. During the study the subjects were invited to maintain their normal dietary habits, to reduce the intake of polyphenols-rich food every dinners before blood draws, and to daily update the diet diaries provided before the trial. Blood was obtained after an overnight fast at days -10 (baseline 1), -1 (baseline 2), 0, 4, 8, 12, 16 with days 0-16 representing the period of strawberries supplementation, and one month after the end of the study (wash-out 1). Heparinized plasma

samples were immediately stored at -80°C until analysis, and 600 μl aliquots were sent on dry-ice to the host institution.

Plasma samples were extracted by diluting plasma 1:3 (v/v) in the extraction solution, consisting of 0.25% formic acid in 60% methanol and 40% acetone. 37.5 μM fluorotyrosine was also added to the extraction solvent as internal standard, in order to reach the final concentration of 75 ng fluorotyrosine/injection volume (10 μl). After vortexing, the samples were placed in the dark on ice for 1 hour, to facilitate proteins precipitation, and centrifuged at 14000 rpm for 15 minutes at 4°C . The supernatant was removed, frozen for 2 hours and then spinned to dryness by rotary evaporation. Immediately before the LC-MS analysis, a first set of dry serum samples were resuspended in 200 μl 5% acetonitrile in ultrapure water (final solution 2 times more concentrated than the starting plasma), vortexed for 20 seconds and sonicated at 8°C for 10 minutes. The resuspended samples were left to cool down for 1 hours at -20°C , to facilitate flocculation and successive elimination of remaining proteins and peptides, and centrifuged at 14500 rpm for 10 minutes at room temperature prior to LC-MS injection. In a second trial, we introduced some slight modifications to the sample preparation procedure, in order to reduce as much as possible the signal noise due to the still present peptides: a set of serum dry residues were resuspended in 100 μl cold 10% acetonitrile in ultrapure water (final concentration 6 times higher than the starting plasma samples), vortexed, sonicated and then kept overnight at -80°C . The following day, the samples were thawed, centrifuged at 14000 rpm at 4°C and subjected to LC-MS analysis.

Liquid Chromatography-Mass Spectrometry (LC-MSⁿ). The resuspended serum samples were analyzed on a Finnigan LTQ system, controlled by the XCALIBUR software (2.0, ThermoFinnigan). The system comprised of a Surveyor autosampler, pump and photodiode array detector (PDA), and the Finnigan LTQ ion trap, a ThermoFinnigan mass spectrometer of new generation. In particular, the Finnigan LTQ is the first 2-dimensional linear trap available, and the advanced linear design allows in the same time to maintain the main fruitful characteristics of the last versions of the ion trap instrumentation, and to achieve faster scan rates, enhanced sensitivity, better trapping efficiency and higher trap capacity (5 μl of injection volume are required).

The photodiode array collected spectral data in wavelength range 200-600 nm, and scanned three discrete channels at 280, 360 and 510 nm. The column (Hydra RP with polar end capping, 2.0 mm x 150 mm x 4 μm , Phenomenex Ltd.) temperature was kept at 30°C , and the autosampler (Surveyor AS, ThermoFinnigan) tray temperature control was set at 6°C during sequence run. As first trial, a 2-98% linear gradient with 0.1 % formic acid in ultrapure water (Eluent A) and 0.1% formic acid in acetonitrile (Eluent B) at a flow rate of 200 $\mu\text{L}/\text{min}$ over 45 minutes was used for separation,

followed by further 10 minutes for the re-equilibration of the column prior to the next injection. In a second attempt, a less steep gradient was adopted.

The Finnigan LTQ 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 50-2000 m/z followed by data dependent MS/MS of the five most intense ions, were used for compounds detection and identification. The data-dependent MS/MS used collision energies (source voltage) of 45% in wideband activation mode. The capillary temperature was set at 275 °C with sheath gas at 60 psi and auxiliary gas at 10 psi. Before the analysis, the instrument was tuned by flow-injection of 50 µg/ml cyanidin-3-glucoside and gallic acid aqueous solutions, for the positive and negative ion mode detection, respectively.

