Discovering Barley Intake Biomarkers in Urine by UPLC-MS Based Untargeted Metabolomics

Project outside coursescope, MSc (15 ECTS)

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9th, Nov

Outline

Background

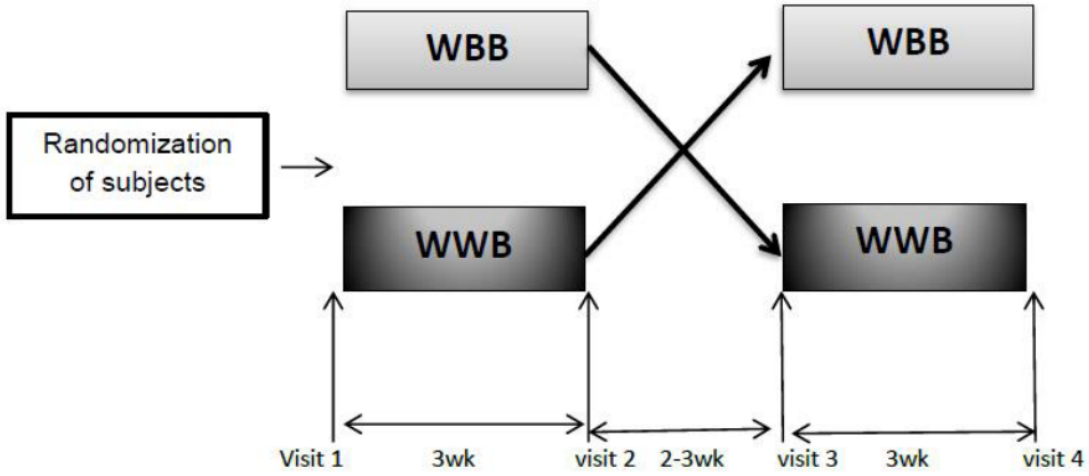


Figure 1: Schema of Study Design (WBB=whole barley bread; WWB=whole wheat bread)

- randomized cross-over intervention design
- 2 bread rolls/day during intervention period
- 14 healthy volunteers (6 men, 8 women)
- blood & urine samples collected each visit
- Conclusion: No significant changes of CVD risk factors and other health statue factors (before & after intervention; after barley & wheat)

Barley: from farm to table

Barley in the farm

- **4th** most produced cereal grains (maize, rice, wheat, **barley**)
- 143 million tones in 2016
- Widely adapted species (drought, cold and salt tolerant) - food security

Barley on the table

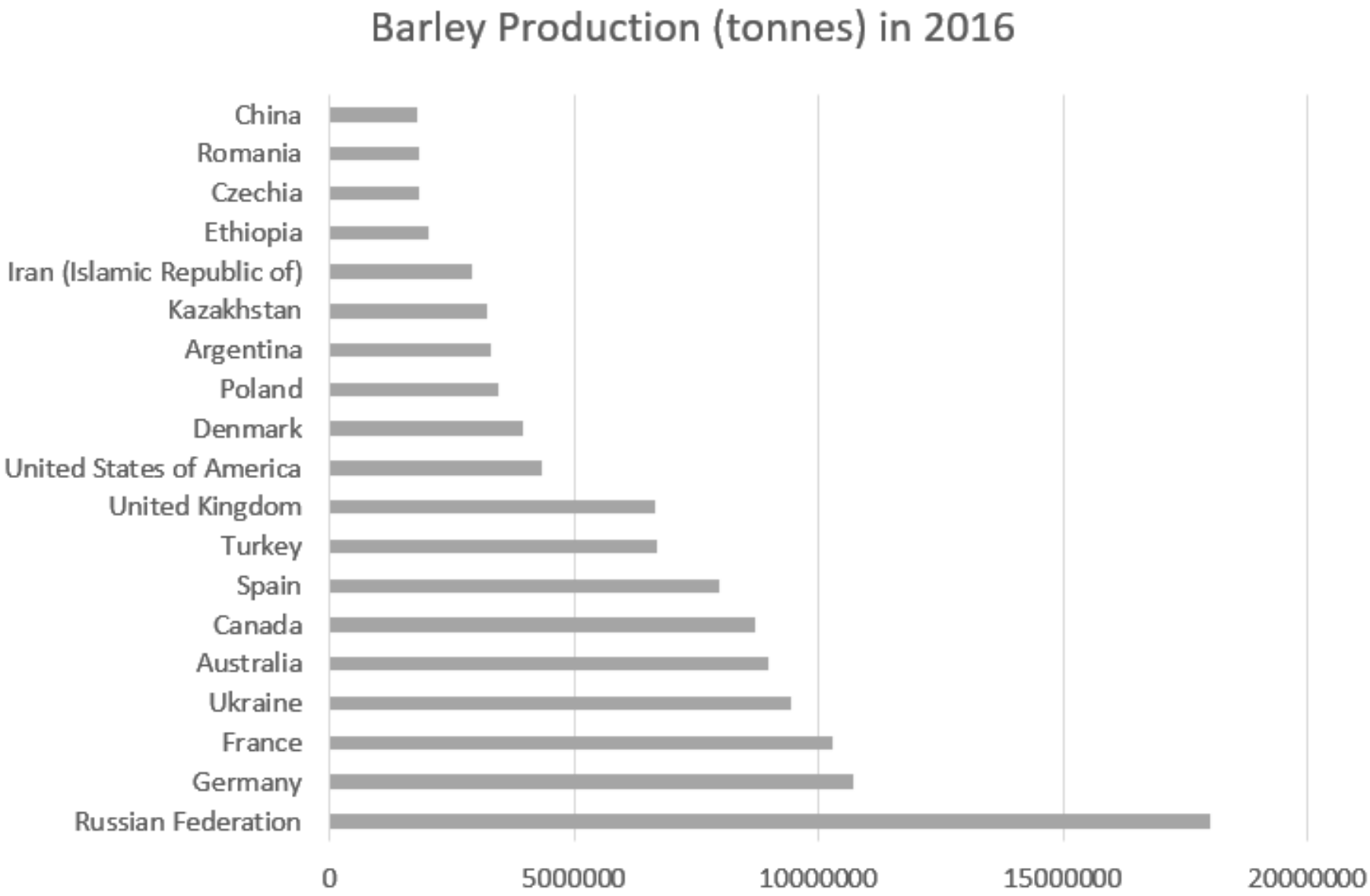
- 1/3 for malting and brewing (beer)
- 2% for direct food use
- Majorly for animal's feed
- Rough mouthfeel, texture
- No systematic breeding
- No quality/ usage standard

