Department of Agrifood Chemistry and Biochemistry
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METABOLOMIC STUDIES PN SEABUKTHORN LEAVES AND FRUITS –

TOWARDS RAPID FINGERPRINT OF BIOLOGICAL AND GEOGRAPHICAL ORIGIN

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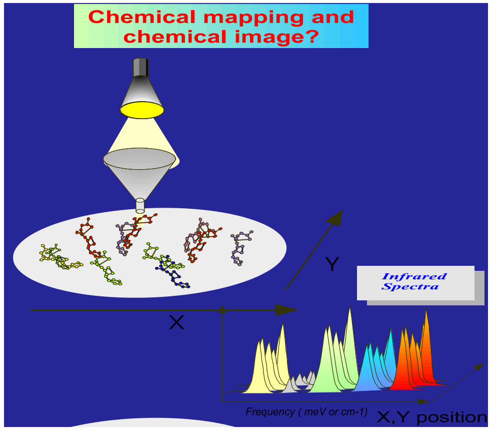

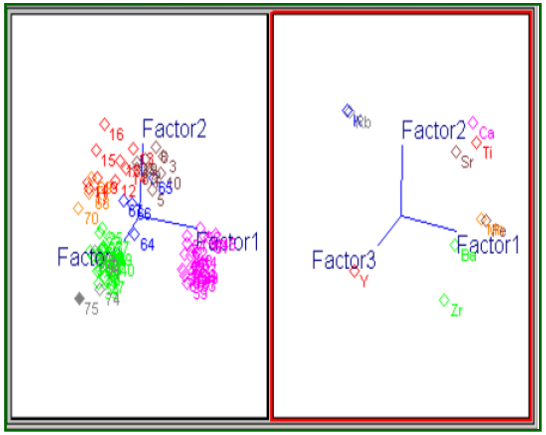

Outline

I. Metabonomics in postgenomic era: fingerprint of phytochemicals *vs* quantitation of metabolites - NETWORK

II. Phytochemicals as indicators of seabuckthorn (SB) composition (leaves vs. fruits)

III. Analytical steps and advanced techniques -BIOMARKERS

IV. Case studies: determination of biological and geographical origin by metabolomic analysis + chemometrics

V. Impact of metabolomics studies

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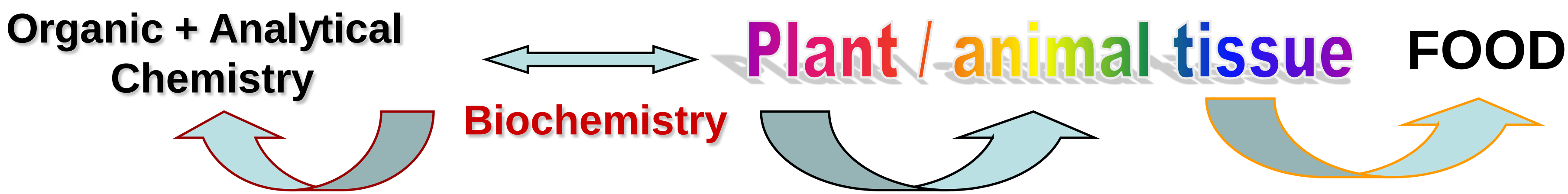
Metabolomics = systematic study of chemical **fingerprint to realize a metabolite profiling (small molecules)** in a specific matrix (plant, food)

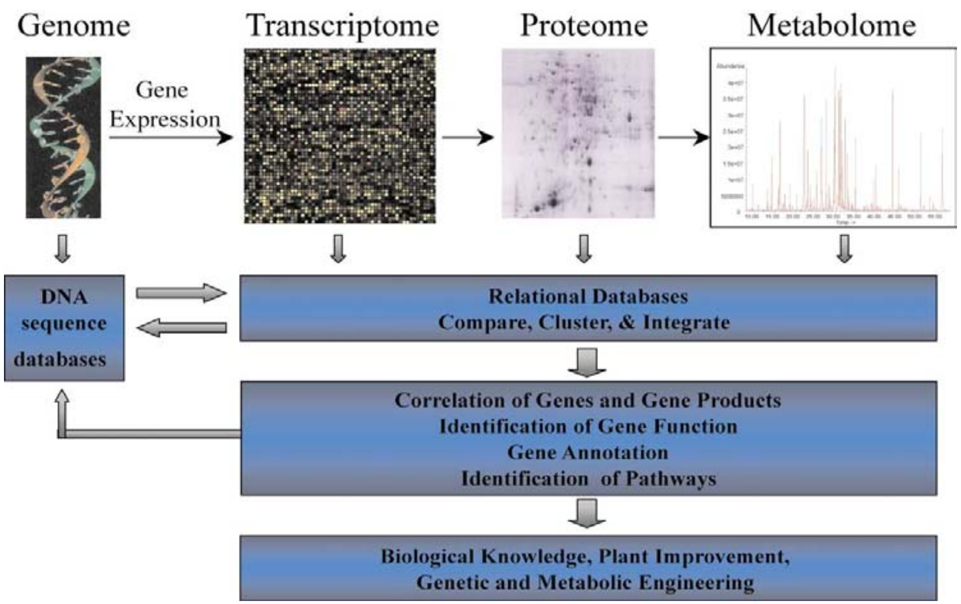
***M*etabonomics = quantitative measurements to identify a specific metabolic response** (by key-molecules, e.g. phytochemicals)

METABOLOME = complement of all metabolites expressed in a cell, tissue or organism



Metabolomics: An INTEGRATED Tool for Studying SYSTEMS BIOLOGY

GENOTYPE + ENVIRONMENT → **PHENOTYPE**



Transcriptomics

Metabolome

METABOLITES

Primary

Proteomics

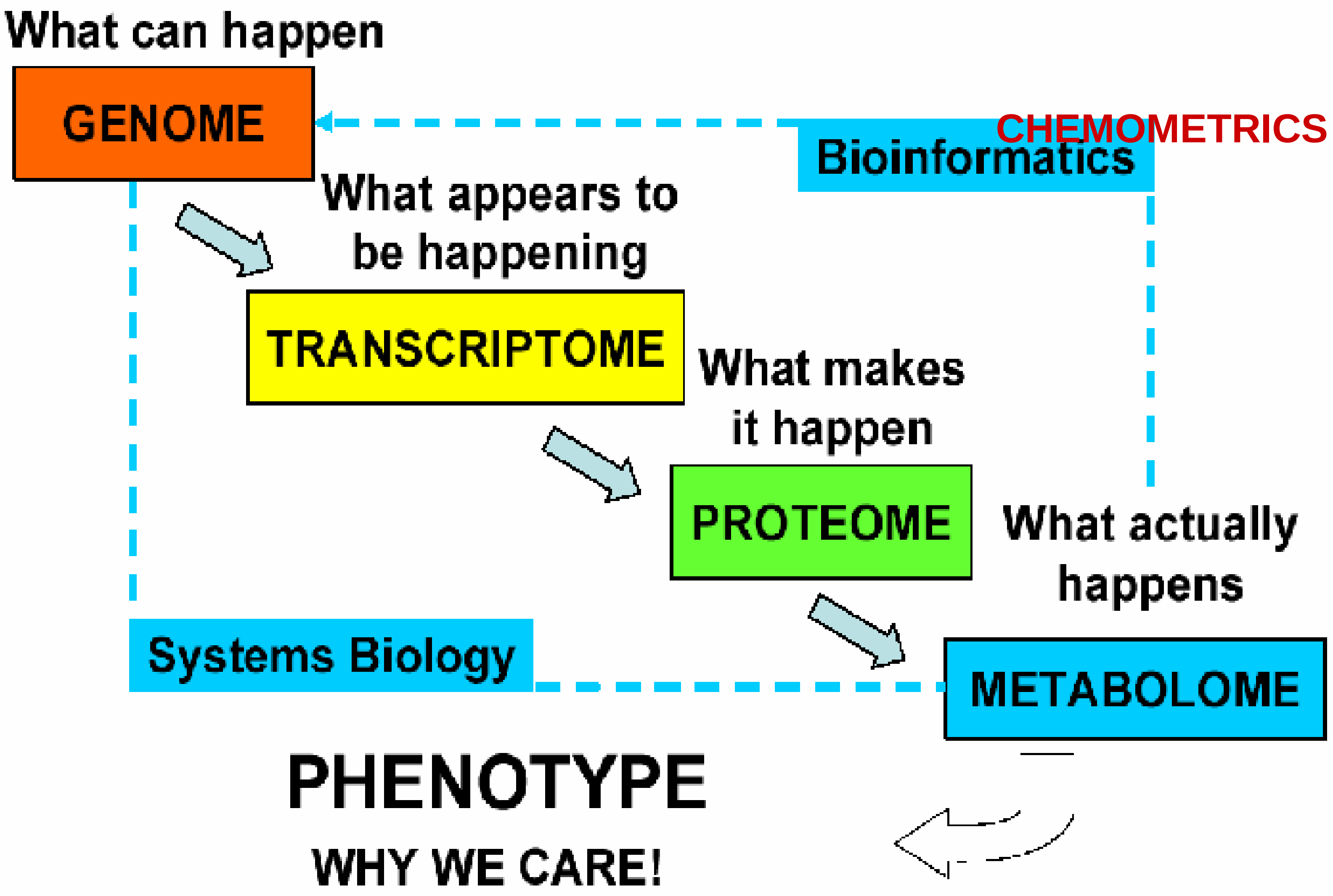
Secondary
(small molecules)

Metabolites = end products of gene expression and enzymatic activities

Metabolomics – reflect the activity of a certain NETWORK

- complementary method to the large-scale gene transcript analysis (transcriptomics) and proteins fingerprint (proteomics)
- explain and identify the differences between sets of organisms (e.g. differences in genotypes) CHEMOTAXONOMY
- elucidate environmental factors that influence biomolecules fingeprint

The Omics-Cascade



WHAT WE NEED

Plant metabolome

Processing

Chemistry

Isolation
Purification
Structure
Elucidation

Individuals

IT

Advanced Statistics

High performance

Equipments

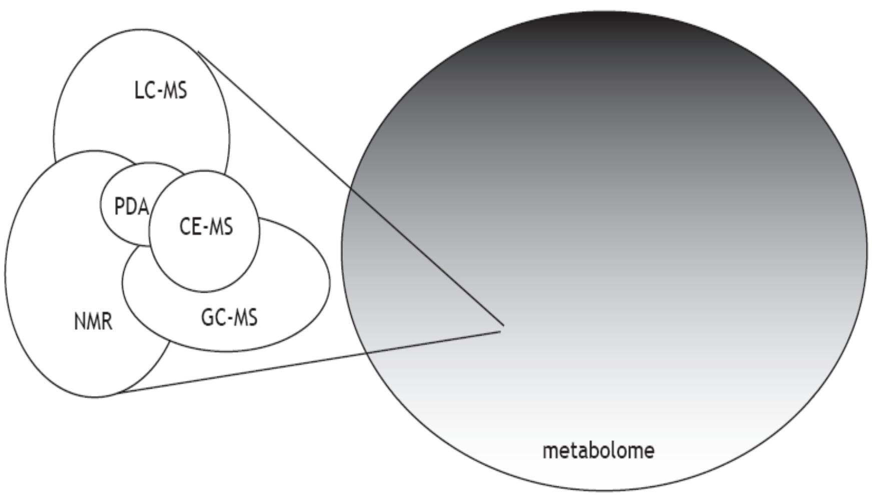
Applied BIO-Chemistry

- Direct measurement of a physical property (e.g. color)
- Selective solubilization from a complex matrix
- Group identification and quantification (spectrometry)
- Fingerprint of a certain group (comparisons)
- Individual characterization (MS, NMR)