Plant material and extracts preparation. Strawberry fruits from the selected cultivar were previously harvested at the Azienda Acciarri Bruno in Ripatransone (Ascoli Piceno), as for the human study, and at the experimental field of the Faculty of Agriculture of Marche Polytechnic University (Ancona). Whole strawberries were sent to the host institution on dry-ice, and stored at –80°C until analysis. Then the fruits were thawed, weighted and extracted by homogenizing (Waring Blender, 5 sequential times for 10 seconds each) 100 g of fruit samples in 100 ml of extraction solution, consisting of 1% (v/v) formic acid in acetonitrile, in order to reach 50-60% acetonitrile in the final extracts, due to the high water content of strawberries. The mixture was filtered two times through muslin, the final collected volume was recorded and centrifuged at 8 °C in two sequential times (15 min at 4000 rpm). The supernatant was stored at –80°C until analysis.

Folin-Ciocalteu and Anthocyanins assays. The total phenolic content of the 1% strawberry extracts was determined using the Folin-Ciocalteu colorimetric method as modified by Slinkard e Singleton. Quantifications were calculated through a calibration curve previously prepared with known concentrations of gallic acid standards. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh weight (FW) of strawberry, and reported as a mean value ± standard deviation (SD) for four measurements.

The total anthocyanin content of the extracts was determined using a modified pH differential method previously described, with some modifications. Absorbance readings were converted to quantifications by using the molar extinction coefficient of 12100 for cyanidin-3-glucoside, previously calculated. Results were expressed as milligrams of cyanidin-glucoside equivalents per gram of fresh weight, and reported as a mean value ± standard deviation (SD) for four measurements .

Liquid Chromatography-Mass Spectrometry (LC-MSⁿ). Samples containing 500 µg GAE by

Folin-Ciocalteu assay were aliquoted and the solvent was removed by rotary evaporation. The dry matter, once resuspended in 250 μ l 5% (v/v) acetonitrile in ultrapure water, was analyzed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan). A Surveyor HPLC system (ThermoFinnigan, USA) was used. This comprised an autosampler set at 10°C, a pump, a photodiode array detector (PDAD) collecting data from 200-600 nm and scanning three discrete channels (at 280, 365 and 520 nm), and a ThermoFinnigan mass spectrometer ion trap. Separation was carried out using a 4.6 mm x 150 mm x 4 μ m Synergi Hydro C18 column with polar end capping (Phenomenex Ltd.), heated to 30°C and eluted at a flow rate of 400 μ L/min over 75 minutes. A 5-100% step gradient of 0.1 % formic acid in MilliQ water (Eluent A) and in acetonitrile (Eluent B) was used for separation. 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 five most intense ions, were used for compounds detection and identification. The data-dependent MS/MS used collision energies (source voltage) of 45% in wideband activation mode. 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 gallic acid (negative mode) in ultrapure water.

Results and Discussion

Liquid Chromatography-Mass Spectrometry (LC-MSⁿ) of plasma samples. Until now, very few studies have been focused on the detection of phenolic metabolites in human plasma and urine after the intake of phenolic-rich food, and most of these limited the search to the first 2-3 hours after the food consumption. A recent work (Duthie SJ et al., 2006), similarly designed to our study, observed that a daily consumption of anthocyanin-rich cranberry juice did not seem to alter plasma antioxidant activity and biomarkers of oxidative stress, when blood and urine collection are obtained after an overnight fast. Furthermore, no flavonoid metabolites were detectable in plasma. The beneficial effects on the erythrocyte resistance to oxidative stress, observed during our strawberry consumption study (data not shown), suggested us to focus our attention to any putative metabolic variations detectable in human serum and to detect the presence of phenolic components from strawberry, once absorbed and biotransformed. The detectable presence of polyphenols in human serum would be an important data to confirm the possible role of these phytochemicals in the observed improvement of antioxidative defenses.