Facts (2016)[1]

EU produced **63%** barley.
Turkey (**9th**) & Denmark (**11th**) & China (**19th**) ranked by barley production

[1] statistics from FAO

Barley production



Barley: increasing interest and health benefits

Increased interest as an healthy ingredient

- Customer & food industry

- **Selling points**
- Beta-glucan
- Dietary fiber
- Whole grain

Health beneficial effects

- **Beta-glucan** content: 4.6%
- capable of reducing cholesterol; regulate blood glucose
- **Phytochemicals** (polyphenols, sulfur compounds, lignin and phytic acid)
- antioxidant activites and other unknown effects

Conclusions on health benefits

Controversial. Both positive and negative results were reported.
Lack of objective measurement of barey dietary exposure.

Biomarkers of Food Intake (BFIs)

Traditional way to measure dietary exposure

- Self-reported: 24-h dietary recalls, food-frequency questionnarires
- subjective (recall bias, difficult to assess portion size ...)
- alcohol or tobacco consumption (affected by cultural or societal attitute)
- 'pressurized' to pretend a healthy diet

Biomarkers of Food Intake

- Objective
- Precise, detailed, informative, dynamics
- Metabolic & signaling pathway

Alkylresorcinols: biomarkers of whole grain cereal intake

Current status

- Widely reported and validated biomarker for whole grain cereals (Table)
- Detected both in urine and plasma

Limitations

- Taking account of all whole grain cereals.
- Not specific to individual grain type (wheat, rye, oats, barley...)
- No barley biomarkers reported

Alkylresorcinols

No	Authors	Experimental methods	Food types	Compounds	Subjects	Matrix	Reference
1	Wierzbicka, R etc.	three-day weighed food record	Whole grain cereals	alkylresorcinol metabolites	69 Swedish	urine	[15]
2	Zhu, YD etc.	Diet Intervention	Whole grain wheat	alkylresorcinol metabolites, benzoxazinoid derivatives,phenolic acid derivatives	12 healthy participants	urine	[16]
3	Garcia-Aloy, M etc.	Self-reported food frequency questionnaires	whole grain bread	phytochemicals (benzoxazinoids, alkylresorcinol metabolites)	155 subjects	urine	[17]
4	Magnusdottir, OK etc.	controlled diet	whole grain rye	alkylresorcinol C17:0/C21:0 ratio	93 metabolic syndrome patients in Nordic countries	plasma	[18]
5	Lappi, J etc.	Diet Intervention	whole grain and fibre riched rye bread	alkylrecorsinol		plasma	[19]
6	Ma, JT etc.	Self-reported food frequency questionnaires	whole grain cereals	alkylrecorsinol	407 olders	plasma	[20]
7	Ross, AB etc.	Diet Intervention	whole grain food (including wheat, oats, brown basmati rice, corn, rice, barley)	alkylrecorsinol	316 overweight and obese participants	plasma	[21]
8	Andersson, A etc.	Food records	whole grain wheat and rye	alkylrecorsinol	72 Swedish adults	nonfasting and fasting plasma	[22]
9	Landberg, R etc.	semi-quantitative food frequency questionnaires	rye bread	alkylrecorsinol	360 postmenopausal women	plasma	[23]
10	Montonen, J. etc.	Self-reported food frequency questionnaires	Whole grain food	alkylrecorsinol	100 healthy adults	plasma	[24]
11	Guyman, LA etc.	three-day food record and food frequency questionnaires	Whole grain food	3-(3,5-dihydroxyphenyl)-1-propanoic acid		urine	[25]
12	Landberg, R etc.	Diet Intervention	whole grain wheat and rye	alkylrecorsinol	22 women and 8 men	plasma	[26]

Workflow of BFIs discovery by Untargeted Metabolomoics

- Metabolome profiling (**LC-MS**, GC-MS, 1-NMR)
- Data preprocess
- Data analysis
- **Compound identification**
  - Most difficult part in all areas of metabolomics study
  - **Expert opinions** (1st Copenhagen Clinical Metabolomics Conference)
- Validation

Compound identification: expert opinions

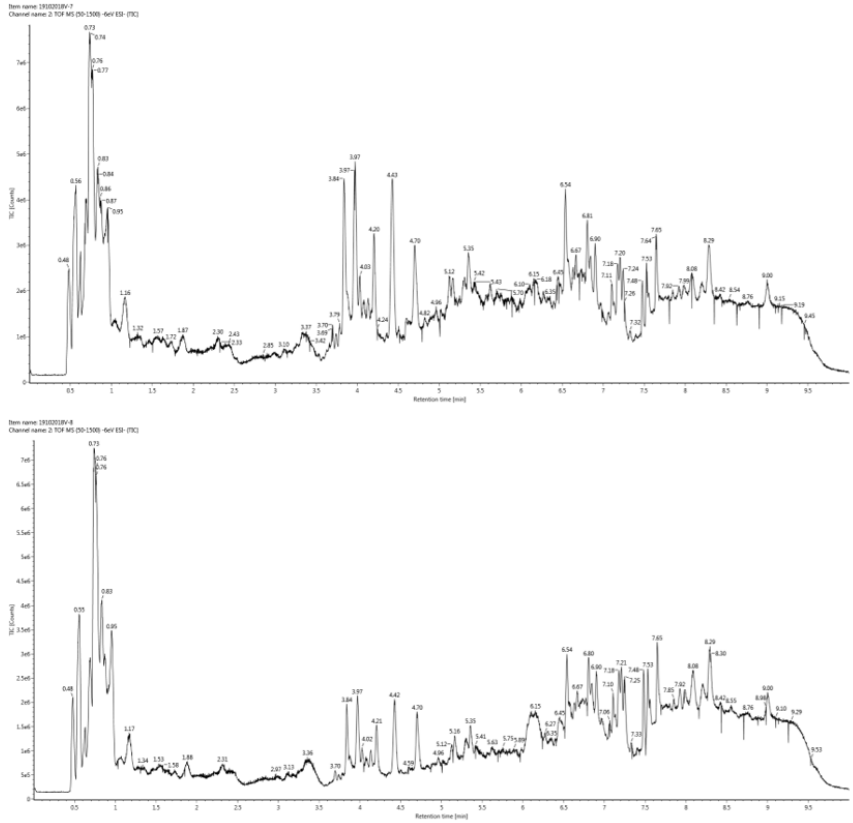
- **Improvement of analytical instruments for metabolome profiling:**
  e.g TIMF-PASEF (ion mobility, rt, m/z and intensities)
- **Bioinformatics, database and data sharing:**
  MS/MS prediction, HMDB, GNPS, molecular networking
- **Deepening the understanding of biological aspect of metabolism**
- **'Correctly' identify the identifiable compounds:**
  strict experimental conditions, in-house database (lipidomics)