Fingerprint

Metabolomics Technologies

- UPLC, HPLC
- CE/microfluidics
- LC-MS
- FT-MS
- QqQ-MS
- NMR spectroscopy
- X-ray crystallography
- GC-MS
- LIF detection



Widely used methods for plant metabolite analysis:

❑GC /MS and LC/MS *(Sumner, Mendes & Dixon (2003) and Dunn & Ellis (2005).*
❑LC/PDA/MS (*Huhman & Sumner, 2002),*
❑Capillary electrophoresis/mass spectrometry (CE/MS (Soga *et al.*, 2003; Sato *et al.*, 2004)
❑Fourier Transformed IR Spectroscopy (*Socaciu, 2009*)
❑Fourier-transform ion-cyclotron mass spectrometry (FT/MS) *(Tohge et al., 2005)*
❑Nuclear magnetic resonance (NMR) (*Ward et al., 2003; Wiklund et al., 2005)*

II. Phytochemicals as indicators of seabuckthorn (SB) composition (leaves vs. fruits)-BIOMARKERS

1. Carotenoids & chlorophylls
2. Vitamins C and E
3. Unsaturated -fatty acids
4. Phytosterols
5. Polyphenols- flavonoids, antocyanins, phenolic acids, tannins

Plant secondary metabolites (more than 10000 molecules known yet…)

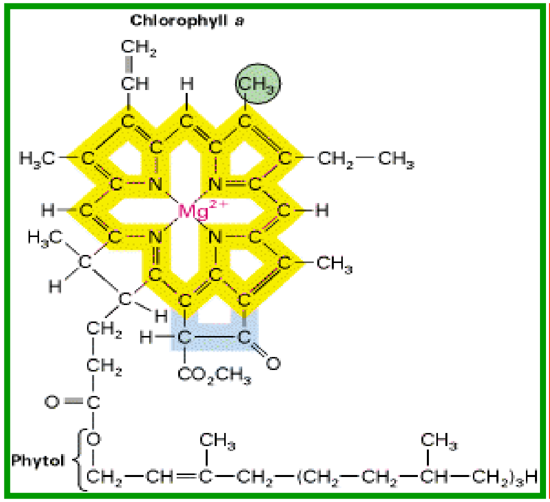
✓Attraction/defence molecules
✓Antioxidant/antibiotic action
✓Beneficial for plant, animal & human health.

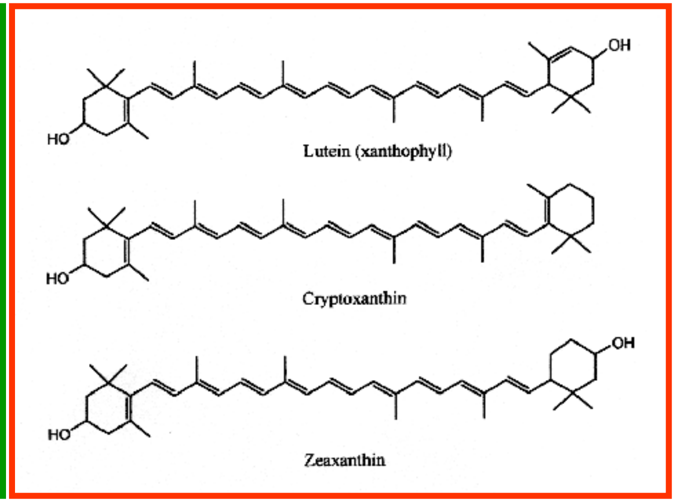
Functionality- dependent on solubility, stability, bioavailability & redox potential

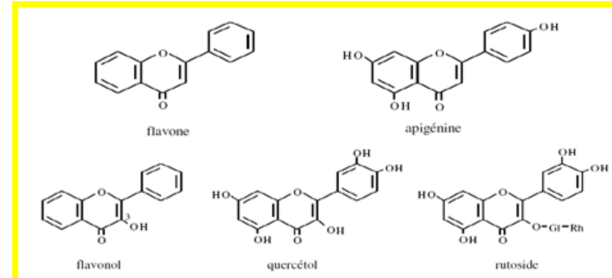
AIMS – specific localization and stability of biomolecules related to their solubility

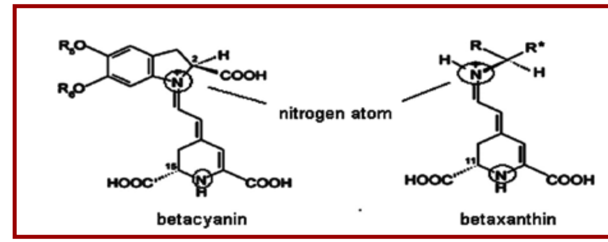
Lipophilic

Hydrophilic







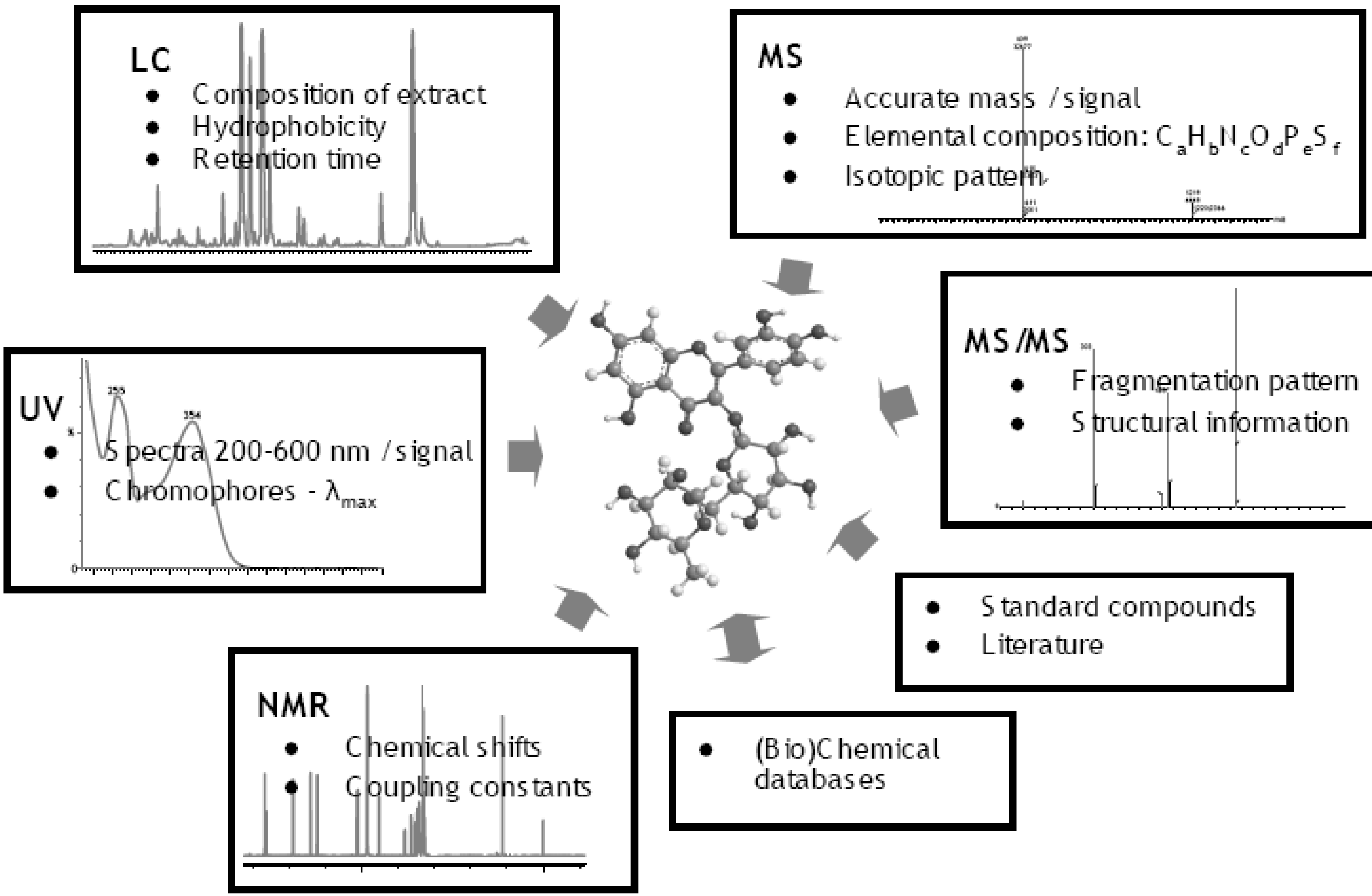


Comparative composition of *H.r. ssp. carpatica* varieties from România and Germany (Hergo, Leikora)

Determination of metabolic profiles of carotenoids- polyphenols by HPLC-PDA, LC-MS, GC-MS, FTIR UV-Vis spectrometry

III. Analytical steps and advanced techniques

NEED FOR COMPLEMENTARY METHODS/TECHNIQUES/KNOWLEDGE



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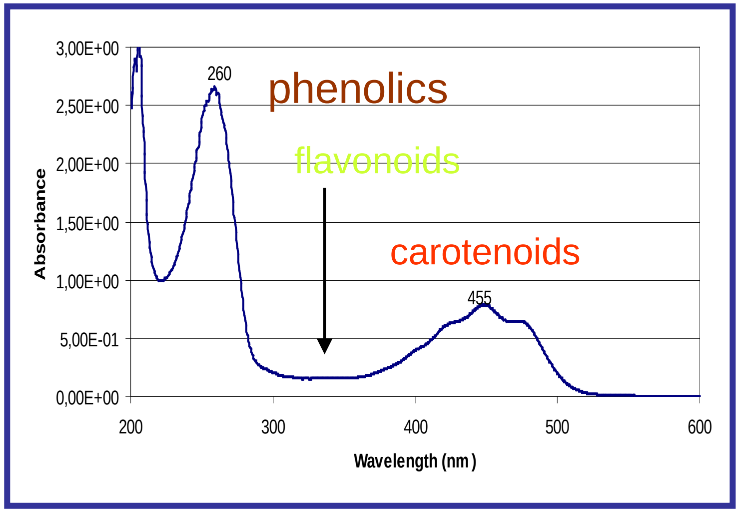
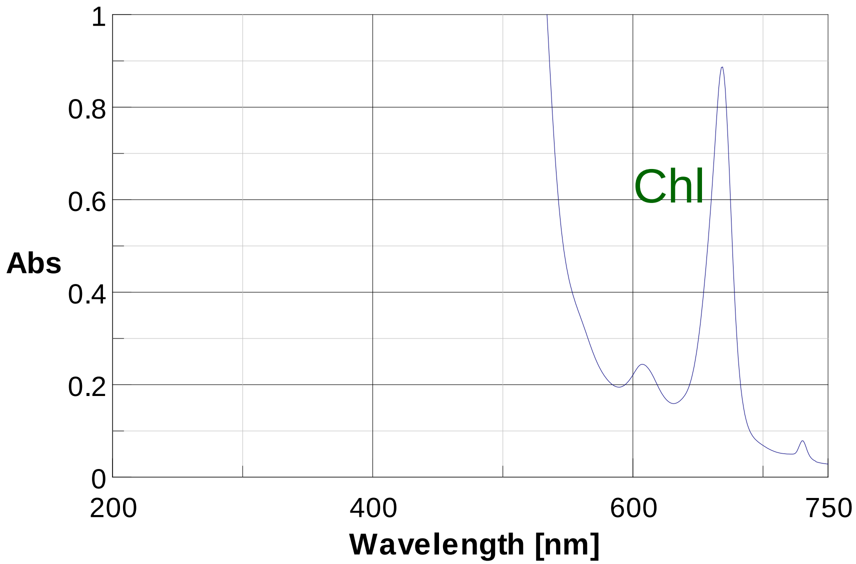
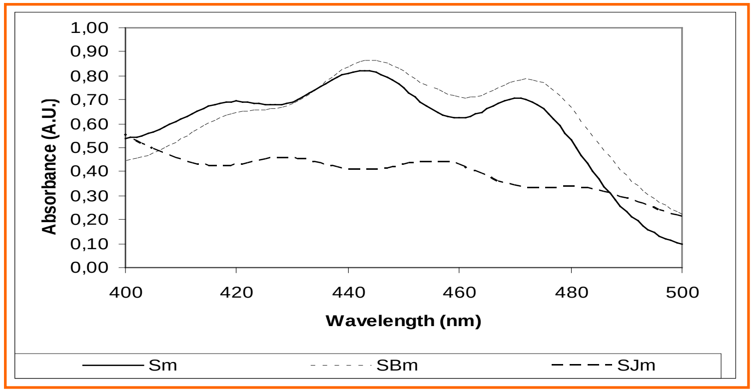
1st step = UV- VIs analysis (for both LE and HE)