One goal of the present STSM was to develop and adapt a procedure for the preparation and the analysis of human plasma samples at our disposal. The main difficulty consisted in the low volume

of plasma at our disposal for this specific analysis (less than 1ml), which limited the possibility to concentrate serum samples prior to injection. A further challenge was the ability to almost completely get rid of plasma proteins and peptides which otherwise could interfere with the fine LC-MS analysis, by masking the compounds of interest and increase the signal interferences.

In the first attempt, the procedures for plasma deproteinization and extraction, such as the LC-MS analysis method were developed by adapting existing protocols, available in literature, for GC/MS and LC/MS plasma metabolome analysis (Jiye A et al., 2005; Mauri PL et al., 1999; Mazza G et al., 2002; Cooke DN et al., 2006; Rechner AR et al., 2002). Two sequential attempts were conducted to optimize the procedures: the main modifications in the sample preparation procedures consisted in the increase of the degree of concentration of the samples during preparation (from factor 3 to 6), the increase of % acetonitrile in the resuspension solvent, and the promotion of protein precipitation and sample purification by giving attention to the low temperature maintenance and the centrifugation step. For what concerns the chromatographic conditions, in the second trial we opted for a less steep elution gradient, in order to facilitate the separation and detection of the compounds of interest.

In both the attempts, no evident differences were observed in serum metabolic profiles that could be correlated with the strawberry intake. Looking at the PDA and MS chromatograms, no discernible variations were found among the baselines and the samples collected during the study (Fig.1). The incomplete removal of proteins during extraction may have masked other potential co-eluting metabolites, and a future revision of the extraction procedure is aimed. Also the low degree of concentration could have played a role in the failure of detection of the putative metabolites, which could be present but below the limit of detection of the analysis system. For this reason, in parallel to our work we prepared, runned and analyzed higher volume of starting plasma samples coming from a similarly designed feeding study; anyway, also in this case the problem of protein transfer into the serum persisted, and no detectable phytochemical metabolites from berries were observed in serum during the period of berry intake (data not shown).

Interestingly, in all the subjects analyzed a 360 nm-absorbing compound (RT = 18.3), absent in the baselines samples, was detectable in serum during the strawberry intake period, while again disappearing after one month from the study (Fig.2). The clear identification of the molecule was not possible, due to the very low concentration and the lack of MS and MS/MS informations; the compound, in fact, didn't show to have easily ionizable chemical groups in its structure, at least at the LC-MS conditions used in this study.

Using the present experience, another future attempt to observe the bioavailability of the phenolics ingested and absorbed from strawberries is hoped. For this reason, a new modified feeding study

based on the prolonged consumption of strawberries has already been planned, and a future collaboration with the host institution would be aimed. Anyway, we are aware that the sample preparation and LC-MS conditions optimization are the most crucial steps for this fine analysis, and that the development of a method should take more than 4 weeks.

