METABOLOMIC APPROACH FOR GREEK HONEY ORIGIN DISCRIMINATION MAKING USE OF ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

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ABSTRACT

Honey is a foodstuff which is subjected to various deceitful practices, such as addition of syrups or mislabelling due to its high price in the market. Many honeys in the market, sold as unifloral, are often adulterated with others inferior in quality products, on account of great demand and high production cost. It is widely known that honey obtained from specific plants is strictly associated with unique organoleptic and/or health beneficial properties [1]. Hence, the evaluation and verification of honey authenticity is a task of paramount importance for the producers, consumers and regulatory bodies. Untargeted metabolomics using UPLC-ESI-QTOF MS is a powerful approach for the simultaneously analysis of many compounds as well as identify new biomarkers which can discriminate the samples according to their origin. A generic extraction protocol was utilized in order to obtain the whole metabolic profile of the samples. The developed method was applied to 135 Greek honey samples from 5 different botanical origins. Most of the samples are unifloral while some other are polyfloral. The non-target screening approach was performed using Bruker Metaboscape 3.0 software which incorporates sophisticated tools for profiling, statistical analysis and compound identification. New compounds which differentiate the samples according to botanical and geographical origin were finally identified. The same samples were deeply investigated by a uniquely developed methodology and screening workflow called "AutoSuspect" making use a novel MS-ready database containing hundreds of thousands of naturally occurring compounds [2], and the results were compared.

Experimental

1 g honey + 5 ml acidified water (pH<2) 2% NaCl solution → Extraction with 5 ml EtAc x 3 times → Centrifugation 5 min between each extraction → Dry near dryness in N2 stream → Reconstitution to 0.2 ml MeOH:H20 (1:1)

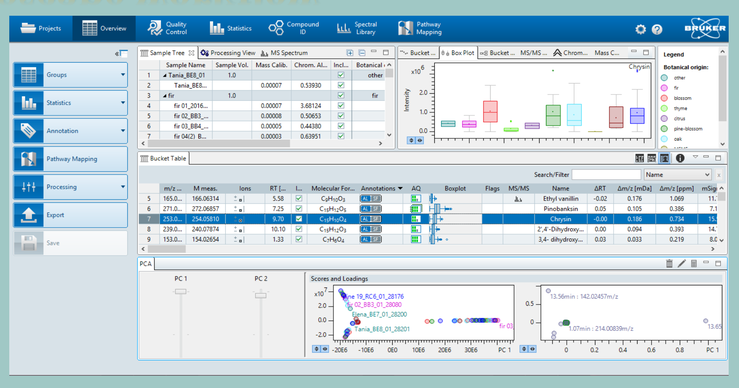
- UHPLC-ESI-MS/MS: (QTOF-MS)(Bruker MaXis Impact)
- Column: Acclaim C18 (Dionex-Thermo Scientific)
- RP chromatographic system
- Elution gradient program
- ESI mode: negative
- Full scan MS and bbCID mode in a single run

Data Treatment

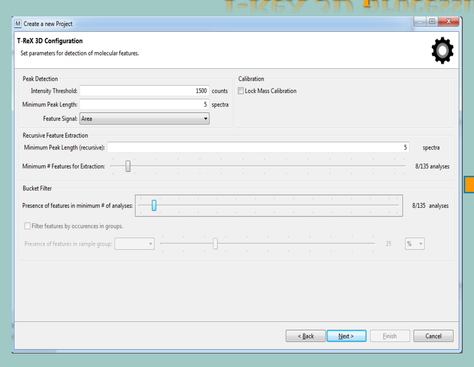
Metaboscape workflow

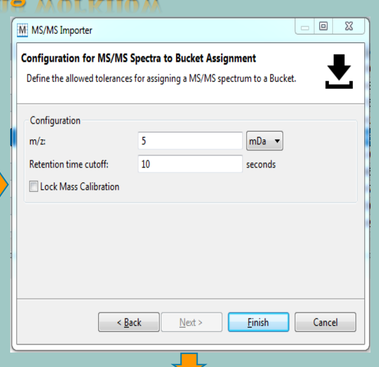
T-ReX 3D algorithm (Time aligned Region Complete eXtraction algorithm).

