Short Scientific Report on the STSM (COST Action FA1403)

STSM Topic: Analysis of the metabolic markers of dietary phytosteryl conjugates in plasma

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Background and purpose of the STSM:

Cardiovascular disease is one of the leading causes of death worldwide. It is well known that regular intake of phytosterols can reduce total cholesterol and low-density lipoprotein cholesterol, and further reduce the risk of cardiovascular disease (European Food Safety Authority, 2010). Phytosterols occur in the nature as free sterols, as well as conjugates of fatty acid esters, phenolic acid esters, glycosylated sterols and acylated steryl glycosides (Nyström, 2012). Some data showed that phytosteryl conjugates had high potential to reduce cholesterol level (Berger et al., 2005; Chavez-Santoscoy et al. 2014). However the exact mechanism is still not clearly understood. The overall goal of the project is to study the inhibition of the risk of cardiovascular diseases with phytosteryl conjugates. Metabolomics techniques can explore not only the metabolites of phytosteryl conjugates after intake, as well as other features that are related to their cholesterol lowering effect. In this STSM, the main task was to learn untargeted LC-MS based metabolomics techniques to explore the metabolites after food intake, including the sample preparation, LC-MS analysis and data analysis, which could be applied in the future phytosteryl conjugates project.

Work carried out:

1) Sample preparation

Biological samples were used for the training of sample preparation, including plasma, urine and fecal samples (provided by the group of Prof. Dr. Lars Ove Dragsted).

For the plasma sample, after adding the internal standard, plasma was filtered through a protein precipitation plate (Sirocco™ plates, Waters) after precipitation with 50%MeOH: 50%ACN. The filter was subsequently washed with the same solvent twice. The sample was evaporated to dryness by vacuum centrifugation followed by redissolving in 95%H_2O:5% (70%ACN:30% MeOH).

For the urine sample, the urine was centrifuged at 3000 × g for 2 min at 4°C. Then the internal standard was added in the urine.

For the fecal sample, fecal sample was firstly diluted with H_2O (e.g., 1:14 with H_2O for rat fecal sample). After adding the internal standard, the sample was extracted by MeOH. After centrifugation, the supernatant was collected. The residue was further extracted with 80% MeOH. The supernatants were combined and evaporated to dryness by vacuum centrifugation followed by redissolving in 95%H_2O:5%(70%ACN:30% MeOH).

2) LC-MS analysis

UPLC-Q-TOF (Waters) was used for sample analyses. The UPLC column was HSS T3 C18. The mobile phase was 0.1% formic acid in H_2O (A) and 0.1% formic acid in 70% ACN:30%MeOH (B). The chromatographic method and the Q-TOF parameters were according to the method described recently (Rago et al., 2015).

3) LC-MS data processing

The raw MS data (rat urine data of apple and apple-pectin intake) was converted to NetCDF format and preprocessed by MZmine. The feature set list (compounds characterized by retention time, m/z, and peak area) was imported to Matlab for chemometric analysis. Matlab firstly was used to do the data arrangement and error reduction of the dataset. Then PLS-Toolbox was applied to do the data exploration by PCA and classification analysis by PLS-DA. A permutation test (1000 interations) was applied to assess the classification performance of the PLS-DA model. A list of potential markers was selected from these models.

4) Metabolite annotation

Chemical identification of metabolite could be through MS/MS fragmentation, NMR or through data match with related database. In this STSM, the identification was through online database, namely, Human Metabolome Database and METLIN Metabolite Database.

Outcomes from STSM:

1) Sample preparation

After the training, the repeatability of sample preparation was acceptable. An example of the chromatograms from a rat fecal sample was shown in Figure 1.