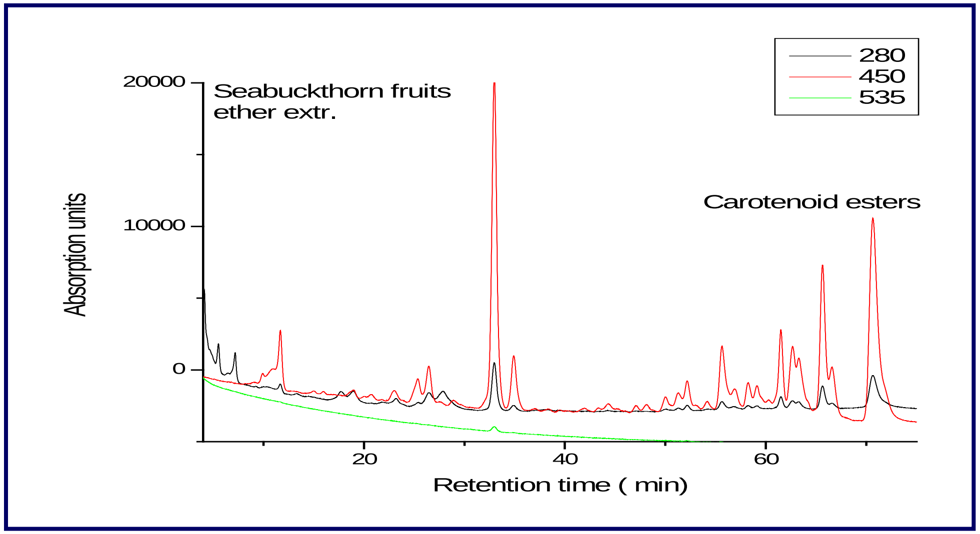
Biomarkers group identification
Quantitative evaluation

LE – hexane
HE- methanol-water

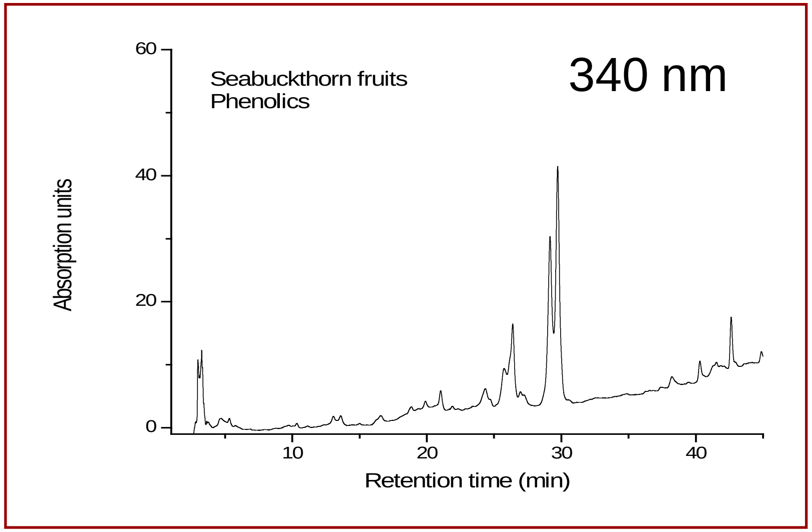
2nd STEP- advanced methods HPLC-PDA or LC-MS

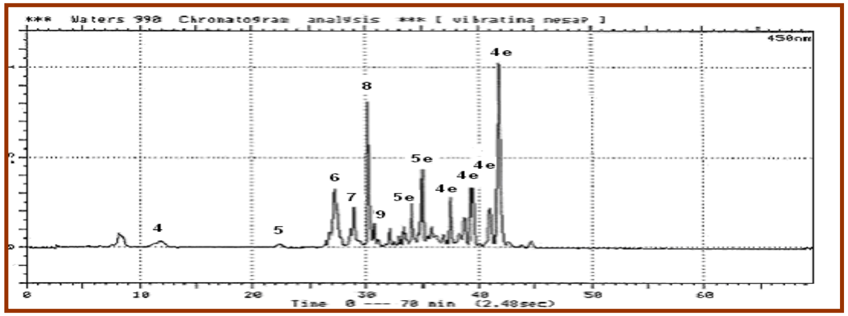
BERRY Profiling

LE



HE





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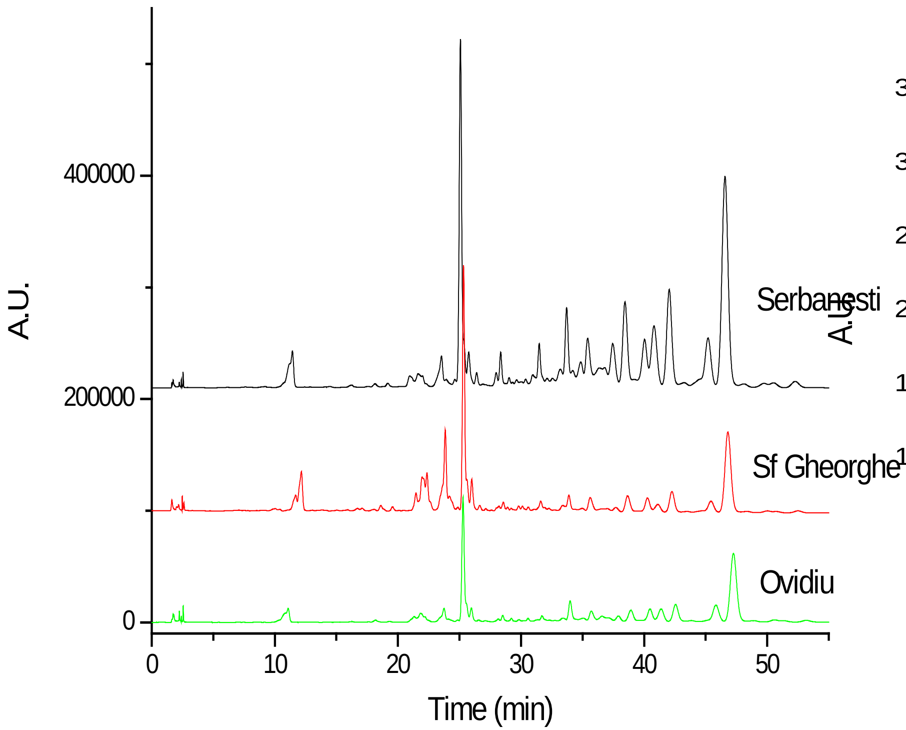
2nd STEP- advanced methods HPLC-PDA or LC-MS

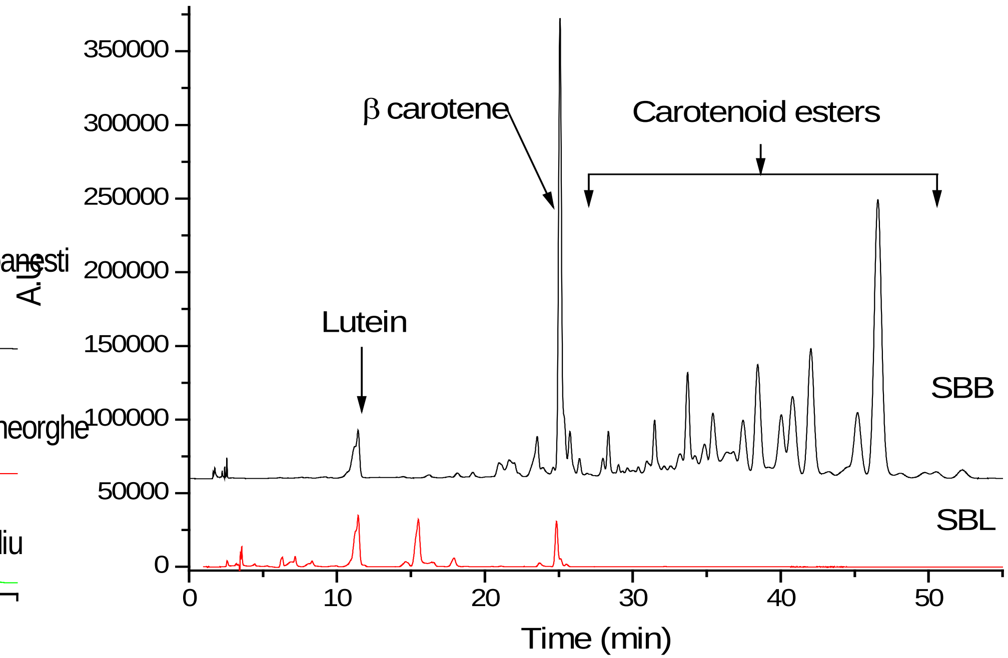
Profiling and quantitation of LE - carotenoids

BERRIES

SBB carotenoid fingerprint at 450 nm



Carotenoid fingerprint at 450 nm, Serbanesti variety



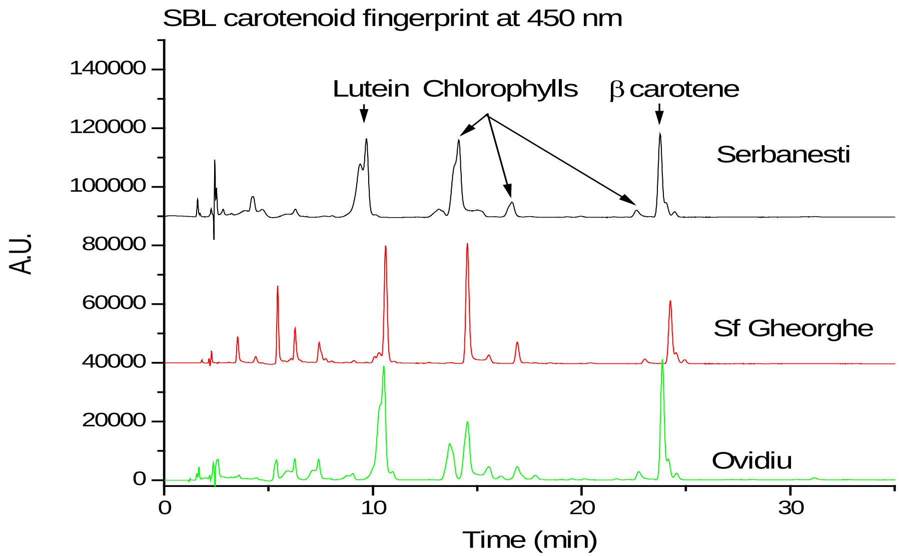
Fingerprint and quantitative evaluation – discrimination of variety and geographical env.