Materials & Methods: highlights

- 2 sets of **LC-MS** system were used; 2 **LC methods** were used (7 min, 10 min)
- 2 fractions of whole grain barley powder: **bran (brownish) from outlayer**, **endosperm (white) from inner**

LC-MS analysis of Whole Grain Barley

Negative mode



Bran (**top**) vs Endosperm (**bottom**) powder chromatograms

Same pattern. Retention time shift within 0.02 min. Higher intensities of **bran** powder in some peaks resulted from (1) experimental error (2) 'whole grain' dissolved more in ethanol/water.

Data Preprocess

Mode	Number of feature detected
positive	1719
negative	3304

Possible reasons for more features were detected in negative mode

- Negative mode had better resolving power
- Metabolites from urine (e.g. glucoronate conjugates) were easier ionized in negative mode.

PCA modeling

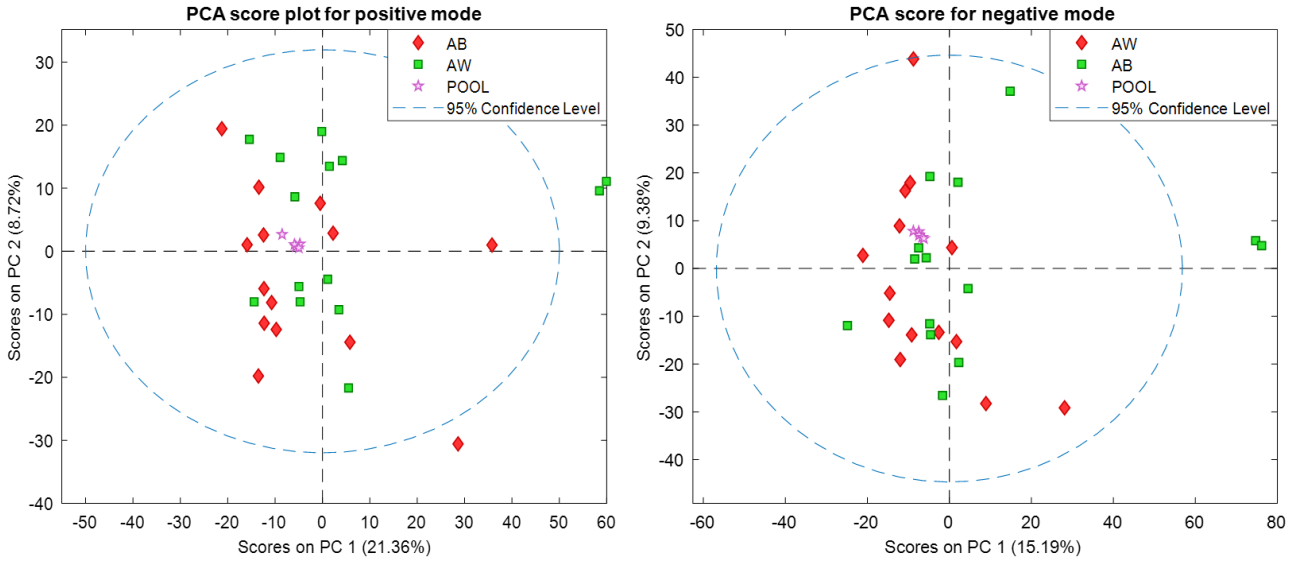


Figure 2: PCA score plot (AW= After Wheat, AB= After Barley, POOL= pooled samples)

- AB & AW were not separated in score plots
- POOL located tightly near the center: high quality data
- Outliers: because of too concentrated urine (high intensities of all variables)

PLSDA Modeling and Variable Selections

Mode	Variables selected (out of)
positive	72 (1719)
negative	86 (3304)