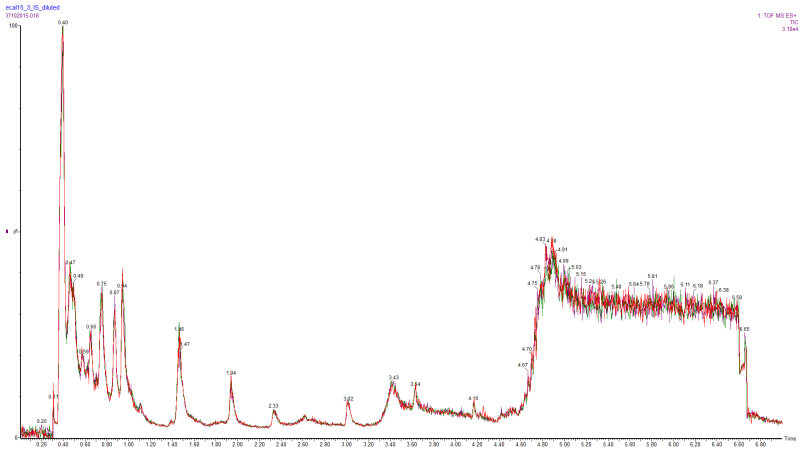


Figure 1 chromatograms overlapping of sample 15 (triplicates): internal standards at adding level 8.3 µg/ml PABA and 5.5 µg/ml hippuric acid (1-13C) (final concentration in the 96 well plate) in positive mode.

2) Data analysis

The MZmine resulted in 3499 features. They were subjected to PCA (Figure 2). Data were mean centered, and the model used was venetian blinds.

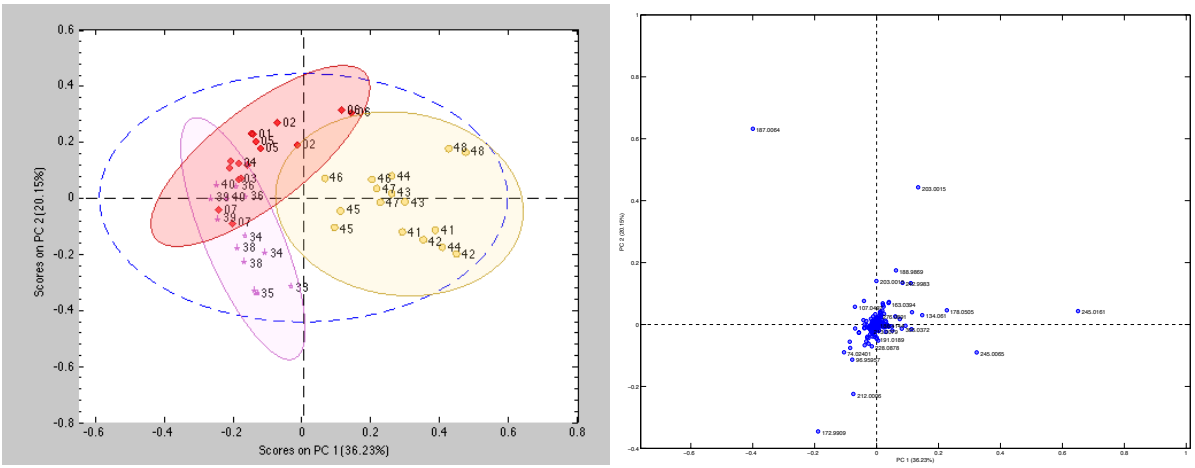


Figure 2 PCA score (left) and loading plot (right) of PC1/PC2 with all the features measured in negative mode (n=3499). In the score plot, the labels were 'subjects'; red: control group; yellow: whole apple group; pink: apple pectin isolate group. In the loading plot, the labels were 'm/z'.

In order to reveal differences between urine metabolites in control rats vs. whole apple rats, control rats vs. pectin rats, whole apple rats vs. pectin rats, PLS-DA was performed in autoscaled datasets using PLS_Toolbox in matlab. An example of variable selection by VIP scores from control rats vs. whole apple rats was shown in Figure 3. The model and prediction information was shown in Table 1.

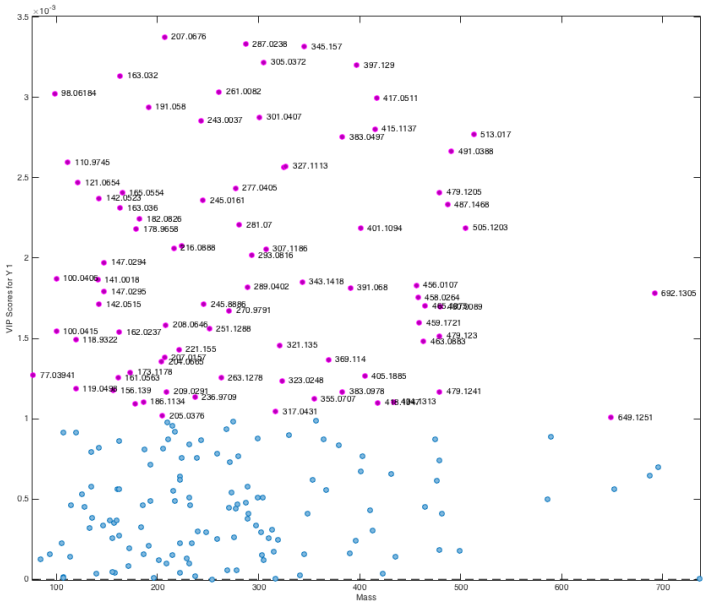


Figure 3 Variables/Loadings plot for variable selection by VIP scores from control rats vs. whole apple rats.

