URINE AND FECES METABOLOMICS-BASED ANALYSIS OF CAROB TREATED RATS

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Graphical Abstract

Carob treated

Control

Sample Collection

Sample Analysis

Biochemical Correlation

Data Processing
Abstract

*Ceratonia siliqua* L. *Fabaceae*, commonly known as the carob tree, is native to the eastern Mediterranean countries and its products are widely used in the diet of people living in Mediterranean Europe, Middle East and North Africa. Carobs are considered to be of high nutritional value, as they are virtually fat-free, rich in proteins, antioxidants, vitamins and contain several important minerals. Different types of carob products are available in the local market, such as carob syrup, powder, flour, snack, cream, etc. However, the potential positive health effects of carob-containing products are largely unknown and have not been extensively studied. The aim of this study was to determine significant urine and fecal metabolome alterations in 8 rats treated with carob powder for 15 days as compared to 8 non-treated ones (controls) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to underline specific metabolites that changed according to the treatment.

Urine and fecal samples were collected in five time points during a 15 day period of treatment with carob powder throughout water consumption (10 g powder / L). A targeted HILIC-UPLC-MS/MS method was applied for the determination of 101 polar metabolites (sugars, amino acids, organic acids, amines, etc) in a single run of 40 min in both rat urine and feces. Chromatographic separation was performed on an Aquity BEH amide column (2.1 x 100 mm, i.d. 1.7 μm); the mobile phase was consisted of A: Acetonitrile:H₂O 95:5 v/v (+ 10 mM ammonium formate) and B: H₂O:Acetonitrile 70:30 v/v (+10 mM ammonium formate). The solvents flow rate was set at 0.5 mL/min. Mass spectrometry parameters were optimized for each of the 101 pre-selected analytes.

Approximately 55 urinary and fecal metabolites were identified in both specimens. Data were further processed with multivariate (SIMCA 13) and univariate statistics (ANOVA). The differentiation of treated rats and controls was highlighted using discriminant multivariate models.

**Acknowledgements:** The authors would like to thank the “Black Gold” project financially supported by the University of Cyprus

**Keywords:** targeted metabolomics, carob, rat, urine, feces, LC-MS/MS
Carob tree, *Ceratonia siliqua* L., (native to the eastern Mediterranean countries) is widely used in the diet of people living in Mediterranean Europe, Middle East and North Africa.

**Effects**
- Anticancer
- Antiviral
- Antidiabetes
- Antioxidant
- Digestive
- Antidiarrheal
- Control hyperlipidemia
- Gastroesophageal reflux (in infants)
- Weight loss

- High nutritional value & fat-free (rich in proteins, antioxidants, vitamins & several important minerals).
- Different types of carob products available in the local market (carob syrup, powder, flour, snack, cream, etc.).

However, the potential positive health effects of carob-containing products are largely unknown and have not been extensively studied.

Systematic study of the unique chemical fingerprints that specific cellular processes leave behind

The study of their small-molecule metabolite profiles


Mass spectrometry (MS) dominates in holistic metabolite profiling due to its sensitivity and wingspread availability.