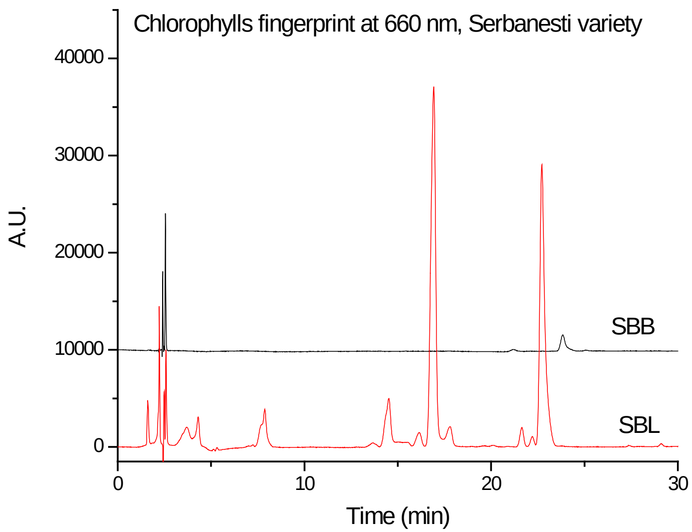
2nd STEP- advanced methods HPLC-PDA or LC-MS

Profiling and quantitation of LE - carotenoids

LEAVES 3 diff. varieties of leaves

Chlorophyl (leves vs berry) at Serbanesti variety





Fingerprint and quantitative evaluation – discrimination of variety and geographical env.

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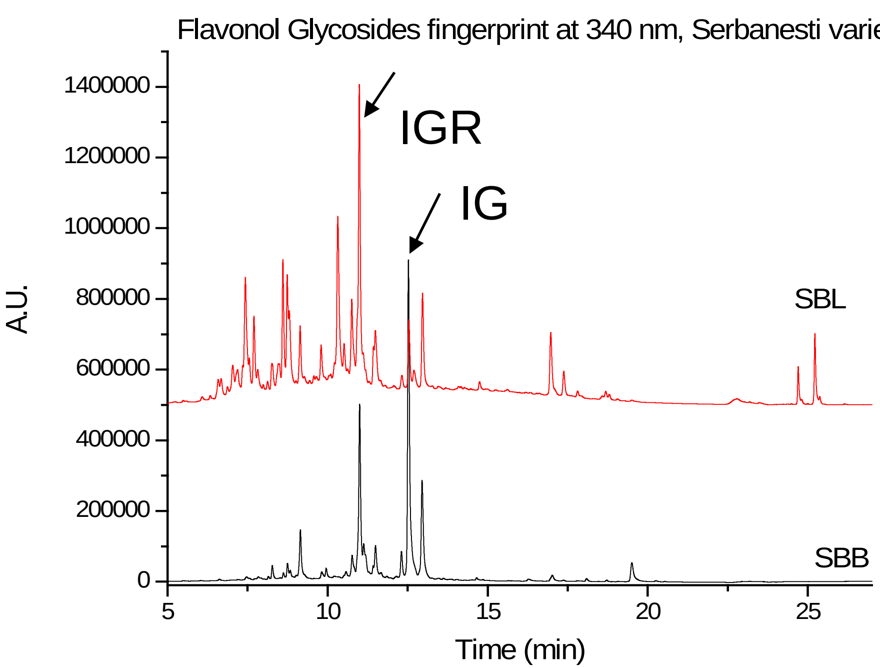
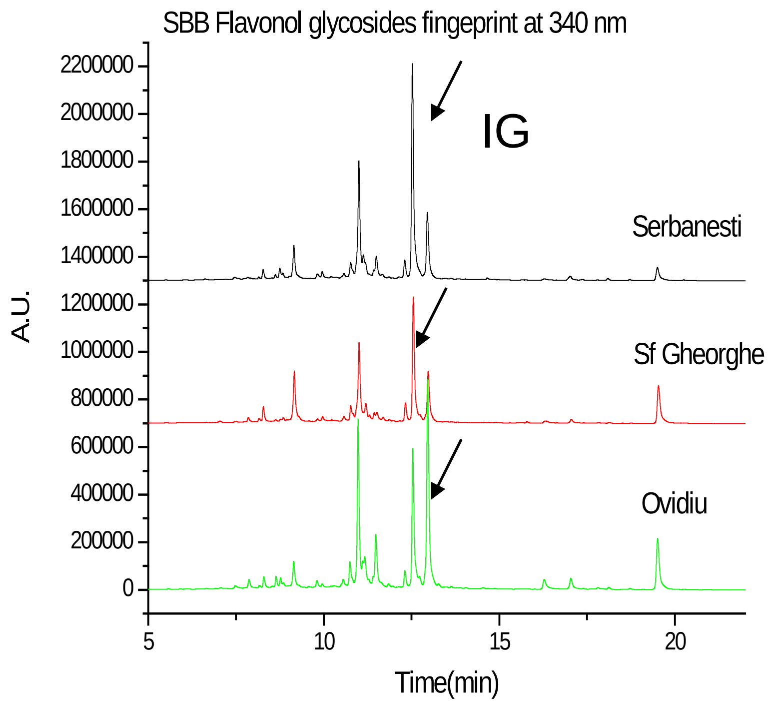
2bd STEP- advanced methods HPLC-PDA or LC-MS

Profiling and quantitation of HE - phenolics

Leaves vs fruits for 1 variety

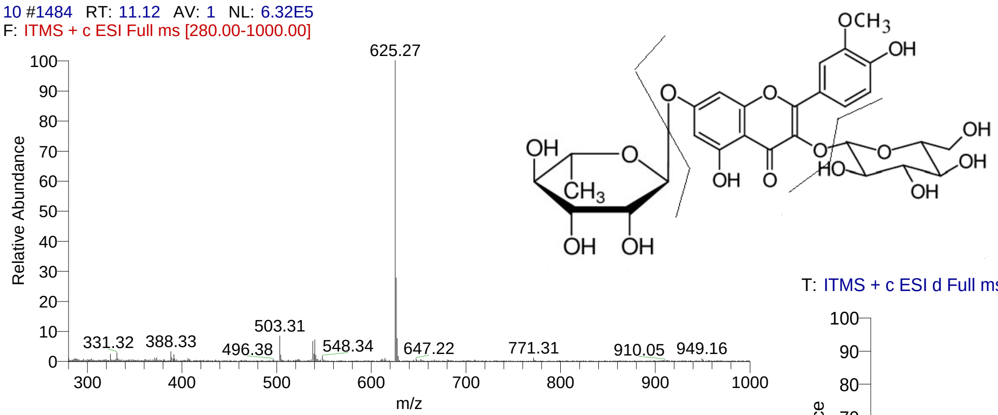
3 diff. varieties of berries

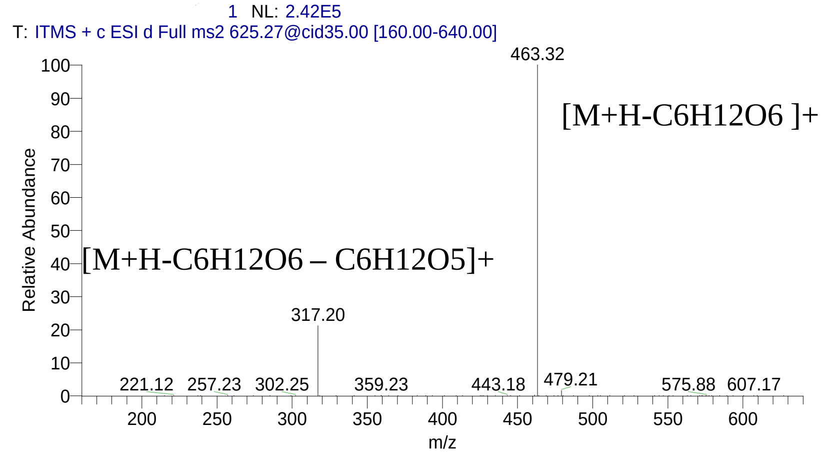


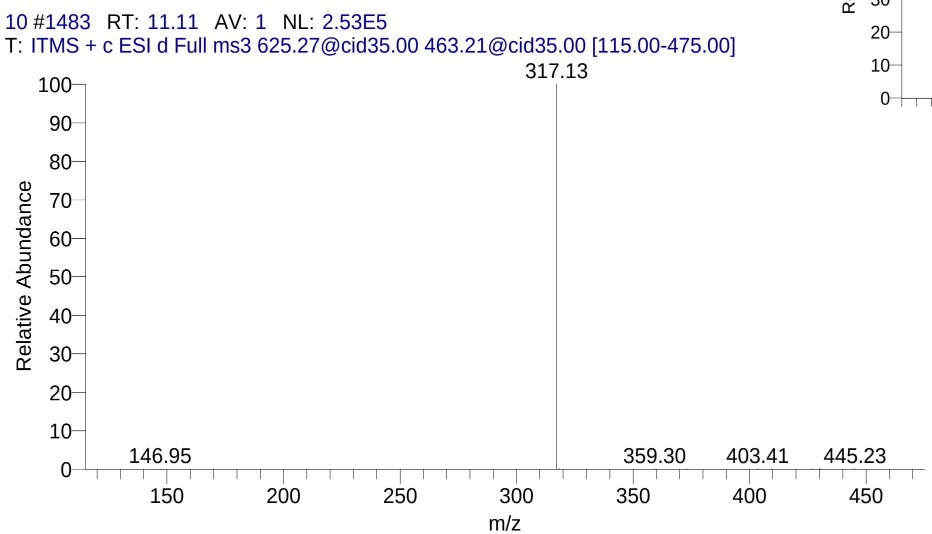


Peack	t_R (min)	UV (nm)	$[M+H]^+$ m/z	MS^2	MS^3	Structure Assignment
1	7.59	227,269, 350	627(100)	465(100), 303(24)	303(100)	Q di glucoside
2	8.29	227, 351	733 (100)	611(20), 449(100), 303(35)	303 (100)	Q sopho-rhamno
3	8.64	227, 350	641(100)	479(100), 317(20)	317(100)	I diglucoside
4	8.77	227, 350	787(100)	641(38), 479(100), 317(36)	317(100)	I diglucoside rhamnoside
5	9.14	227, 353	787(100)	625(22), 463(100), 317(54)	317(100)	I sophoroside rhamnoside
6	9.77	227, 340	611(100)	465(2), 449(100), 303(27)	303(100)	Q glucoside rhamnoside
7	10.74	227, 351	625(100)	479(7), 463(100), 317(28)	317(100)	I glucoside rhamnoside
8	10.98	228, 254, 354	625(100)	463(92), 479(13), 317(100)	317(100)	I glucoside rhamnoside
9	11.48	227, 254, 353	465	303(100)	257(100), 304(78), 229(68), 165(52), 286(45)	Q hexoside
10	12.31	226,352	625(100)	479(32), 463(3), 317(100)	302(100), 285(43), 317(14), 257(13)	I glucoside -rhamnoside
11	12.54	227,254,354	625(100)	479(19), 463(3) ,317(100)	302(100), 285(35), 314(14), 257(10)	I glucoside -rhamnoside
12	12.96	228, 254, 353	479(100)	317(100)	302(100), 285(40), 317(13), 153(4)	I glucoside
13	16.28	226,253,370	303(100)	257(100), 229(76), 303(59), 285(57), 165(55), 137(17), 153(15)	229(100)	Q
14	17.04	226, 254, 370	463(100)	317(100)	302(100), 285(45), 257(10), 153(7)	I rhamnoside
15	18.08	226, 257, 354, 378	709(100)	574(100), 317(33)	317(100)	I-acyl- glucoside-rhamnoside
16	19.51	226, 254, 370	317(100)	302(100), 285(45), 317(17), 257(12), 153(5)	274(100), 285(32), 153(25), 302(2)	I