Table 1 PLS-DA model and prediction information from control rats vs. whole apple rats

Sensitivity (Cal): 1.000	Sensitivity (CV): 1.000	Sensitivity (Pred): 1.000
Specificity (Cal): 1.000	Specificity (CV): 1.000	Specificity (Pred): 0.750
Class. Err (Cal): 0	Class. Err (CV): 0	Class. Err (Pred): 0.125
RMSEC: 0.174741	RMSECV: 0.203978	RMSEP:0.216934
Bias: 1.11022e-16	CV Bias: 0.0010883	Pred Bias:0.0301221
R^2 Cal: 0.877863	R^2 CV: 0.836705	R^2 Pred: 0.820403

2) Metabolite annotation

A list of potential markers was selected from these models. The identification was through online database (Human Metabolome Database and METLIN Metabolite). Relative intensity patterns of some potential markers and identification were shown in Figure 4 and Table 2.

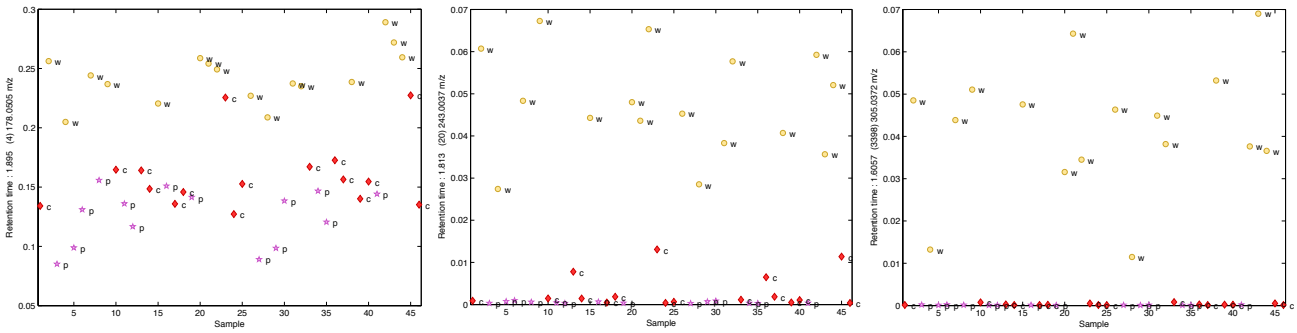
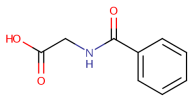
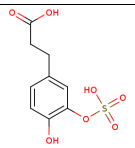
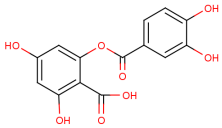


Figure 4 Relative intensity patterns of some potential markers

Table 2 Tentative identification of selected markers through online database

Measured mass	Suggested ion	RT	Theoretical mass	Molecular formular	Annotation	Structure	High in
178.0505	[M-H]-	1.895	179.0582	C8H9NO	Hippuric acid		whole apple
243.0037	[M-H_2O-H]-	1.813	262.0147	C9H10O7S	Dihydrocaffeic acid 3-sulfate		whole apple
305.0372	[M-H]-	1.6057	306.0376	C14H10O8	2-Protocatechoylphlorogl ucinolcarboxylate		whole apple

Outlook:

The training of untargeted LC-MS based metabolomics techniques was finished in this STSM. The phytosterol related markers were not found in these biological samples from rats with apple treatment. Since the apples does not contain high amount of phytosterol conjugates, the outcome of the analysis were acceptable. The metabolomics techniques will be applied in the future phytosteryl conjugates project where the mice will be fed with high dose of phytosterol conjugates, which may reveal some features related to phytosteryl conjugates intake and their cholesterol lowering effects.

References:

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