- Automated calibration
- de-isotoping algorithm
- retention-time alignment algorithm
- de- adducting
- MS/MS spectrum assigned to each individual Bucket automatically

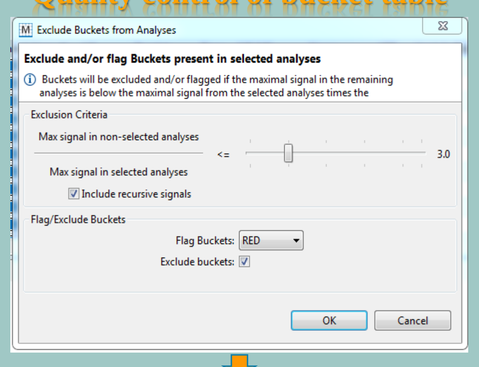


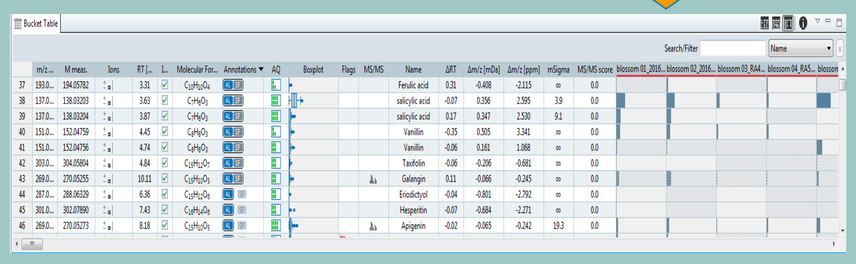
T-ReX 3D processing workflow

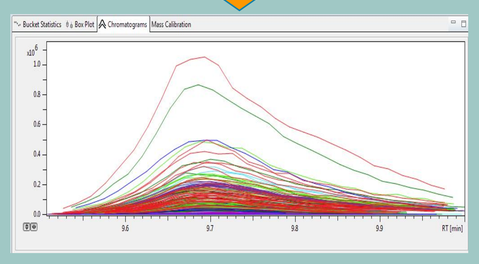




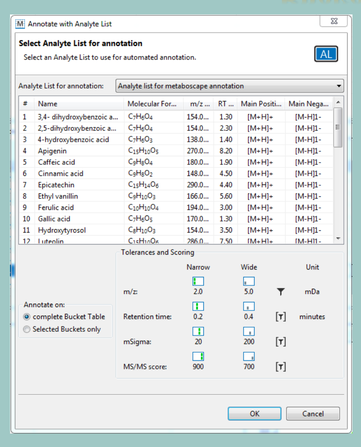
Quality control of bucket table

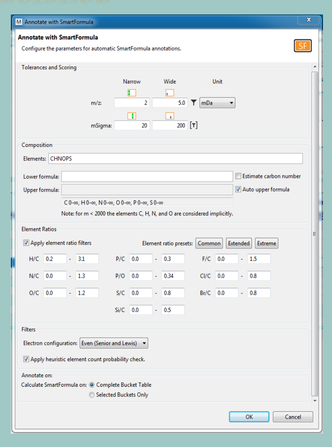




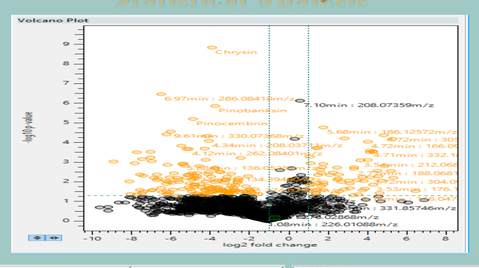


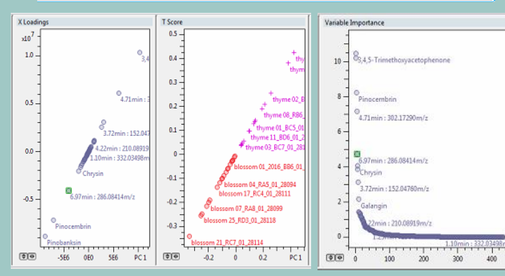
Bucket Annotation





Statistical Analysis

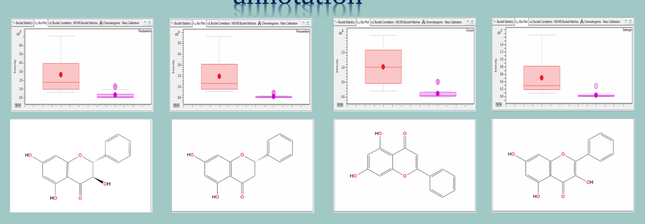




9 buckets seem to be the most influential for the discrimination of Blossom and Thyme honey