ESI MS fragmentation pattern for I 3 glucoside 7 rhamnoside (IGR)



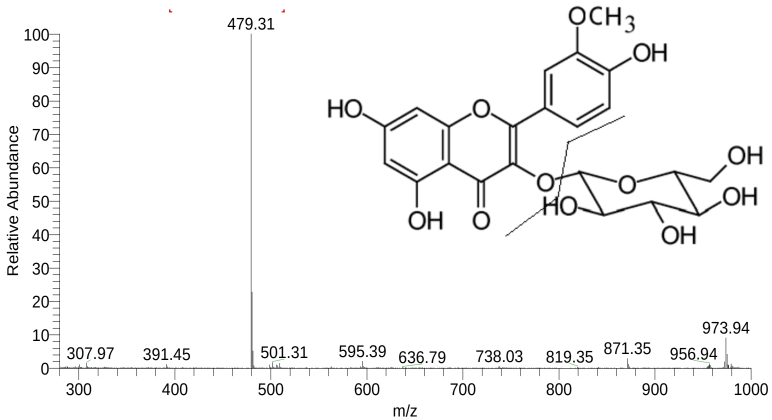




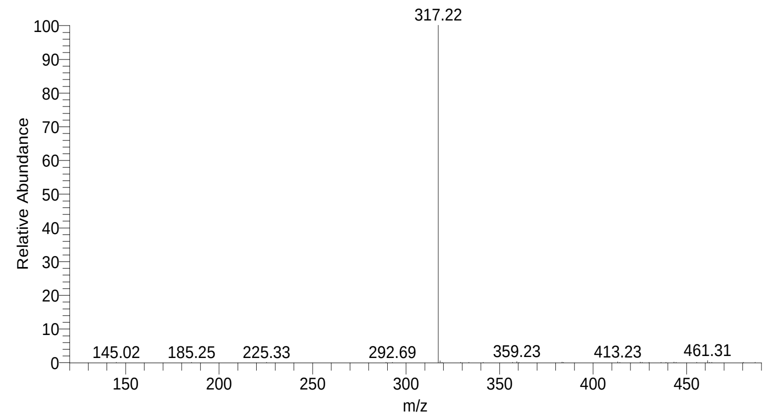
Major biomarker for leaves

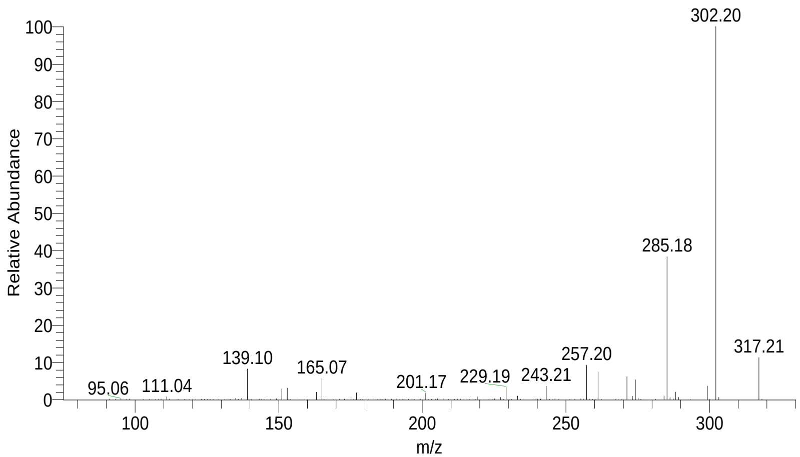
ESI MS fragmentation pattern for Isorhamnetin glucoside (IG)

[M+H]+



[M+H-C6H12O6]+





Fragmentation fingerprint

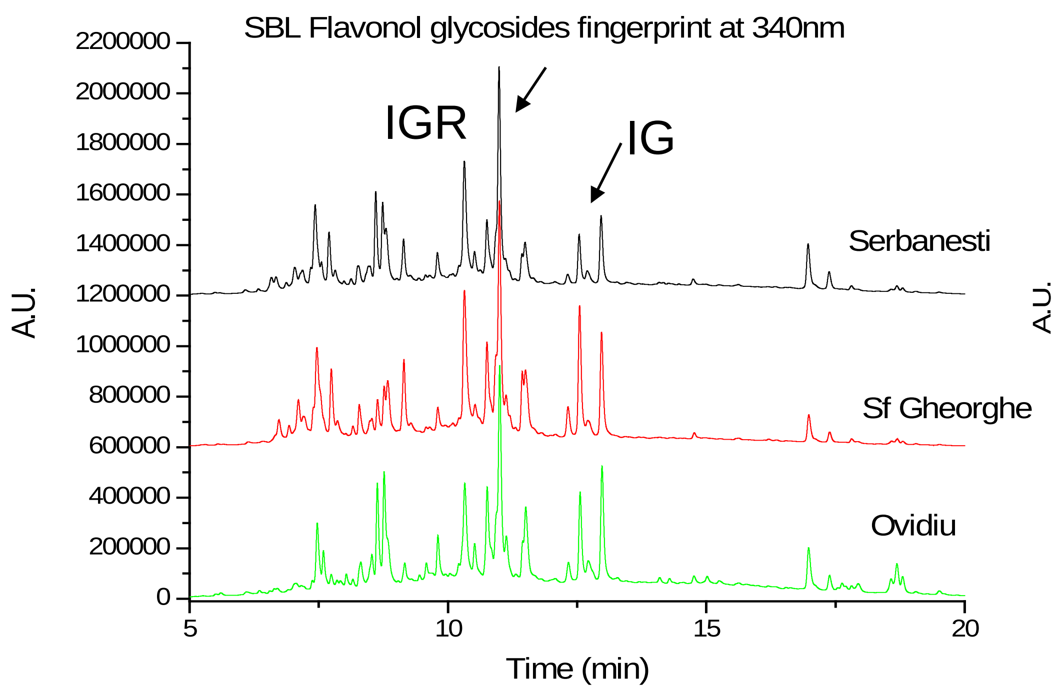
Individual identification of berries, together with IGH (ratio 1-1.5)

Collaboration USAMV Cluj-Napoca & Wageningen University, NL (Pop R.)

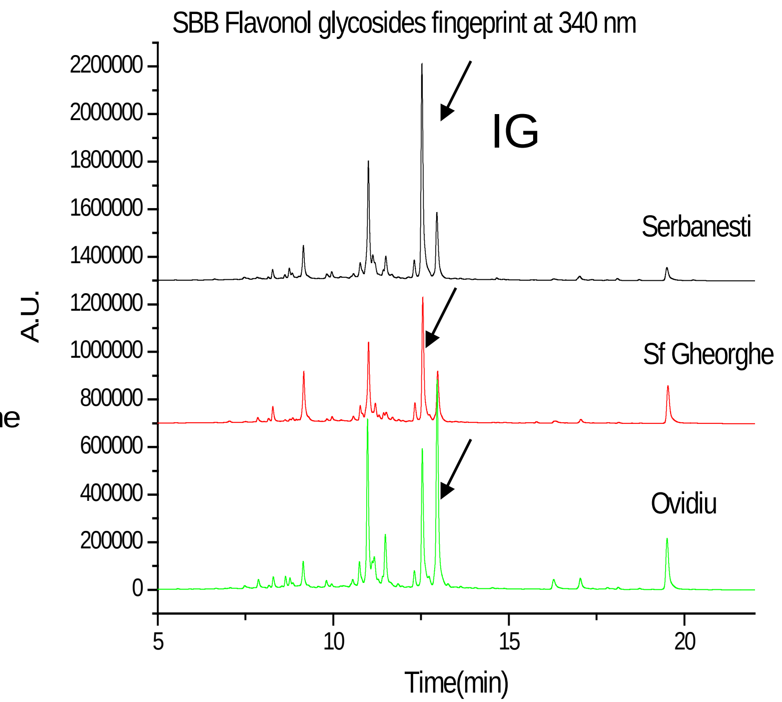
2bd STEP- advanced methods HPLC-PDA or LC-MS

