

Application of LC-MS/MS metabolomics - Quantitative high- throughput method for direct measurements of enterolactone glucuronide, sulfate and free enterolactone

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Enterolactone was discovered in 1980 (article in *Nature*)

Setchell KD, Lawson AM, Mitchell FL, Adlercreutz H, Kirk DN, Axelson M.

In our laboratories, for several years, two phenolic compounds have been detected during gas chromatographic-mass spectrometric analysis of urinary steroid extracts from human and

animal

1. Enterolactone

yl (TMS) ether

derivative

2. Enterodiol

e possibility that

they were a

udies with blanks,

by varying the extraction method and because they were present almost exclusively as

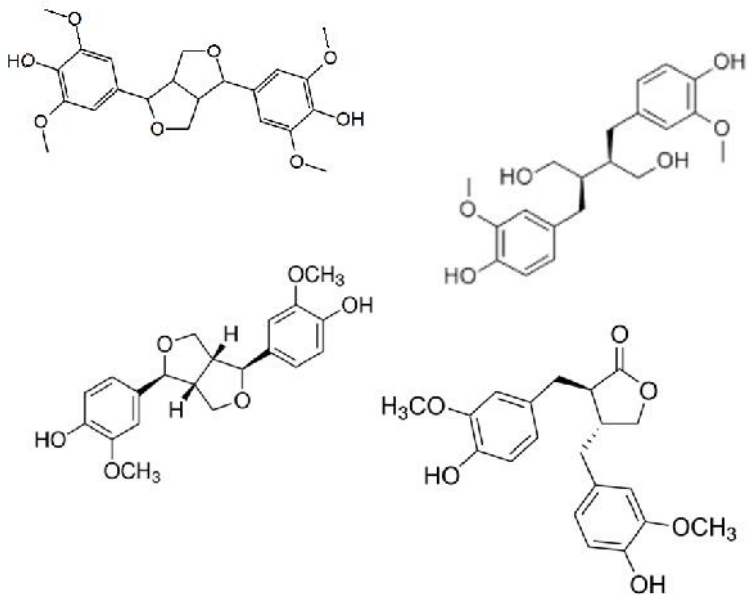
conjugates of glucuronic acid. Several of the general characteristics of the unknown

compounds were reported after one (referred to as compound 180/442) was found to have a

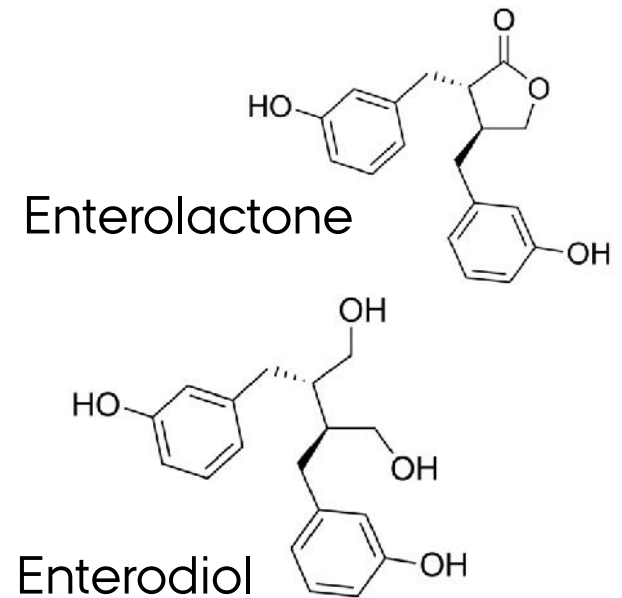
cyclic pattern of excretion during the menstrual cycle of an adult vervet monkey (Fig. 1). ...₂

Phytoestrogens - lignans

Plant lignans



Enterolignans (mammalian lignans)