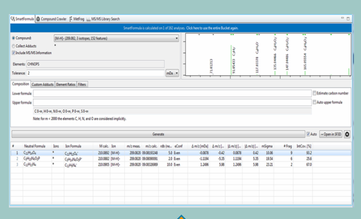
4 buckets are already known from analyte list annotation

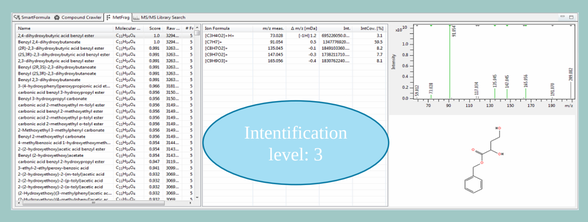
5 important buckets are unknown compounds



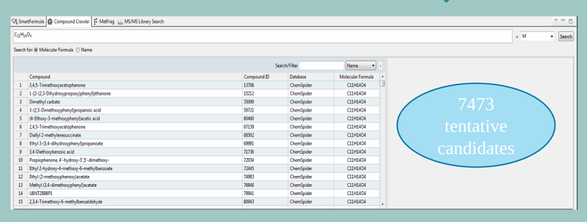
Pinobanksin | Pinocembrin | Chrysin | Galangin

m/z	Rt
209.0819	3.46
301.1656	4.71
285.0768	6.97
287.1499	4.72
151.0403	3.72





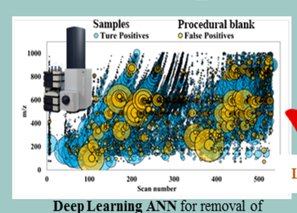
Intentification level: 3



7473 tentative candidates

AutoSuspect workflow

- **AutoSuspect**



Deep Learning ANN for removal of procedural blank peaks

Pretreatment step

- **Data Independent Acquisition mode** (DIA) (Raw data), **MS calibration** + conversion to **mzML**
- **XCMS + IPO package + MS deconvolution by CAMERA and Non-target R package**
- **Subtraction of analytical procedural blank from samples**

Level 5 — **m/z of interest $[M+H]^+$ & $[M-H]^-$**

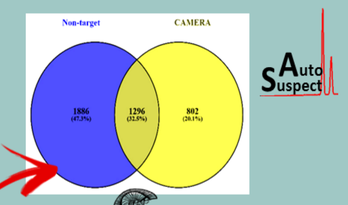
Level 4 — **Mass accuracy threshold (Molecular Formula)** (< 2 mDa); **Isotopic pattern of the precursor ion** (> 50); **Characteristic adduct ions** ($[M + NH_4]^+$, $[M + Na]^+$, etc.)

enviPat for isotopic pattern → **Spectral contrast angle**

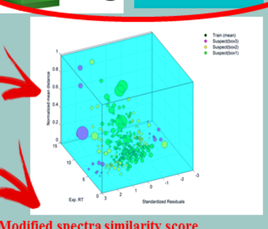
Level 3 — **MS/MS spectral interpretation & prediction** (Recorded by Data Dependent Acquisition mode (DDA)); *In silico* fragmentation platforms (MetFrag); ESI-HRMS spectra prediction via CFM-ID

Level 2 — **2b) LC retention time indices (QSRR models and RTI)** (Matching prediction vs experimental t_R via OTrAMS); **2a) MS/MS spectral match** Literature or library spectrum (MassBank)