Profiling and quantitation of HE-phenolics

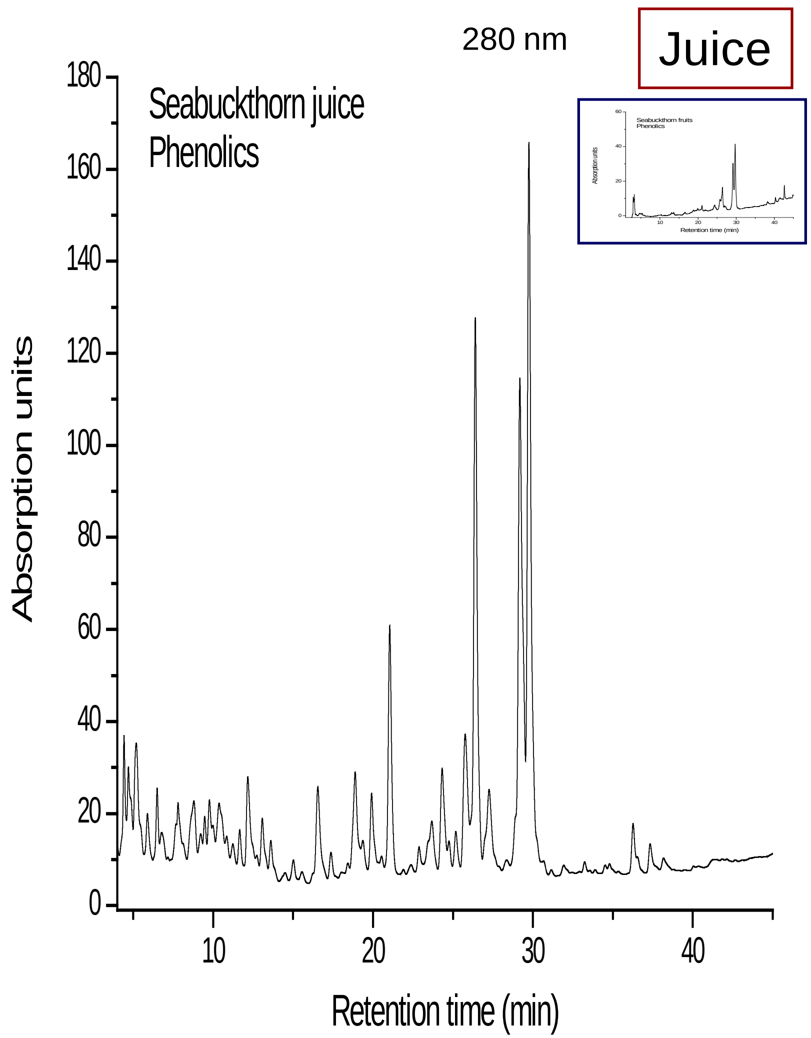
3 diff. varieties of leaves

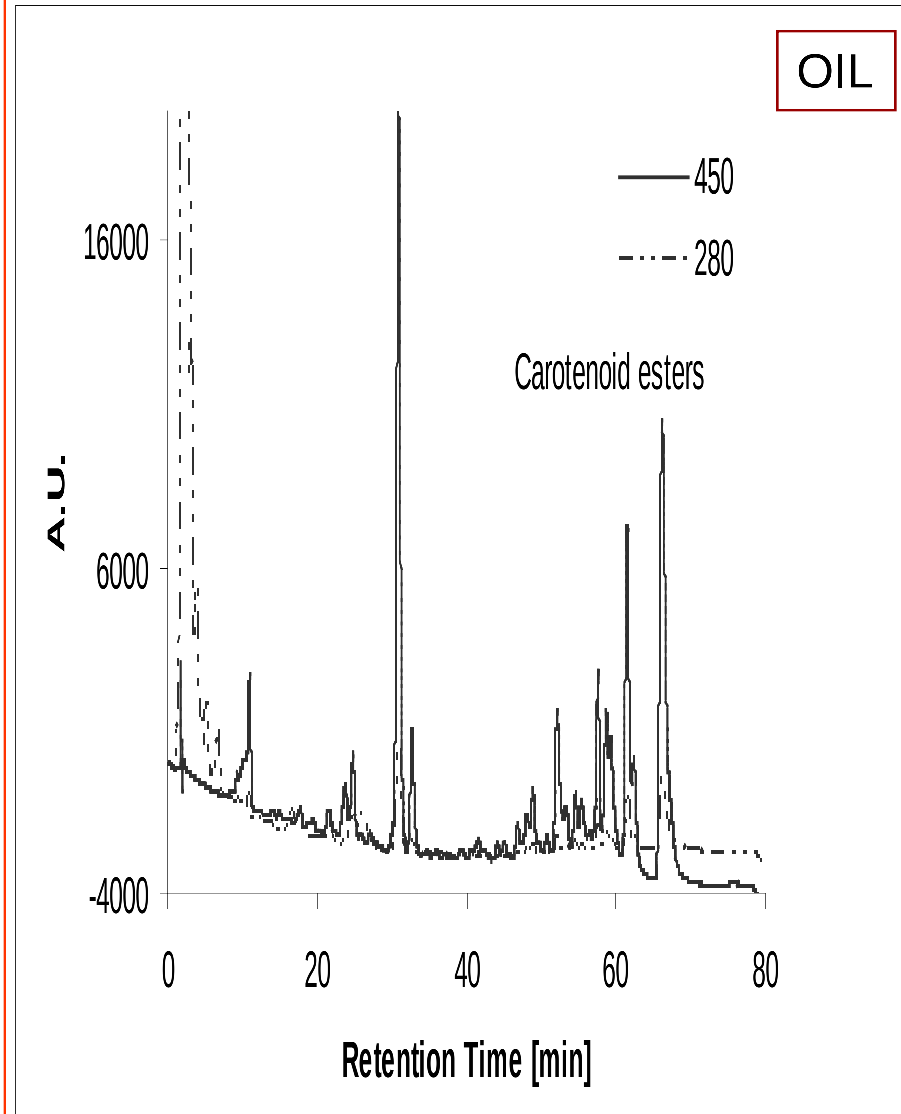


3 diff. varieties of berries

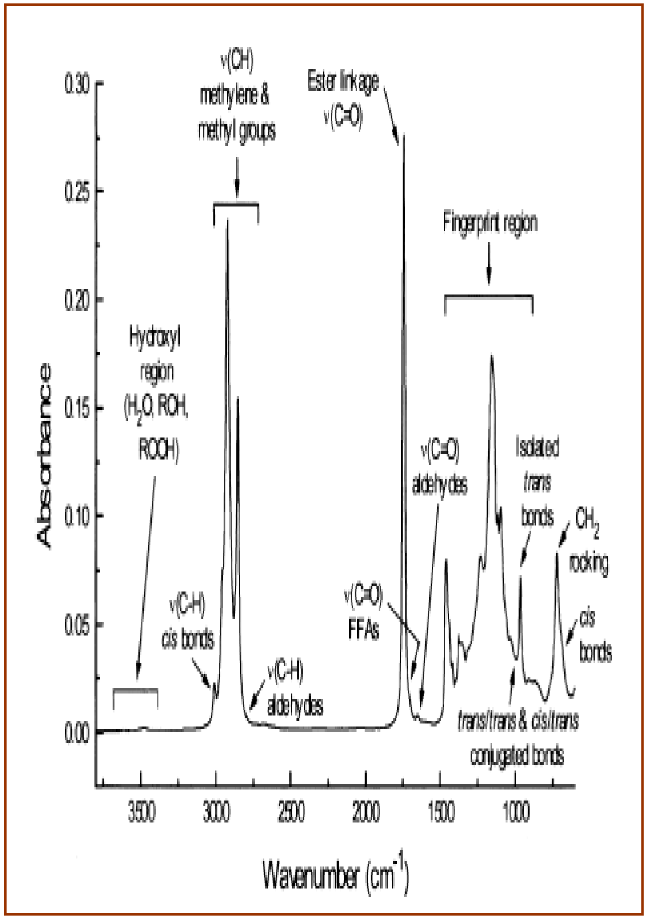


CASE STUDY – SEABUCKTHORN PRODUCTS





3rd Step- FTIR Spectrometric Fingerprint



Rapid, non-destructive, need validation

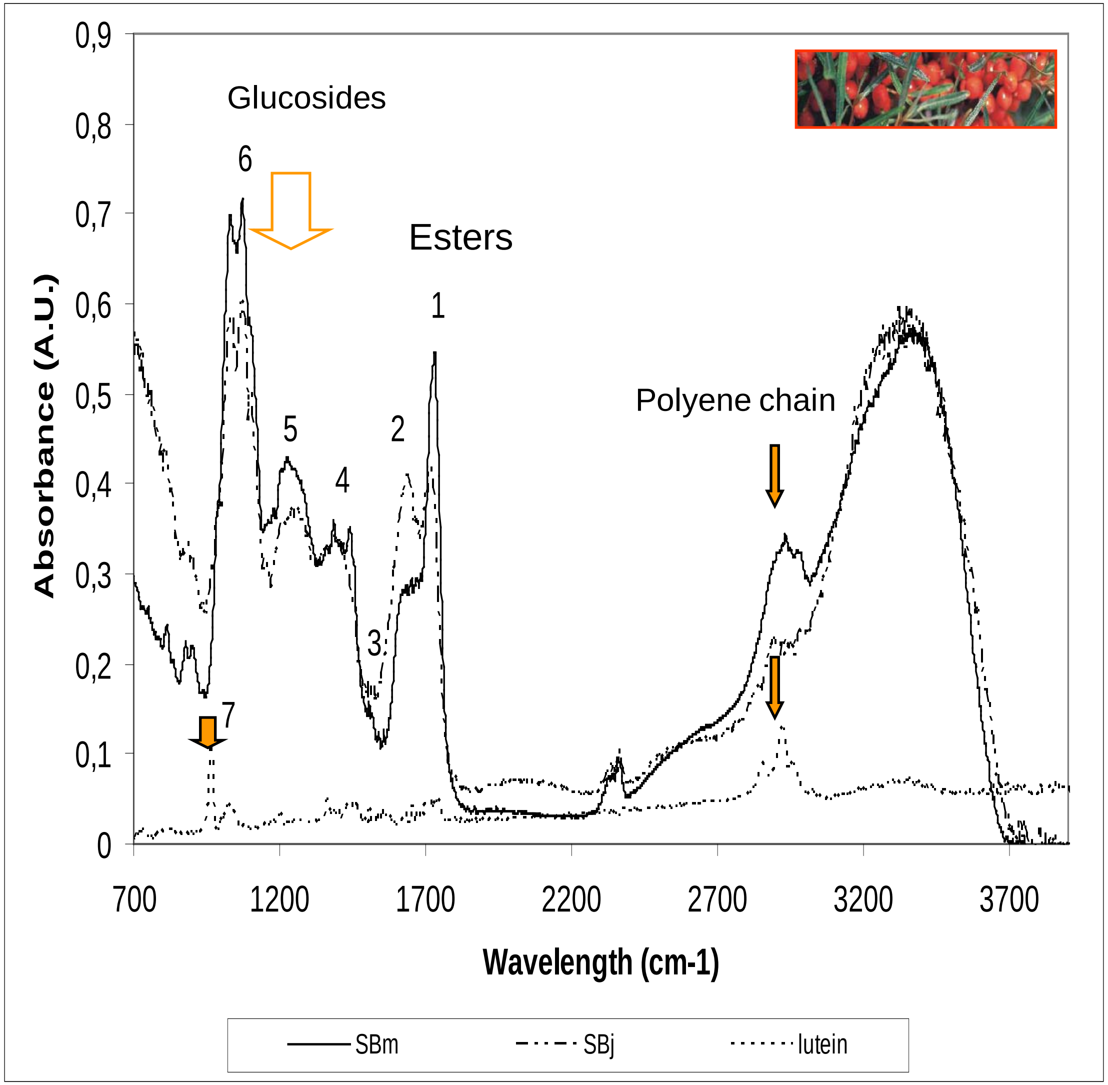
Carotenoids – 965, 1367 and 1450 cm^{-1}
Chlorophylls –1587and 1725 cm^{-1}
Phenolics – 694-849 cm^{-1}

Lypophilic component fingerprint
2800-2900 and 1000-1500 cm^{-1}

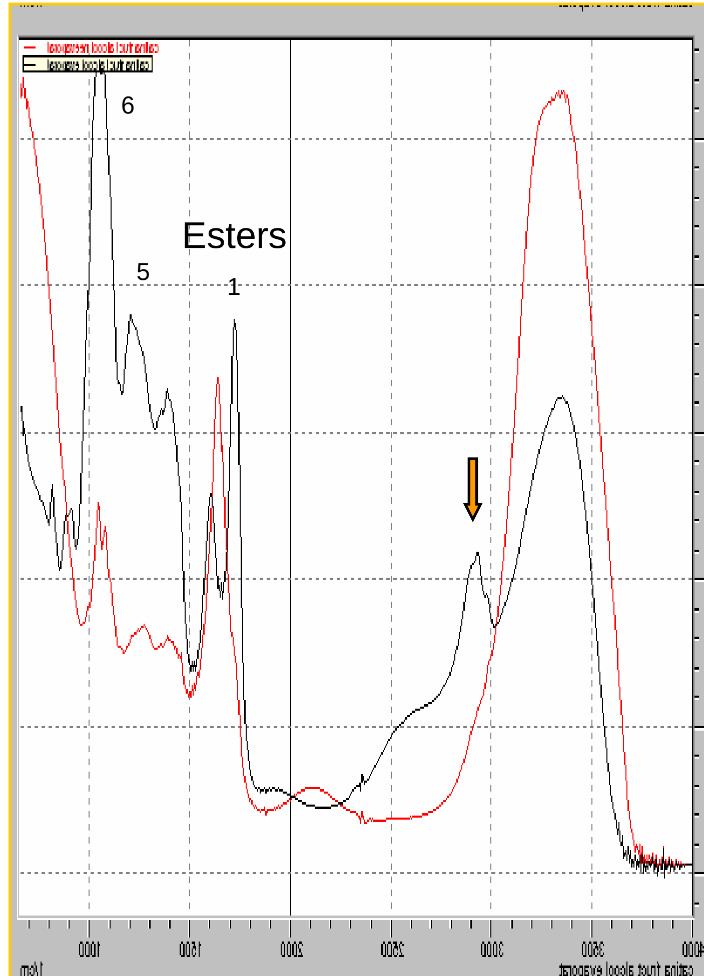
Hydrophilic components–1030-1200 cm^{-1}