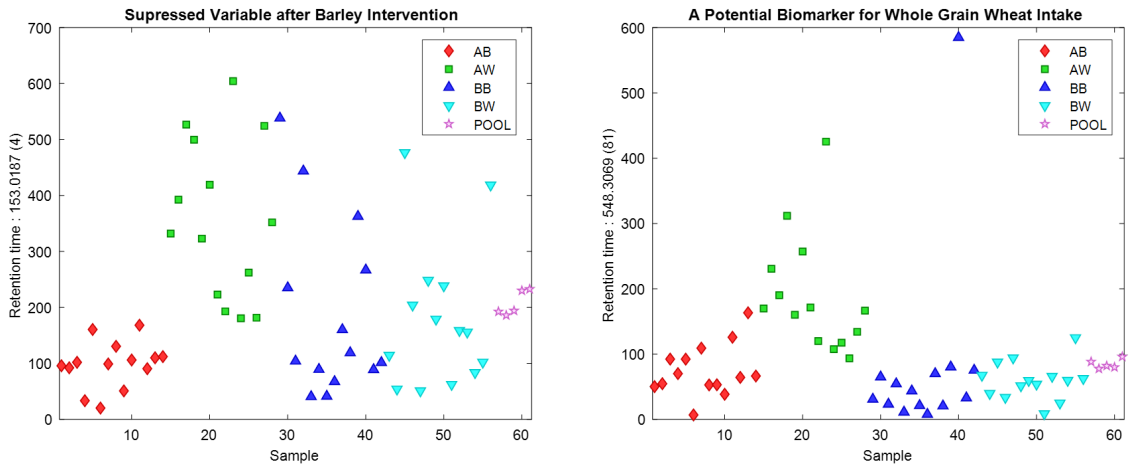
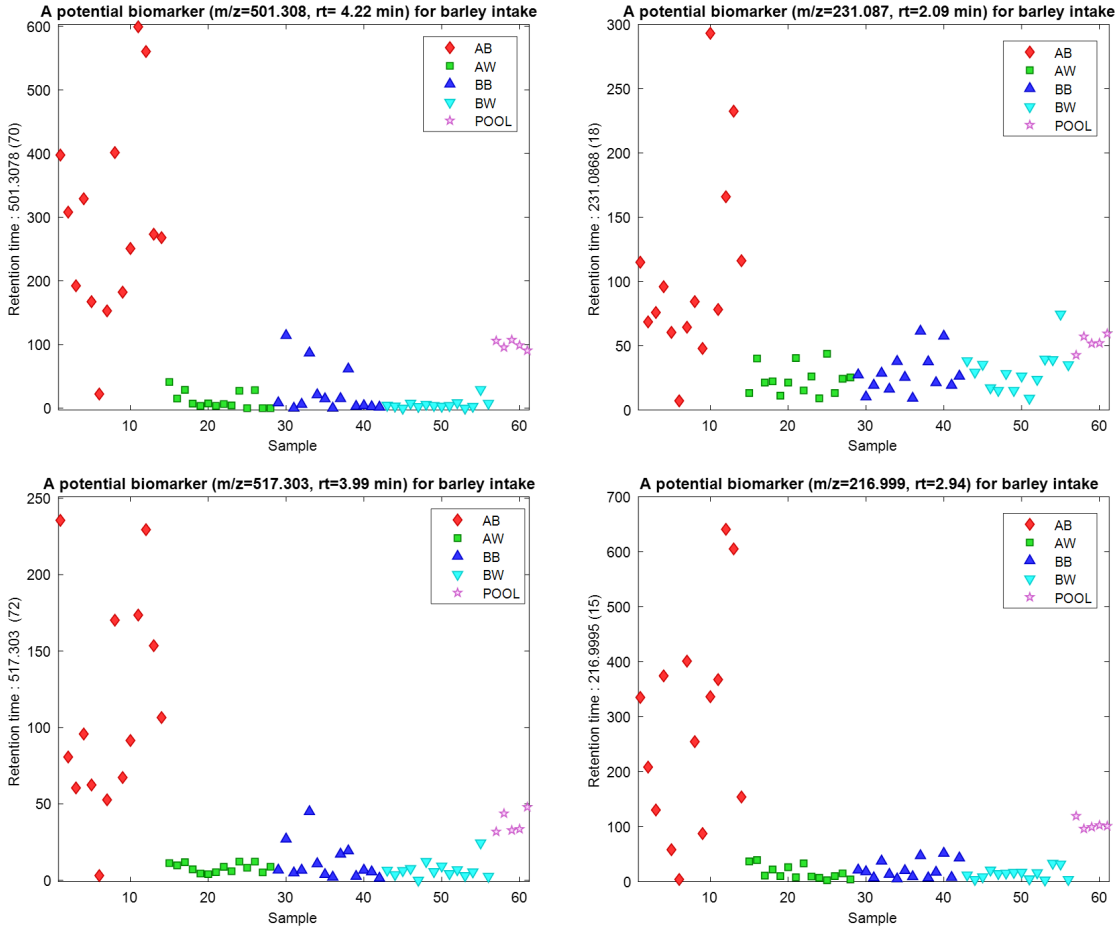


Figure 3: Variables Selected by PLSDA modeling but not classified as potential barley intake biomarkers

- (left) Intensities suppressed after barley intake; could be an endogenous metabolite
- (right) wheat intake biomarker

PLSDA Modeling and Variable Selections



- **Role of thumb**
- Nearly no background signals
- Increased intensities after barley intake

PLSDA Modeling and Variable Selections

Mode	Potential Biomarkers selected for MS/MS
positive	1 (m/z= 291)
negative	5 (m/z= 517, 501, 775, 216)

MS/MS and Identification: m/z 231.0870 (ESI-)

Monoisotopic mass (Da)	Formula	Deviation (ppm)
232.0947	$C_{10}H_{16}O_6$	2
232.0888	$C_{17}H_{12}O$	24
232.10	$C_{16}H_{12}N_2$	25
232.0848	$C_{12}H_{12}N_2O_3$	41
232.1059	$C_9H_{16}N_2O_5$	50
232.1099	$C_{14}H_{16}O_3$	68

Notes: Monoisotopic mass and formula were calculated on neutral molecular. Deviation was calculated based on deprotonated form (ESI^-).

Figure 4: Possible formulas of ion m/z 231.0870

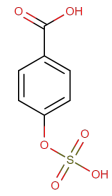
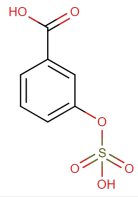
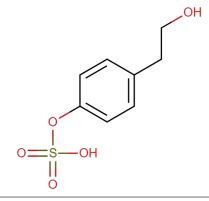
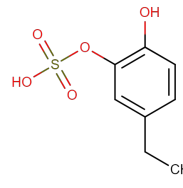
Intensity was too low for MS/MS

MS/MS and Identification: m/z 775.3401 (ESI-)

- Dimer of $[C_{18}H_{28}O_9\text{-}H]^-$ with neutral mass 388.1735
- Intensity was too low for MS/MS