Liquid chromatography-Mass spectrometry (LC–MS) is currently the most widely used mass spectrometric technology, due to its ability to separate and detect a wide range of molecules.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>System</th>
<th>Compounds of interest</th>
<th>Column</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carob Fruits</td>
<td>HPLC-UV-MS/MS</td>
<td>Polyphenols</td>
<td>Aqua C18 (150 mm x 2 mm, 3μm)</td>
<td>Papagiannopoulos et al.</td>
</tr>
<tr>
<td>Carob</td>
<td>LC-MS/MS</td>
<td>Flavonoids</td>
<td>Discovery C-18 column (15 x 4.6 mm, 5 μm)</td>
<td>Vaya et al.</td>
</tr>
<tr>
<td>Carob pod</td>
<td>HPLC-PDA, HPLC-MS</td>
<td>Sugars, amino and organic acids, minerals and phenolic compounds</td>
<td>1. Ion-300 column (300 mm x 7.8 mm, 10 μm)</td>
<td>Ayaz et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Luna Phenyl-Hexyl (250 x 2 mm, 5 μm)</td>
<td></td>
</tr>
<tr>
<td>Carob flour</td>
<td>LC-MS/MS</td>
<td>Phenolic Compounds and Alkaloids</td>
<td>HSS T3 (100 mm x 2.1 mm, 1.8 μm)</td>
<td>Ortega et al.</td>
</tr>
<tr>
<td>Wild carob seed oil</td>
<td>GC-MS, HPLC</td>
<td>Different lipids</td>
<td>CP-Sil 88 (100 m x 0.25mm, 0.2 μm)</td>
<td>Matthaus et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diol phase HPLC column (25 cm x 4.6 mm)</td>
<td></td>
</tr>
<tr>
<td>Carob extracts and mice urine, plasma and cecal</td>
<td>LC-MS/MS, LC-QTOF</td>
<td>Lipids, amino acids, organic acids and phenolic related compounds</td>
<td>C18 Luna 3 n pfp (2) (150 mm x 2 mm)</td>
<td>Jove et al.</td>
</tr>
<tr>
<td>Carob leaves</td>
<td>HPLC-MS/MS</td>
<td>Polyphenols</td>
<td>Zorbax Column Synergi 4 μ MAX-RP 80A (150 mm x 4.6 mm)</td>
<td>Aissani et al.</td>
</tr>
<tr>
<td>Carob Bean</td>
<td>HPLC-RID</td>
<td>D-pinitol and sugars</td>
<td>CARBOsep Coregel 87P (7.8 x 300 mm)</td>
<td>Turhan</td>
</tr>
<tr>
<td>Carob Powder</td>
<td>GC-MS</td>
<td>Volatile compounds</td>
<td>ZB-5ms capillary column (50 m x 0.32mm, 0.25 μm)</td>
<td>Racolta et al.</td>
</tr>
<tr>
<td>Carob leaf extracts</td>
<td>HPLC-MS, GC-MS</td>
<td>Phenolic acids</td>
<td>Kinetex C-18 column, (100 x 3 mm)</td>
<td>Meziani et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ZB-5MS column (30 m x 0.25 mm, 0.25 μm)</td>
<td></td>
</tr>
<tr>
<td>Carob pulp</td>
<td>HPLC-DAD-MS</td>
<td>Proteins, phenolic compounds</td>
<td>C18 Alltima (150 mm x 2.1 mm)</td>
<td>Benchikh et al.</td>
</tr>
<tr>
<td>Carob pod &amp; Carob syrup</td>
<td>TLC, HPLC-RID</td>
<td>Carbohydrates and Sugars</td>
<td>analytical column (300 mm x 8.0 mm)</td>
<td>Fidan et al.</td>
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<tr>
<td>Carob bean</td>
<td>SPME-GC-MS</td>
<td>Volatile compounds</td>
<td>DB5-MS column (30 m x 0.25 mm, 0.25 μm)</td>
<td>Farag et al.</td>
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<tr>
<td>Carob pod</td>
<td>GC-QTOF, LC-QTOF</td>
<td>Different lipids</td>
<td>1. BPX90 SGE column (30 m x 0.25 mm, 0.25 μm)</td>
<td>Nguyen et al.</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>2. Phenomenexkinetex C18 (100 mm x 3.0 mm, 2.6 μm)</td>
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</tr>
</tbody>
</table>
To determine significant urine and fecal metabolome alterations in rats treated with carob powder using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

To underline specific metabolites that are responsible for the differentiations according to the treatment.
In vivo carob study

- 16 male *Wistar* rats
- 2.5-3.5 months of age
- 2 groups, 8 fed rats vs. 8 control ones
- 15 days carob feeding
- 1 week acclimatization period
- Rats were housed in individual cages in standard conditions
- 5 sample collection time points (D0, D1, D5, D10, D15)
- Urine and feces samples were collected
- All samples were analyzed using LC-MS/MS
- Rats body weight and food consumption were measured during the *in vivo* experiment
A notably useful specimen to assess the effect of the study factor

A particularly complex specimen requires optimized sample preparation protocol


Gut microbiota is considered to be responsible for the carobs metabolism partially in rat large intestine

Towle and Schranz, Unpublished report from Hercules Research Center 1975.
10 g carob powder diluted in warm water (10 ppm)

Flasks of rats were filled with 750 ml water

Let them be cooled and place them back to cages

Preparation of fresh solutions and refill every 2 days
**Urine samples**

- Extraction with 1-propanol: water solution, in a ratio of 1:4 fecal sample weight to extraction solvent
- Vortex-mixing
- Sonication for 10 min
- Ultra-centrifugation (20,000 rpm, 4°C, 30 min)
- Filtration through syringe filters PTFE 0.22 μm