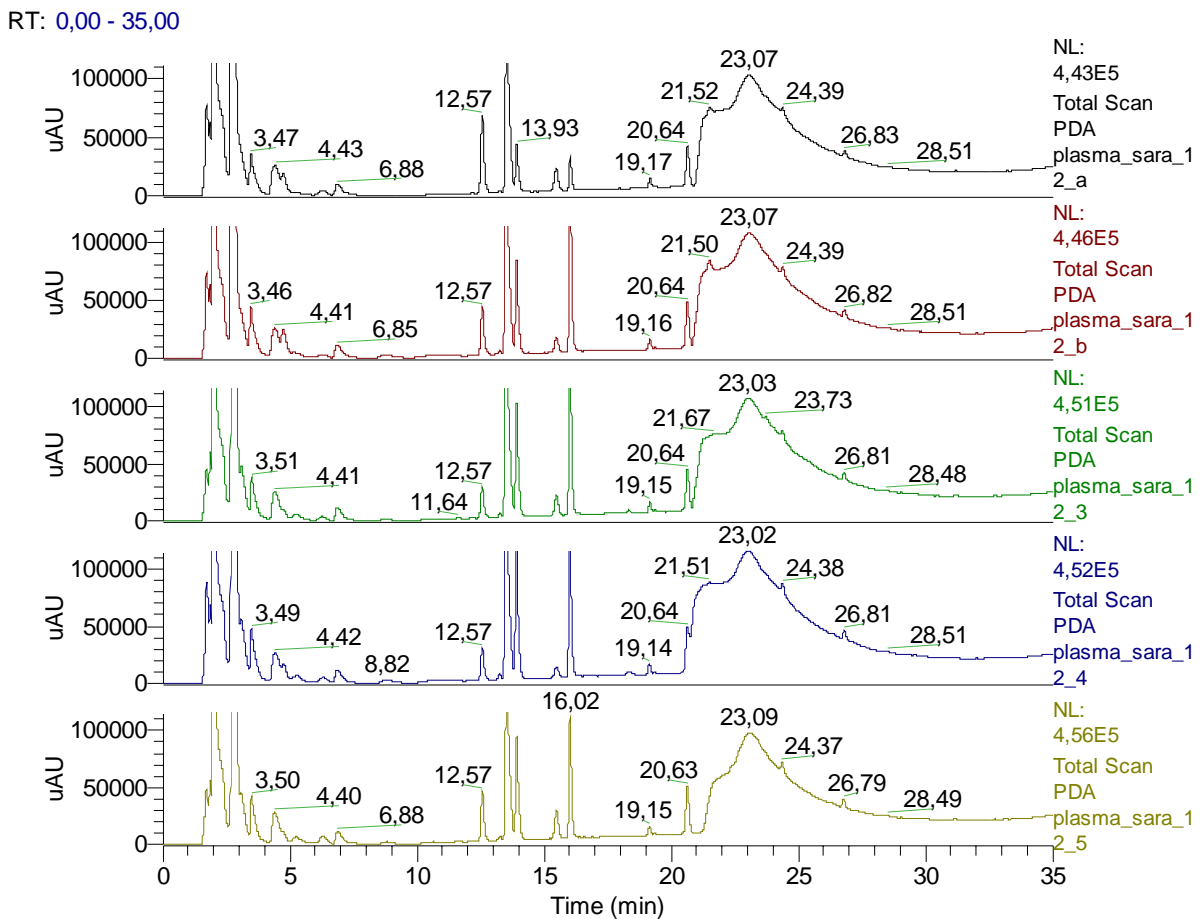


Figure 1. PDA chromatograms of serum samples of one subject, collected before the study (rows 1 and 2), after 12 days (row 3) and 16 days of strawberry consumption (row 4), and one month after the test (row 5). Proteins and peptides noise is evident after 21 minutes of the analysis.