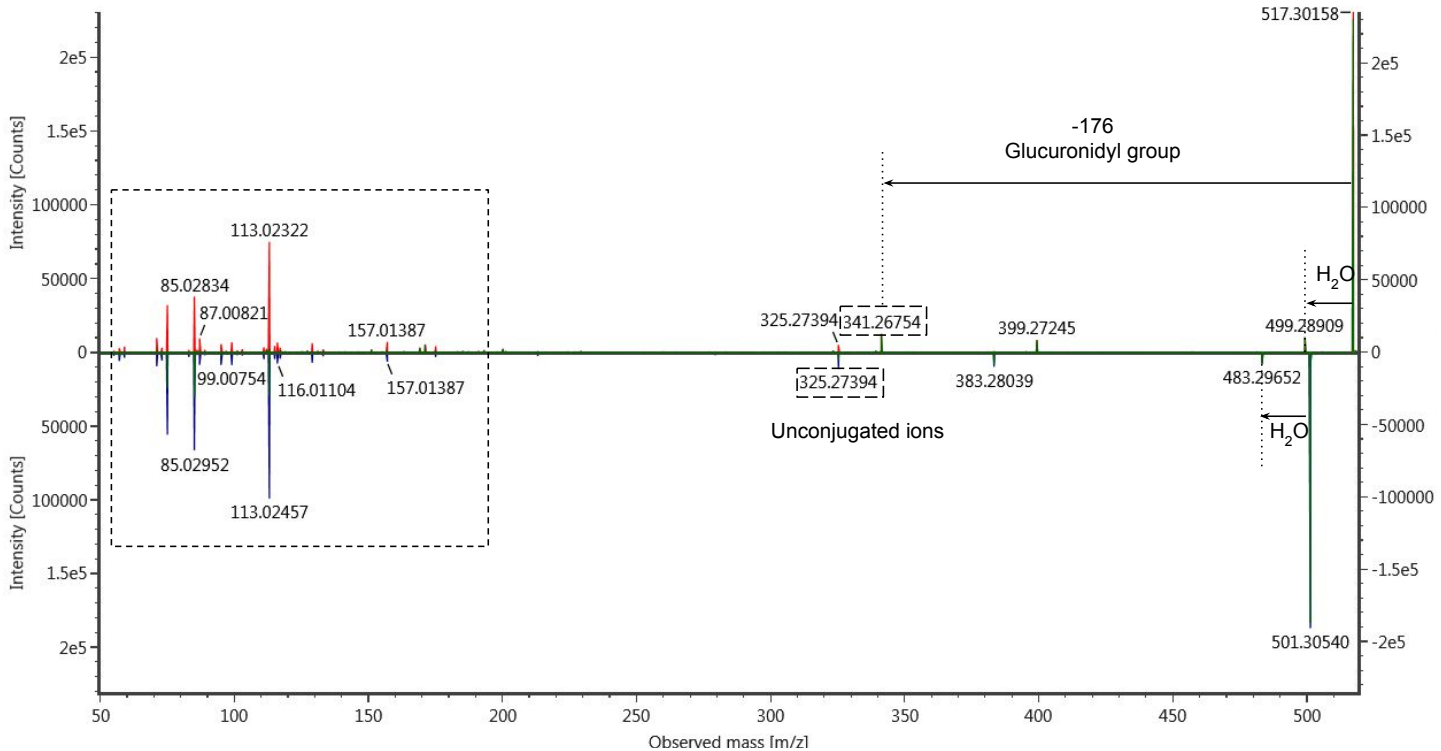
MS/MS and Identification: m/z 216.9995 (ESI-)

- Intensity was too low for MS/MS
- Feasible hints exist in HMDB. Whole grain barley riches in benzoic acid, phenol and polyphenol

Monoisotopic mass(Da)	Formula	Name	Structure	Food Source
217.9885	$C_7H_6O_6S$	4-hydroxybenzoic acid-4-O-sulphate		Tea (metabolite of benzoic acid)
		3-hydroxybenzoic acid-3-O-sulphate		Tea (metabolite of benzoic acid)
218.0248	$C_8H_{10}O_5S$	Tyrosol 4-sulfate		Virgin olive oil (metabolite of polyphenols or phenols)
		(5-ethyl-2-hydroxyphenyl)oxidanesulfonic acid		Predicted (metabolite of polyphenols or phenols)

MS/MS and Identification: m/z 517.3030, 501.3080 (ESI-)

- $C_{30}H_{46}O_7$ and $C_{30}H_{46}O_6$
- Glucuronide conjugates of barley metabolites
- Common lost of glucuronidyl group (-176)
- Unconjugated ions detected (NOT identifiable)
- Low-mass region interfered by 2nd fragmentation of glucuronyl group



MS/MS and Identification: m/z 517.3030, 501.3080 (ESI-)

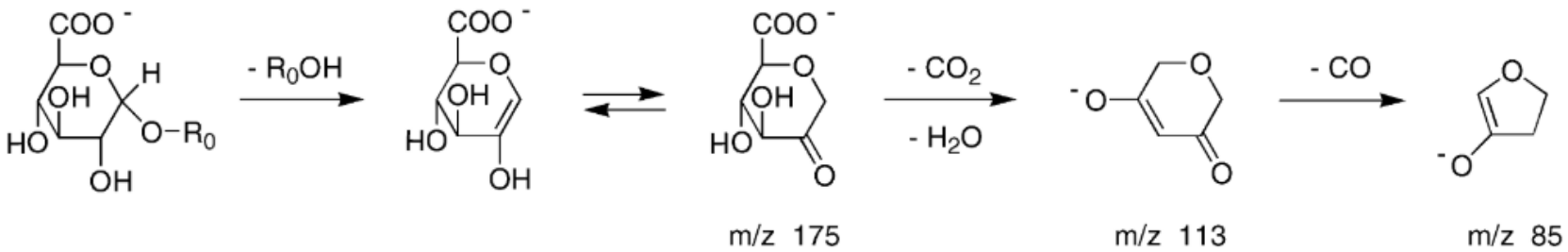


Figure 5: Secondary fragmentation of the glycuronyl moiety in negative-ion MS/MS spectra of glucuronide conjugates.