**Fecal samples**

- Extraction with 1-propanol: water solution, in a ratio of 1:4 fecal sample weight to extraction solvent
- Vortex-mixing
- Sonication for 10 min
- Ultra-centrifugation (20,000 rpm, 4°C, 30 min)
- Filtration through syringe filters PTFE 0.22 μm

**UHPLC-MS/MS Conditions**

- **Column**: Acquity BEH Amide (150×2.1mm i.d., 1.7 μm).
- **Mobile Phase**: A: ACN: H₂O 95:5 v/v, 10mM HCOONH₄, B: ACN: H₂O 30:70 v/v, 10mM HCOONH₄
- **Flow rate**: 0.50 mL/min.
- **Instrument**: AcquityH UPLC class, Xevo TQD.

The mass spectrometry parameters were optimized for each of the 100 pre-selected analytes (amino-acids, organic acids, sugars, nucleosides, amines and other molecules).

**Linear Gradient**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4.00</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>25.00</td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td>30.00</td>
<td>15.0</td>
<td>85.0</td>
</tr>
<tr>
<td>30.01</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>40.00</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**HILIC/MS-MS analysis**

QCs samples & standard mixes to evaluate stability & repeatability
Data handling

Software

- MassLynx (Waters, UK)
- TargetLynx (Waters, UK)
- SIMCA 13.0 (Umetrics, Sweden)
- MS Excel (Microsoft, USA)
- MetaboAnalyst 3.0 (Xia et al., 2015)

Statistical analysis

- Multivariate statistics (PCA, PLS-DA, OPLS-DA), VIP
- Univariate statistics (t-test, fold change)
- Normalization: Log transformation
- Scaling: Univariate (UV) & Auto
- RSD% of QCs to evaluate stability of the system
Urinary and fecal metabolites identified in both specimens

Results
Preliminary results
Fecal samples

PLS-DA scores plot of fecal samples (Day 1)

Carobs fed

Controls

$R^{2}_X[1] = 0.34$
$R^{2}_X[2] = 0.262$
Preliminary results
Fecal samples

OPLS-DA scores plot of fecal samples (Day 15)
Preliminary results
Fecal samples

Box plots of differentiated compounds in day 15 derived by t-test and VIP values

Examples of Hotelling’s line of Tryptophan (1) & Tyrosine (2)
Preliminary results
Fecal samples

- Differentiation between the 2 groups in the day 1 was observed.

- Multivariate statistical analysis managed to separate fecal samples in the day 15.

- Both Multivariate and Univariate statistical analysis demonstrate specific compounds altered in rats fed with carob powder for 15 days (tryptamine, tryptophan, tyrosine, phenylalanine).
Preliminary results
Urine samples

OPLS-DA scores plot of urine samples (Day 1)
Preliminary results
Urine samples

- Mild differentiation between the 2 groups in the day 1.

- Multivariate statistical analysis did not manage to separate urine samples, statistically significantly, in the day 15.

- Univariate statistical analysis demonstrates specific compounds altered in rats fed with carob powder for 15 days (glucose, inositol, thiamine, alanine).
Preliminary results
Urine samples

- Lower number of urine samples (unable to collect from some rats at the specific time point).
- Matrix effect may affect the obtained results.
- Normalization could be applied in raw data from urine samples in order to overcome matrix effect.
Statistically significant differentiations, according to food consumption, were observed between weeks for both fed and control groups.

The metabolomics based analysis manage to separate the analyzed samples according to the treatment.

Carob treated rats showed different metabolic profiles comparing to the controls allowing their discrimination by LC-MS/MS-urine and fecal profiling analysis.

Carobs consumption may affect the fecal metabolome in greater scale than urine metabolome.
Based on our preliminary results, tryptamine was found to be affected in both days 1 and 15 of sample collection.

Affected metabolic pathways derived from fecal sample analysis: aminoacyl-tRNA biosynthesis, phenylalanine tyrosine and tryptophan biosynthesis.

Jove et al., 2011 observed that cecal metabolome was affected more than urine and plasma metabolome in mice fed with carobs.

Based on our preliminary results, and the only relevant metabolomics-based published study (Jove et al., 2011), as well as older studies (Harmuth-Hoene and Schelenz, 1980), it could be considered that carobs greatly affect gut microbiota.
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Images
http://singledesk.in/aims-and-objectives/
We would like to thank the “Black Gold” Research Project financially supported by the University of Cyprus.