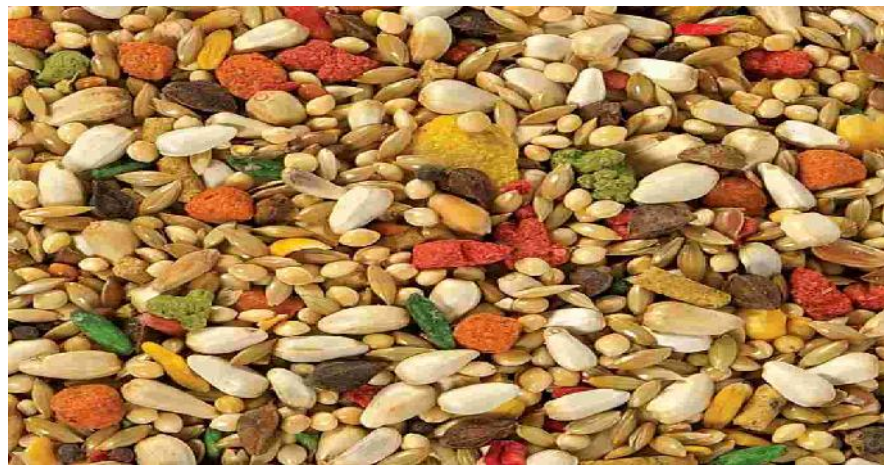


Field of Nutrition/ diseases prevention

Plant lignans are found in different plant based foods



Fruits
Vegetables
Nuts and seeds
Cereals (whole grain)



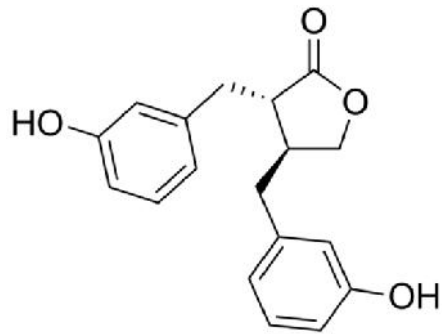
What is healthy about fruits, vegetables, nuts, seeds and cereals

- Dietary fiber → lignans
- Minerals and vitamins
- Unsaturated fatty acids
 - Proteins

Enterolactone: facts and hypotheses

1. Enterolactone is a lignan coming from metabolism of plant lignans in the gastrointestinal tract of mammals
2. Epidemiological studies have indicated that diets high in dietary fiber, containing among other things lignans, are associated with positive health effects.
It is important to find out the role of enterolactone in diseases prevention
3. Plant lignans and especially their metabolite enterolactone, is one of the contributors to the positive health effects
4. Has structural similarity to steroid hormones and has been considered to be protective against hormone-dependent cancers

Danish study
Diet Cancer and Health Cohort
Danish Cancer Society Research Center
6000 participants



Enterolactone

survival
→

After diagnosis:
Breast, prostate and colorectal cancers
CVD
Type 2 diabetes

Enterolactone concentration in plasma and survival
Biomarker of healthy life style