Degradation of lipids – look at 3300-3500 cm^{-1}

FT(ATR)MIR fingerprint regions of SB fruit



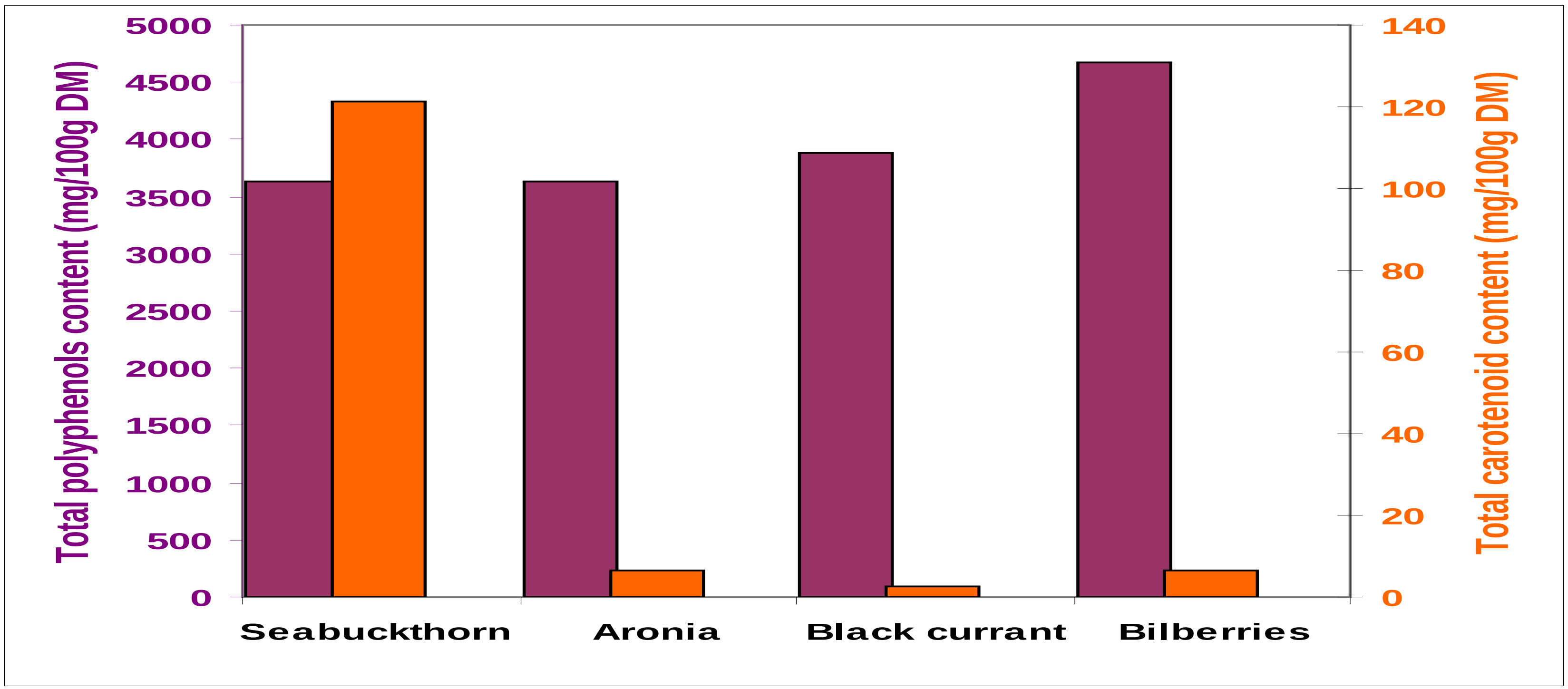
FT(ATR)MIR fingerprint of SB juices (raw vs clear)



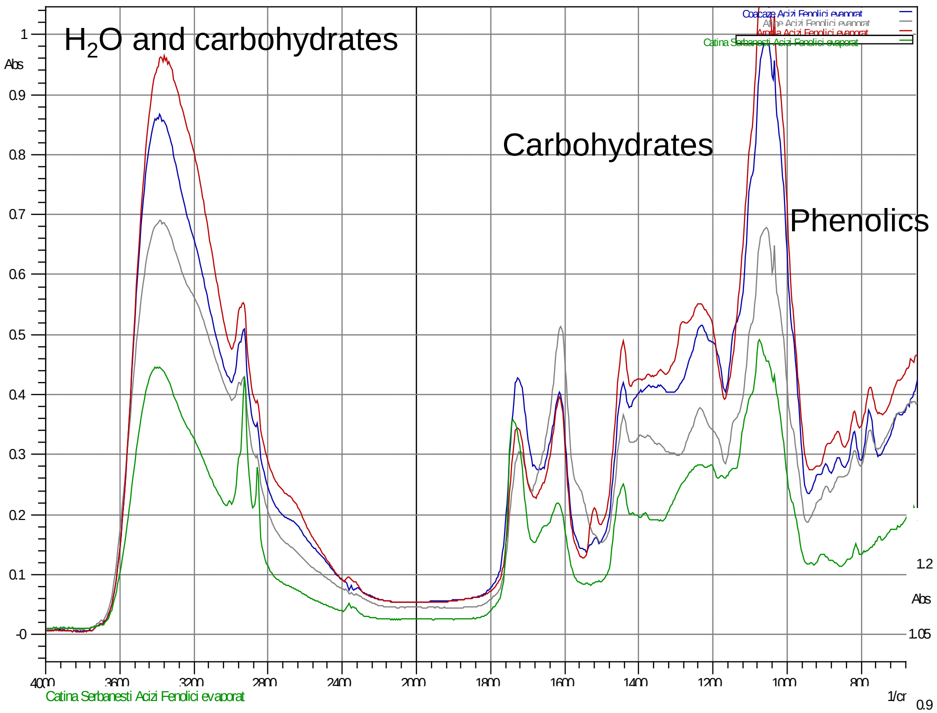
Identification 1720-1734 cm-1 (1), 1620-1695 cm-1 (2), 1516-1550 cm-1 (3), 1238-1396 cm-1 (4), 1132-1134 cm-1 (5), 1024-1029 cm-1 (6), 961-964 cm-1(7).

CASE STUDIES

SEABUCKTHORN-ARONIA_BLACK CURRANT_BILBERRIES

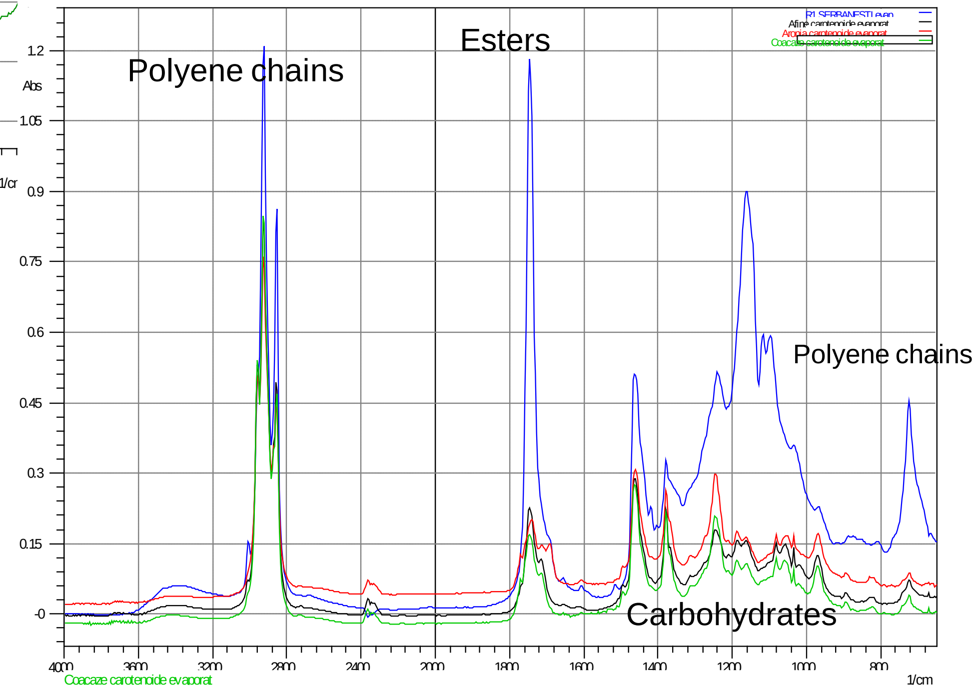


FTIR comparative fingerprint – SB, Bilberry, Aronia, Black currant



HYDROPHILIC EXTRACT

LYPOPHILIC EXTRACT



4^{th} STEP = Statistical correlations = CHEMOMETRY

↑

Profiling and quantitation = INTEGRATION of DATA

↑

HPLC + FTIR (PLS analysis) + PCA to see correlations

Consequences

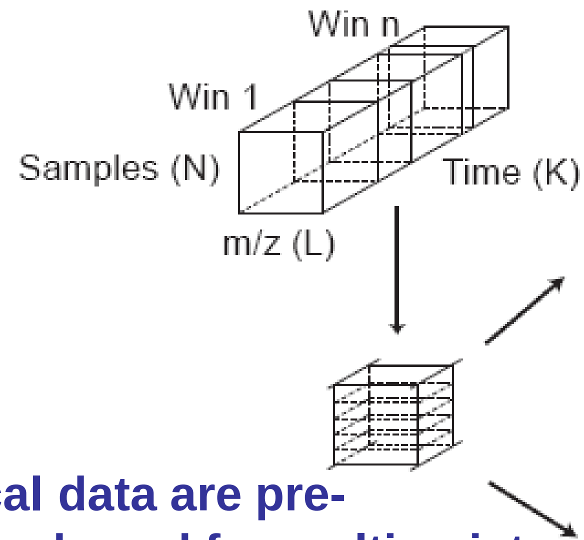
↓

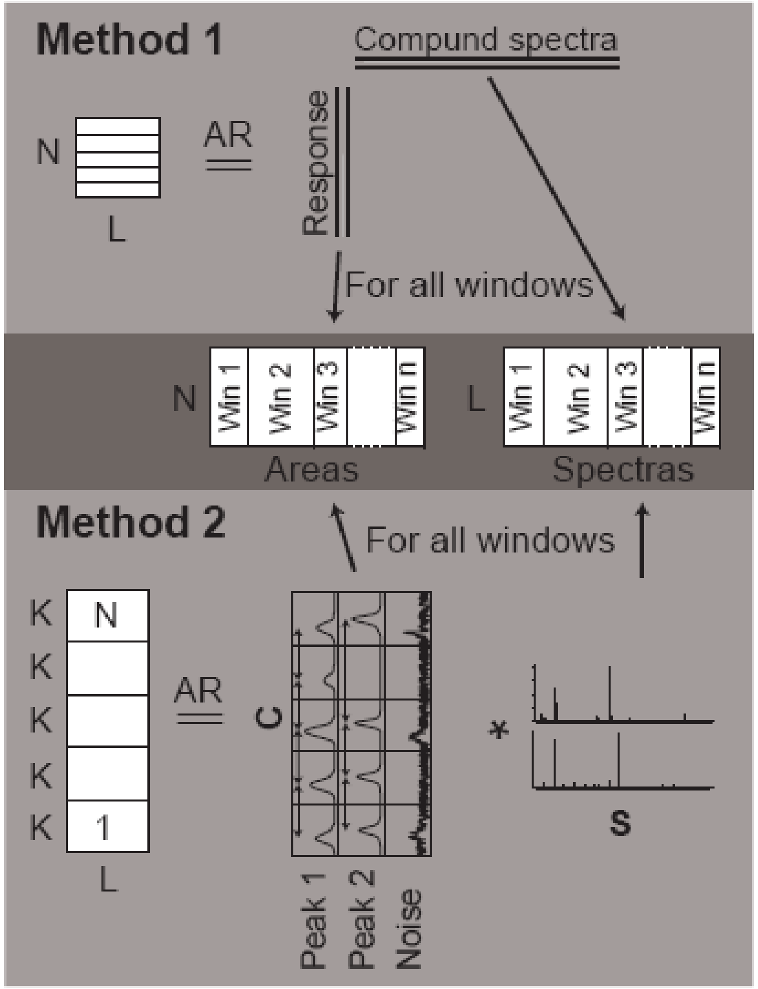
recognize biological / geographical origin

↓

Technological impact on food quality and safety

METABOLOMIC ANALYSIS CONCEPT
=
COUPLING ANALYTICS + CHEMOMETRY





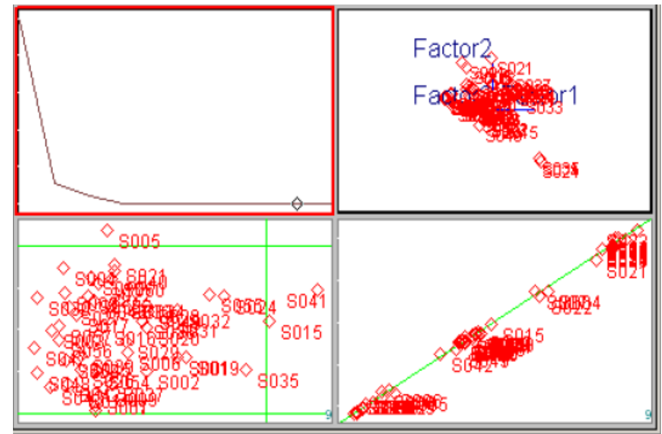
The analytical data are pre-processed and used for multivariate analysis

The differences between samples are used to identify metabolites and make biological interpretation.