Level 1 — Confirmation by **authentic reference standard**



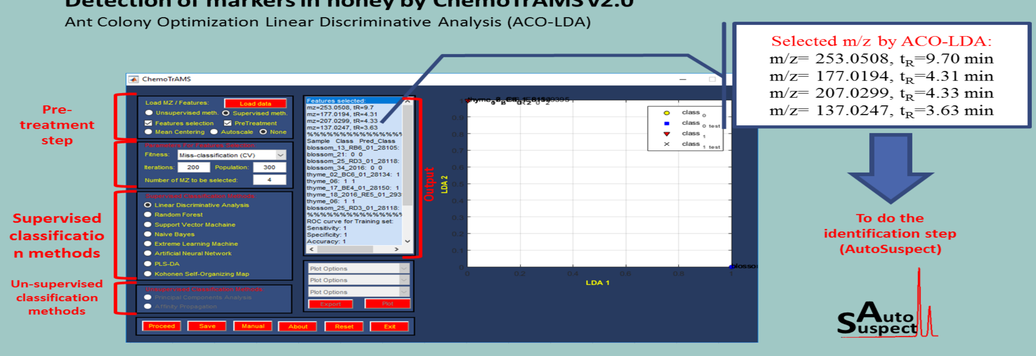
NATURAL PRODUCTS
Over 260,000 compounds
MS-ready Suspect list



Modified spectra similarity score

Detection of markers in honey by ChemoTrAMS v2.0

Ant Colony Optimization Linear Discriminative Analysis (ACO-LDA)



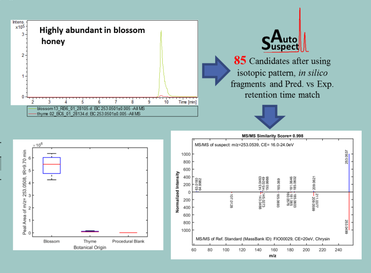
Selected m/z by ACO-LDA:
m/z= 253.0508, t_R=9.70 min
m/z= 177.0194, t_R=4.31 min
m/z= 207.0299, t_R=4.33 min
m/z= 137.0247, t_R=3.63 min

To do the identification step (AutoSuspect)

Auto Suspect

Identification 1

Identification of *m/z=253.0537, t_R=9.70 min*

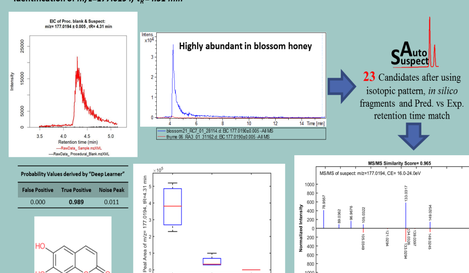


Chrysin

Level of Identification: 1

Identification 2

Identification of *m/z=177.0194, t_R=4.31 min*

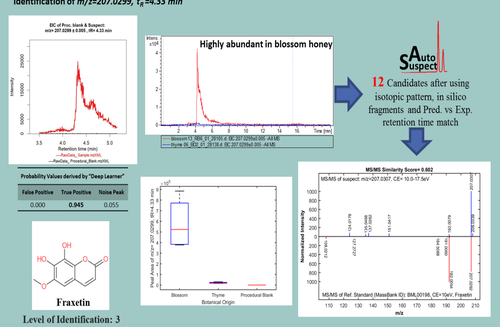


Aesculetin

Level of Identification: 2a

Identification 3

Identification of *m/z=207.0299, t_R=4.33 min*

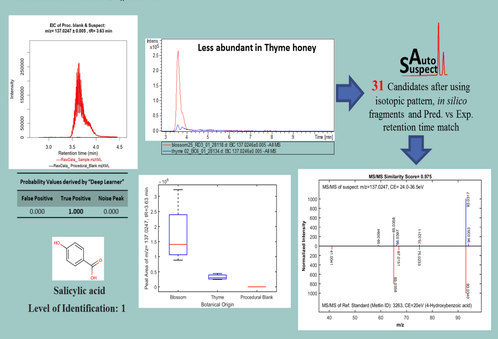


Fraxetin

Level of Identification: 3

Identification 4

Identification of *137.0247, t_R=3.63 min*



Salicylic acid

Level of Identification: 1

Conclusions

- ✓ Authenticity markers were identified using metaboscape 3.0 for the discrimination of blossom and thyme honey samples.
- ✓ AutoSuspect workflow was successfully implemented for the discrimination of blossom and thyme honeys. New biomarkers were identified.
- ✓ Chrysin is common in both workflows.
- ✓ Higher level of identification was reached in metaboscape buckets using AutoSuspect.

Acknowledgments

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Literature

1. M. Ciulu, N. Spano, M. Pilo, G. Sanna,, Molecules (2016) 21, 451.
2. R. Aalizadeh, E. L. Schymanski and N. S. Thomaidis, AutoSuspect: an R package to Perform Automatic Suspect Screening based on Regulatory Databases, 15th International Conference on Environmental Science and Technology, CEST2017.