MS/MS and Identification: m/z 291.2683 (ESI+)

- Originated from ‘whole grain part’ (bran)
- Same structure detected in urine and barley samples
- Annotated as phytosterol or its derivative or its in-source fragment

MS/MS and Identification: m/z 291.2683 (ESI+)

Originated from 'whole grain part' (bran)

- **Intensity higher in bran than endosperm**
- MS/MS showed same structure

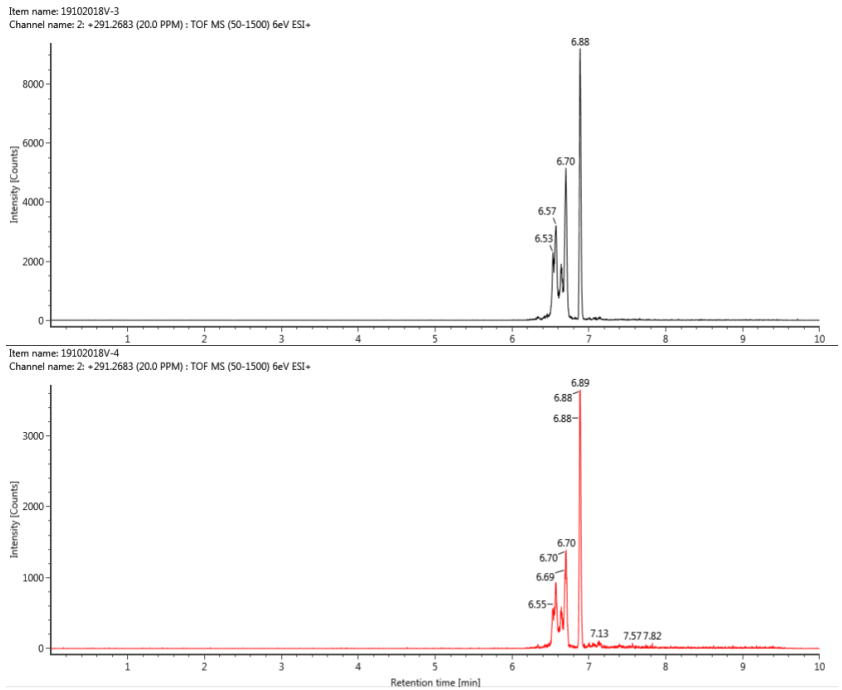


Figure 6: Chromatograms of ion(m/z=291.2683) in bran powder and endosperm powder. Top (bran); bottom (endosperm).

MS/MS and Identification: m/z 291.2683 (ESI+)

Same structure detected in urine and barley samples

- Similar retention time (min) (barley:6.88, urine:6.71)
- Same MS/MS pattern

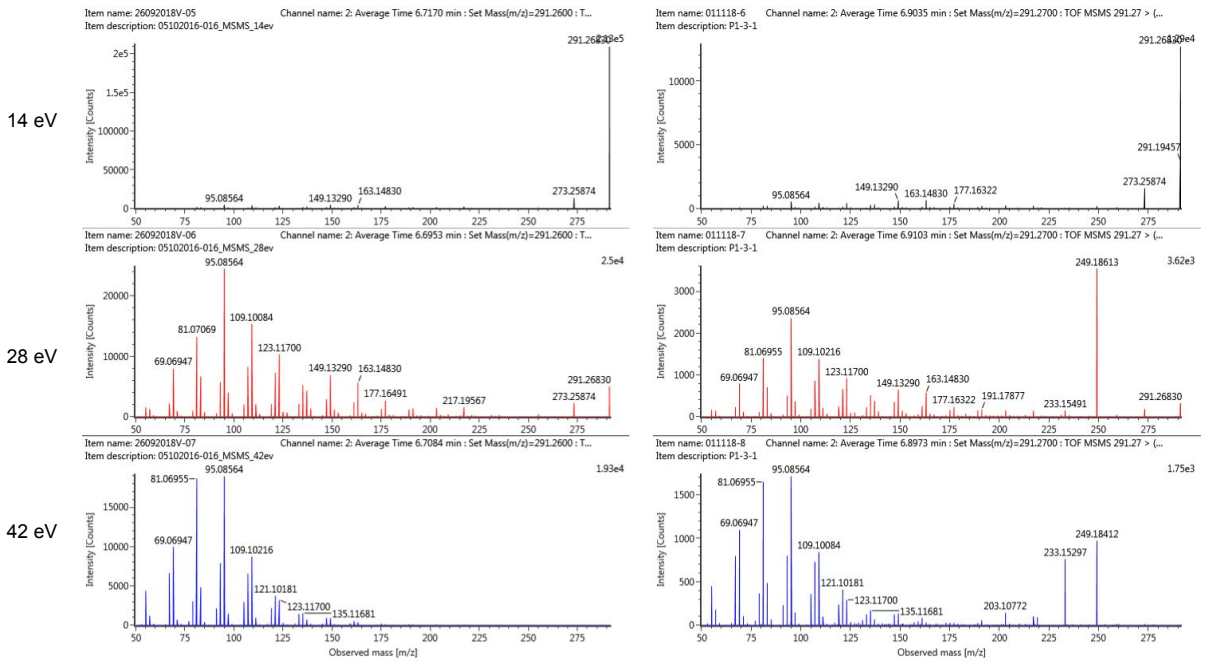


Figure 7: MS/MS spectra of ion (m/z=291.2683) in urine and barley sample with different collision energies

MS/MS and Identification: m/z 291.2683 (ESI+)

Putatively annotated as sterol (or its derivative)

- **MS/MS pattern** matches common sterols
- However, too low mass compared with common sterols