C = Chromographic profiles
P =peaks
S = spectral profiles.

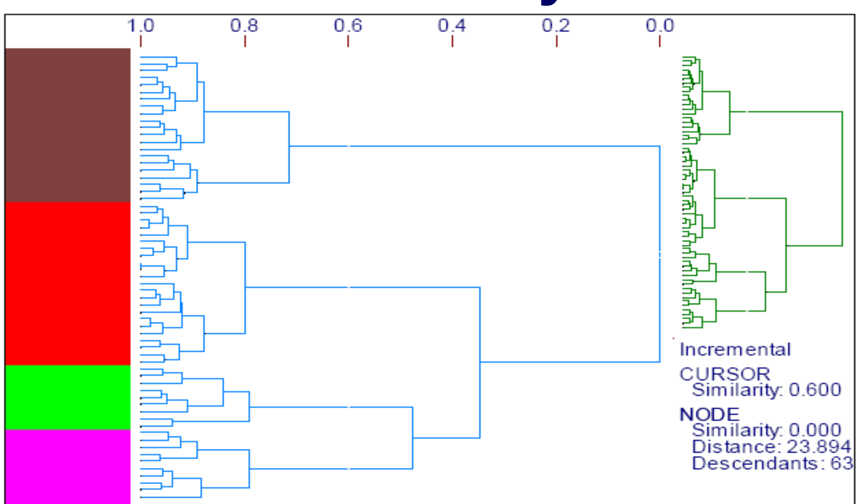
Chemometrics

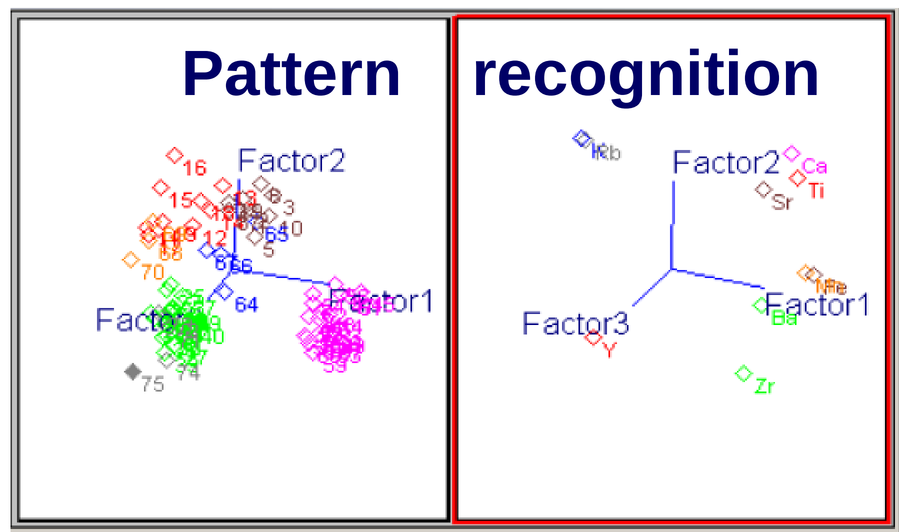
Preprocessing by Metalign
Genemaths
SIMCA
Mathlab
Infometrix:
Pirouette 4.0



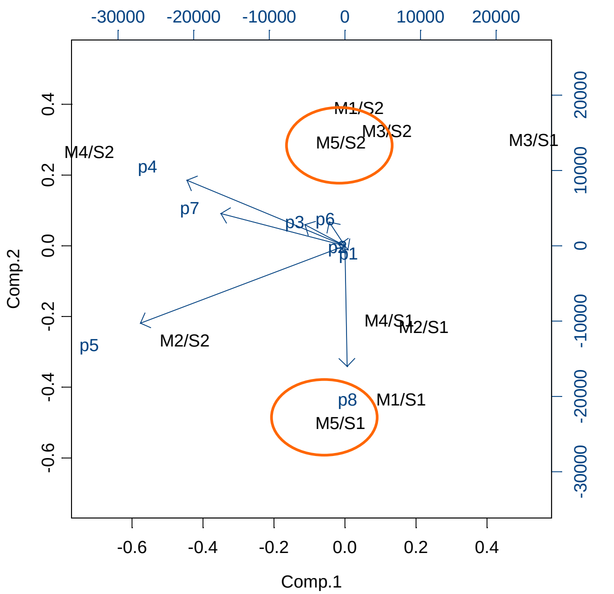
PCA and PLS Analysis

Cluster analysis



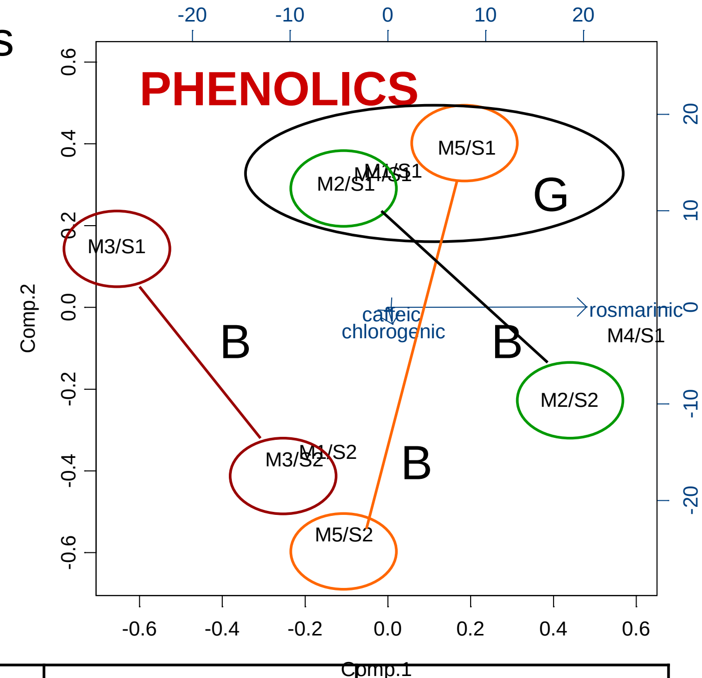


CAROTENOIDS



For bioactive molecules

Biological origin is stronger in SB variability than the geographical origin



Name	Code	Beta-carotene/zeaxanthin esters ratio	Chlorogenic Acid (1) (mg/g)	Rosmarinic Acid (4) (mg/g)
SB Transylvania RO Cluj	M2/S1	0.9	0	21.66
SB Transylvania RO Cluj	M2/S2	1.02	1.42	35.34
SB SouthWest RO Timisoara	M4/S1	1.05	0	23.92
SB South West RO Timisoara	M4/S2	**0.8**	**1.24**	**39.44**
SB Germany (Hergo)	M3/S1	1.4	0	7.58
SB Germany (Leikora)	M3/S2	1.32	1.42	18.45
SB North East RO	M5/S1	1.2	0	29.08
SB North East RO	M5/S2	**1.83**	**1.83**	**21.49**
SB Danube Delta RO	M1/S1	0.7	0	24.56
SB Danube Delta RO	M1/S2	0.9	1.40	20.52

Experimental conclusions

- ✓ We are able to identify different, specific biomarkers realizing the berry vs leave fingerprint, discrimination of biological and geographical origin
- ✓ The technological progress in development of **new instruments** (LC-MS, GC-MS, TOF/MS, FTIR, etc) allows the identification and quantification of each marker (1) as well the plant/food fingerprint/profile (2).
- ✓ **Combinations of different, complementary analytical techniques** completed with **chemometric analysis** are required for comprehensive metabolomic studies.
- ✓ Specific predictions can be established for routine analysis, cheaper and easy to perform

IMPACT OF METABOLOMIC STUDIES

- ✓ Establishment of quality standards based on major biomarkers (carotenoids, phenolics, vit. C) with antioxidant potential
- ✓ Evaluation of technological flow, from raw material to intermediates and final products
- ✓ Authenticity of products and their traceability (origin)
- ✓ Safety aspects (PCH, pesticides)
- ✓ Use of rapid methods (FTIR) which are in good correlation with validated , expensive methods (HPLC, GC)
- ✓ Specific predictions can be established for routine analysis, cheaper and easy to perform for many samples

First Metabolomics program in Romania

Carmen SOCACIU - coordinator

Floricuta RANGA – UV-Vis, HPLC, and LC-MS (plants)
Florinela FETEA – FTIR (plants and food)
Adela PINTEA & Andrea BUNEA- HPLC-PDA (carotenoids and in vitro effects)
Raluca PARLOG: LC-MS and TOF (food metabolomics)
Monica TRIF, PhD – FTIR and NMR (functional oils)
Loredana LEOPOLD: FTIR and Raman spectroscopy
Francisc DULF, PhD : GC-FID and GC-MS (phytosterols)
Constantin BELE and Cristian Matea :GC-FID (fatty acids)

MeT-RO : A major initiative to establish the Centre for Plant and Food Metabolomic Analysis
Metabolomics Society
Reseaux Metabolomics and Fluxonomics –France
MetaboP- EU Project
META-PHOR

More about pigments ….

FOOD COLORANTS: Structural and Functional Properties

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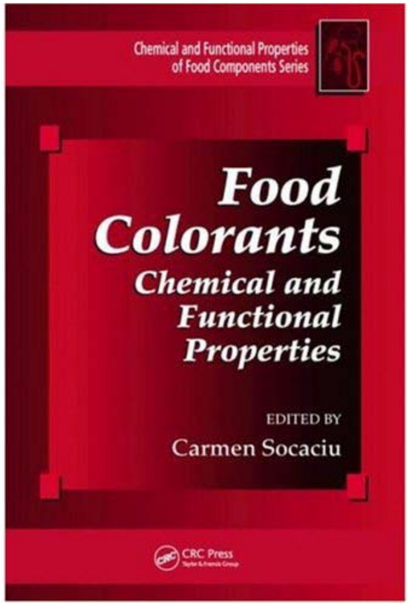
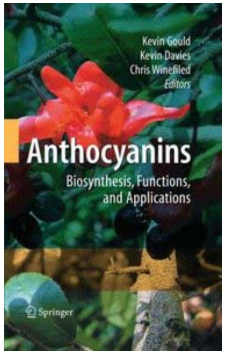


CRC Press, Taylor & Francis, 2008



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Cluj-Napoca - vedere generală
Cluj-Napoca - general view

TRADITION and MODERNITY