RT: 12,00 - 35,00

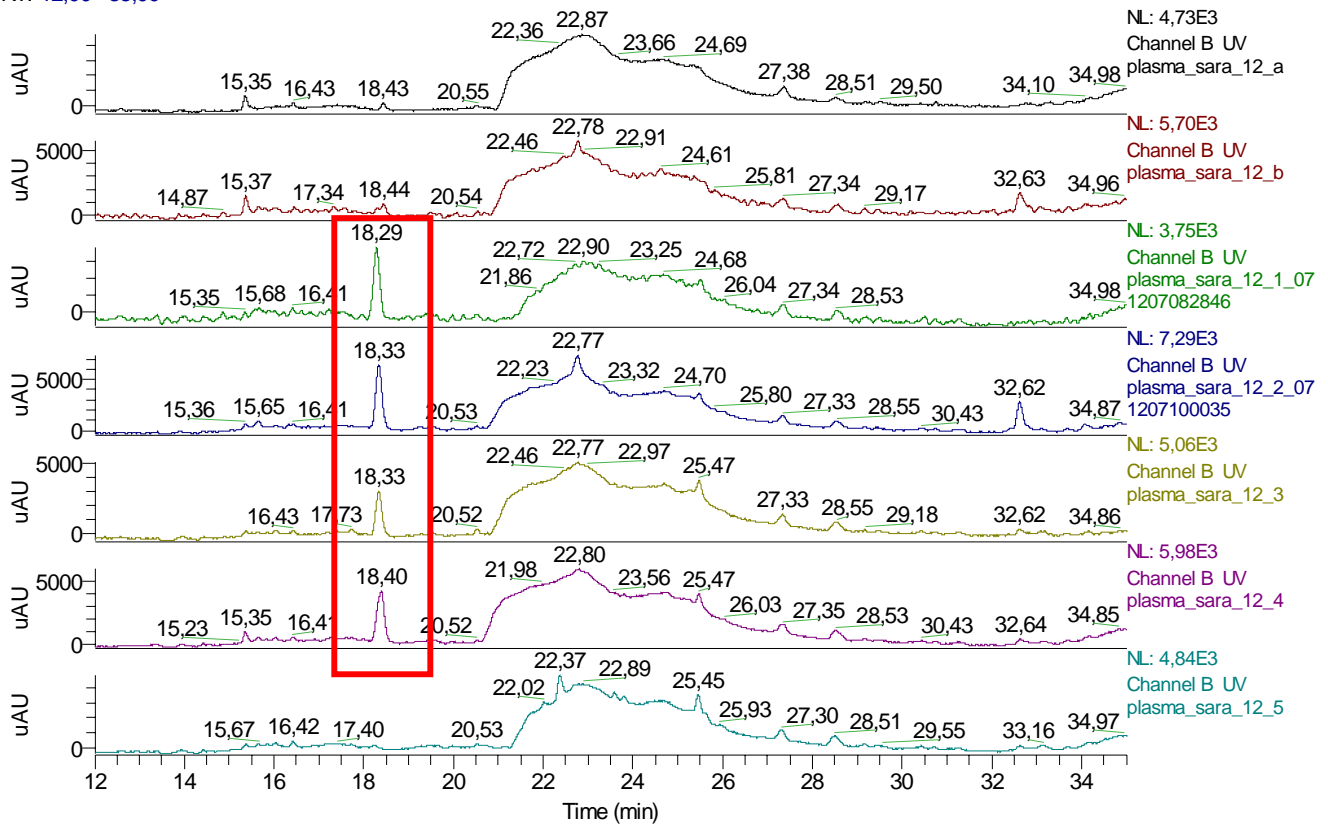


Figure 2. PDA analysis of serum of one of the subjects, recorded at 365 nm. The chromatograms refer to samples collected before the study (rows 1 and 2), after 4 (row 3), 8 (row 4), 12 (row 5) and 16 days of strawberry consumption (row 6), and one month after the test (row 7).

Liquid Chromatography-Mass Spectrometry (LC-MSⁿ) of strawberry extracts. The Folin-Ciocalteu assay showed that Sveva strawberries cultivated at experimental field of my home University have a slightly higher phenol content than strawberries cultivated in the other location (2.93 and 2.63 mg GAE/g FW, respectively); the same difference was observed when considering the total anthocyanin content (0.64 and 0.52 mg Cy-gluc/g FW, respectively). Tentative identities of phenolics were obtained by the aid of the HPLC-DAD profiles recorded at 280, 365 and 520 nm, and by observing their spectral characteristics, and by matching their molecular ions obtained by LC-ESI-MS and LC-MS/MS analyses (in positive and negative mode) with the theoretical molecular weights from data available in literature (Maatta-Riihinen KR, et al. 2004; Seeram NP, et al. 2006; Aaby K, et al. 2007; Lopes-da-Silva F, et al. 2002). Phenolic compounds grouped as hydrolyzable tannins, anthocyanins, flavonols, flavanols and hydroxycinnamic acid derivatives and their esters were detected and identified in both strawberry samples.