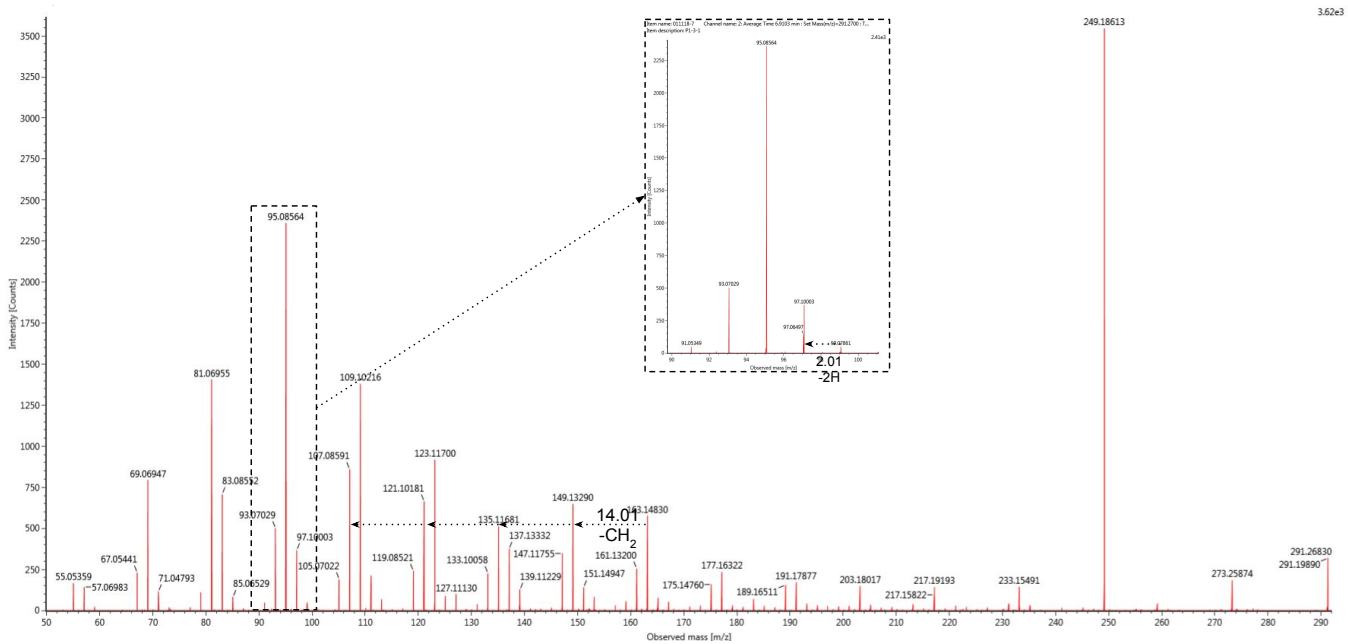
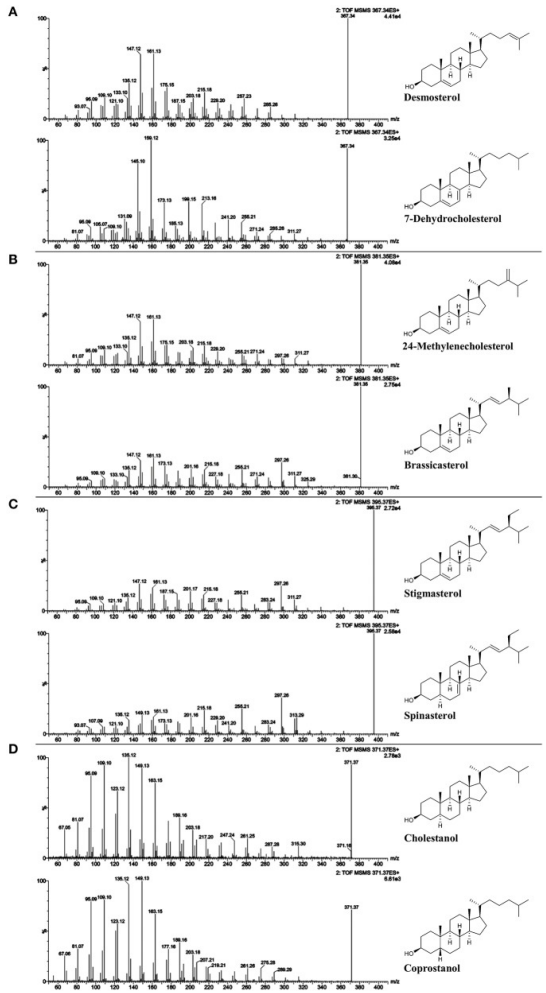


Figure 8: MS/MS spectra of ion(m/z=291.2683) in barley sample (Collision energy = 24 eV)

MS/MS and Identification: m/z 291.2683 (ESI+)

Putatively annotated as sterol (or its derivative)

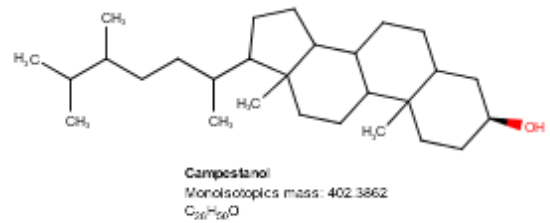
- MS/MS pattern matches **common sterols**



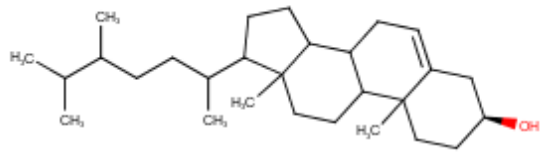
MS/MS and Identification: m/z 291.2683 (ESI+)

Putatively annotated as sterol (or its derivative)

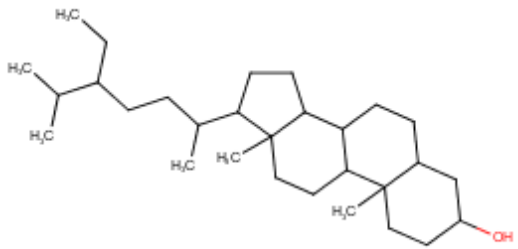
- MS/MS pattern matches common sterols
- However, too low mass compared with **common sterols**



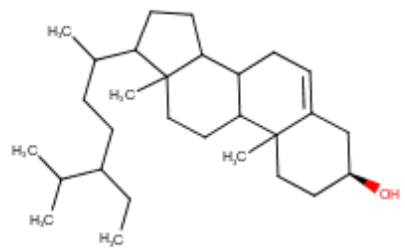
Campestanol
Monoisotopics mass: 402.3862
$C_{28}H_{50}O$