The HPLC-PDA profiles showed mainly quantitative differences between the two strawberry samples, confirming that strawberries harvested from our experimental field are richer in phenolic compounds. Nevertheless, fine qualitative differences were also observed (Fig. 3). Among anthocyanins, differences mainly regarded the presence of cyanidin 3-malonylglucoside and pelargonidin 3-malonylglucoside (see arrows in Fig. 3B). Furthermore, in one of the two strawberry samples, at RT = 46.4 a 365 nm-absorbing compound eluted very close to kaempferol 3-glucoside (RT = 45.7) and kaempferol 3-glucuronide (RT = 46.1), but further investigations will be required for the fine identification (see arrow in Fig.3A).

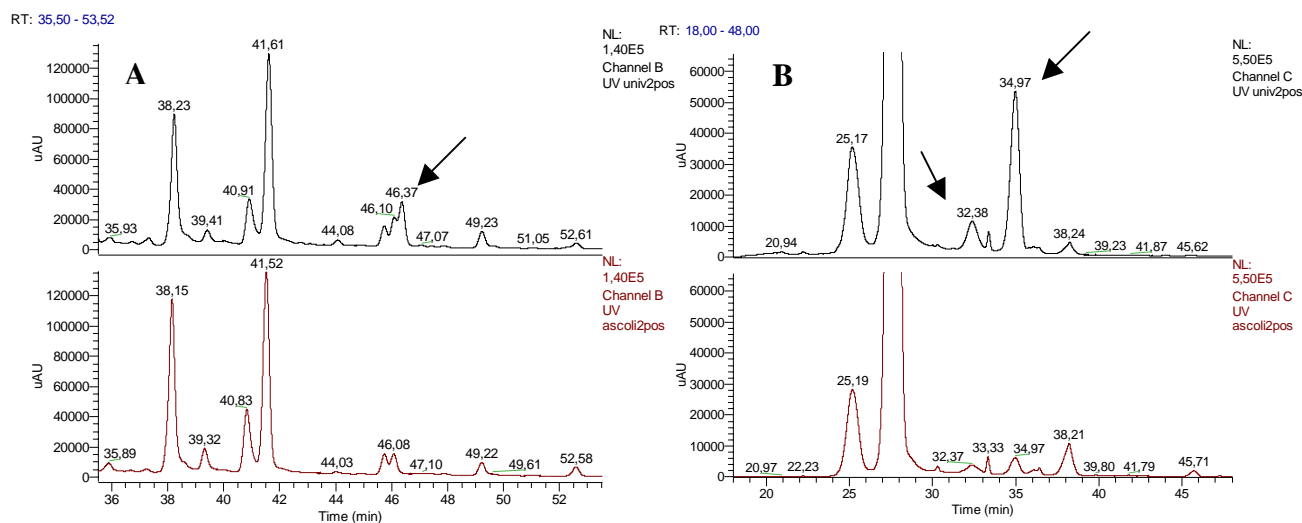


Figure 3. Details of PDA analysis related to Sveva cultivar cultivated at the experimental field of Marche Polytechnic University (upper rows) and in Ripatransone (lower rows), recorded at 365 nm (A) and 520 nm (B).

Concluding Remarks

During the STSM I had the possibility to attend an intensive training on the use of Finnigan LTQ LC-MS facilities, which was very useful in the mass spectrometry operations and data analysis.

The preliminary results obtained with plasma samples from human subjects gave us the opportunity to exchange interesting ideas, to plan a new feeding study focused on long-term consumption of strawberries, and to optimise sample preparation protocols. The analysis of strawberry samples gave interesting results, and a future collaboration for the phenolic profiles of other strawberry varieties and selections cultivated at my home University was planned.

I would like to take this opportunity to thank COST 863 for making this informative and fruitful visit possible. I also thank Dr. Gordon McDougall and colleagues for hosting me at the SCRI.

Essential References

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