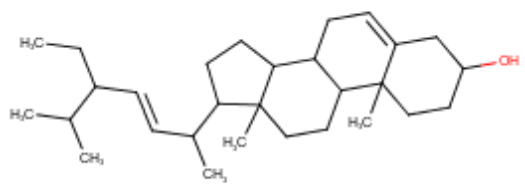
Campesterol
Monoisotopics mass: 400.3705
$C_{28}H_{48}O$



Sitostanol (stigmastanol)
Monoisotopic mass: 416.402
$C_{29}H_{52}O$



Sitosterol
Monoisotopic mass: 414.3862
$C_{29}H_{50}O$



Stigmasterol
Monoisotopic mass: 412.371
$C_{29}H_{48}O$

MS/MS and Identification: m/z 291.2683 (ESI+)

Putatively annotated as sterol (or its derivative)

- MS/MS pattern matches common sterols
- However, too low mass compared with **common sterols**

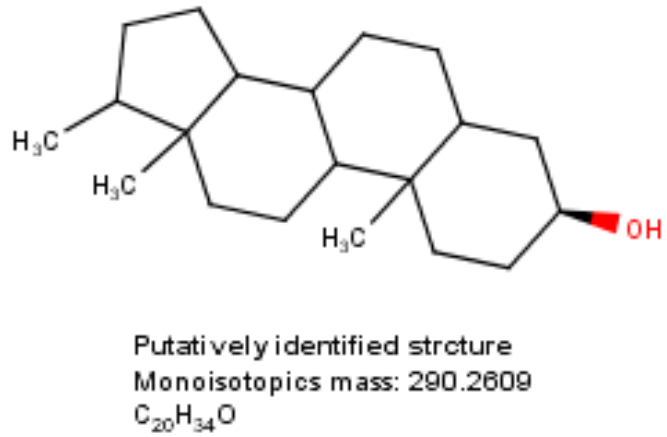


Figure 10: Putatively identified structure

Conclusions & Discussions

summary of identification									
no	m/z	rt (vion qtof)	rt (qtof)	ms/ms fragments	neutral formula	annotation	mode	detected in	origins
1	517.3030	6.48 6.52	4.22	116.0096 224.0600	$C_{30}H_{46}O_7$	[Unknown1+O+glucuronate-H]-	ESI-	urine	phase II metabolites (glucoronate conjugate)
	341.2675	N/A			$C_{20}H_{38}O_4$	[Unknown1+O]-	ESI-	N/A	
	343.2815					[Unknown1+O+H]+	ESI+	N/A	
2	501.3080	6.70	3.99	116.0110 169.1222 171.1372 325.2739	$C_{30}H_{46}O_6$	[Unknown1+glucuronate-H]-	ESI-	urine	phase II metabolites (glucoronate conjugate)
	325.2739	N/A			$C_{20}H_{38}O_3$	[Unknown1-H]-	ESI-	N/A	
	327.2879					[Unknown1+H]-	ESI+	N/A	
3	775.3401	N/A	3.78	N/A	$C_{36}H_{56}O_{18}$	[2Unknown3 - H]-	ESI-	urine	
	387.1666	N/A	3.78	N/A	$C_{18}H_{28}O_9$	[Unknown3]-	ESI-	urine	
4	216.9995	5.31	2.94	N/A	$C_7H_6O_8$	[M - H]+	ESI-	urine	metabolites of polyphenol/phenol
5	231.0870	N/A	2.10	N/A	Figure 5	[M - H]+	ESI-	urine	
6	291.268	6.71	4.21	69.06947 81.07079 95.08564 109.10084 123.117	$C_{20}H_{34}O$	[M + H]+	ESI+	urine & barley	metabolites of sterol

Conclusions & Discussions

Metabolome of phytochemicals could indicate plant-source food intake

- Annotated compounds appeared to be phytochemicals (and their metabolites).
- Identification, quantification and database of phytochemicals NOT mature.
- More phytochemicals could be studied.

Phytochemicals

- a general term for chemicals produced by plants
- functions for human not clarified
- varied a lot of between species
- metabolome of plants, controlled by plants' gene expression

Perspectives (Next step/6 month)

- Barley - Urine/Plasma ; Wheat - Urine/Plasma
- Get hints from other dataset (Mediterranean, New Nordic)
- More perspectives
  - **Foodomics (Foodome)**
  - New concept initiated from 2009; even more 'emerging' than metabolomics
  - Omics methods for food
  - We have a lot of data of food (barley and wheat's MS profile)
  - **Be critical**

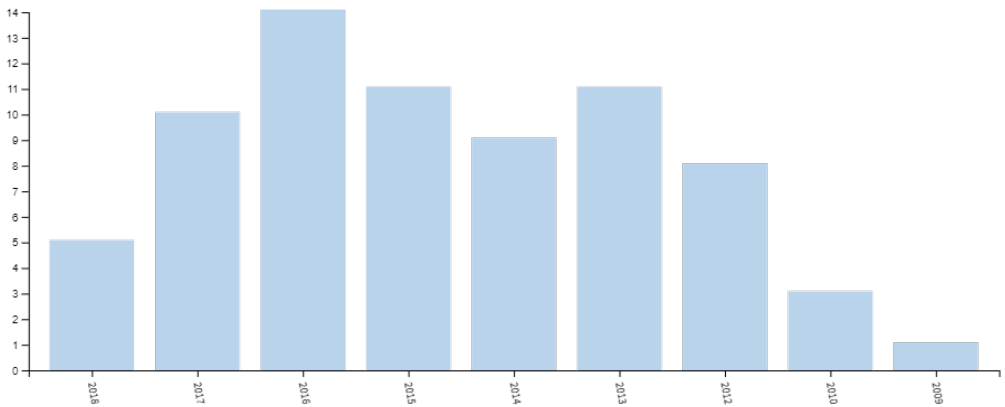


Figure 12: Articles with 'foodomics' and 'foodome' as topic in Web of Science

Perspectives (Next step/6 month)

- **Bioinformatics Tools (open source)**
- MetaboDiff (comparative metabolomics) - R package
- MAIT (Metabolite Automatic Identification Toolkit) - R package

Acknowledgements

My supervisor Gözde.
Henrik, Lars
All fellows and technicians