Usual way to measure enterolactone

Enzymatic hydrolysis for 19 hours
Total concentration of enterolactone

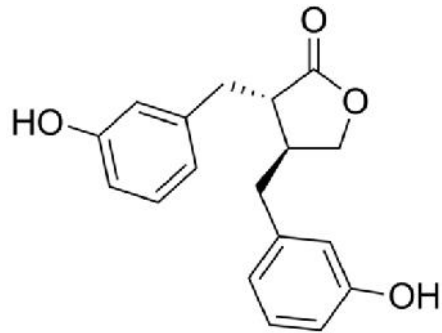


Extraction SPE or LLE



HPLC, LC-MS/MS or fluoroimmunoassay

Enterolactone and conjugation



Enterolactone

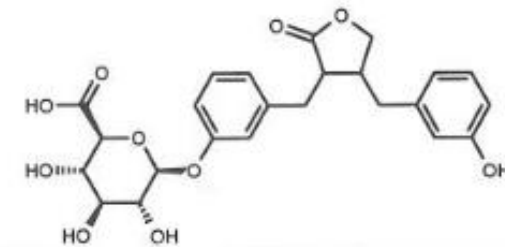
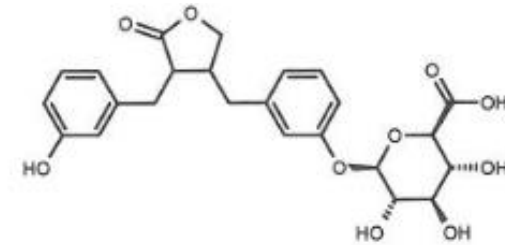
conjugation



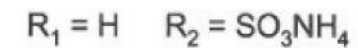
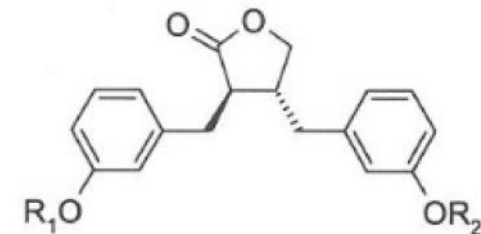
In plasma and urine app. 95 % enterolactone glucuronide, 4 % enterolactone sulfate and 1 % is free enterolactone

Enterolactone can be measured as free or conjugated

(±)-Enterolactone mono-β-D-glucuronide
Mixtures of Regioisomers



(±)-Enterolactone monosulfate
Mixtures of Regioisomers

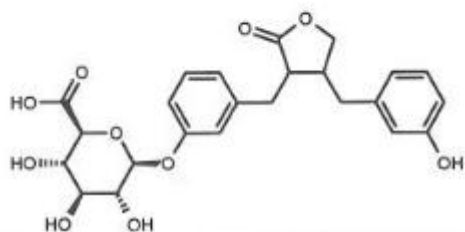
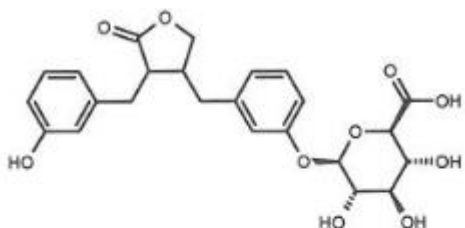


Advantages of measuring enterolactone in intact forms

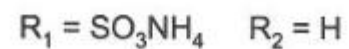
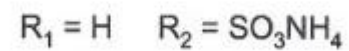
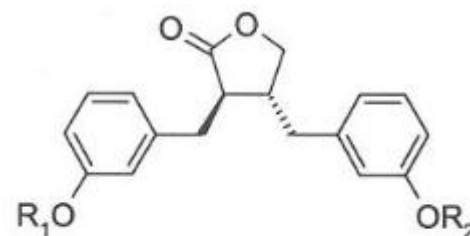
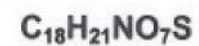
1. Simple sample preparation (save time)
2. High throughput methods
3. No modification of the sample
4. Better ionization/higher sensitivity
5. Higher stability of standards

New standards

(±)-Enterolactone mono-β-D-glucuronide
Mixtures of Regioisomers



(±)-Enterolactone monosulfate
Mixtures of Regioisomers



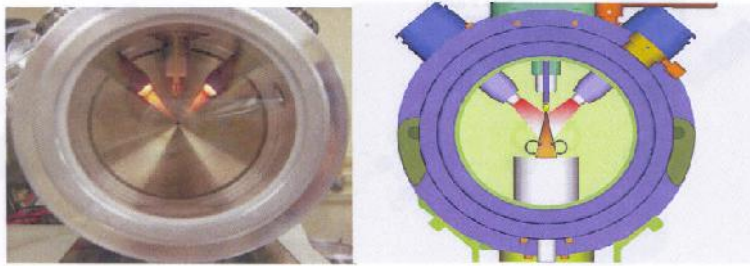
reseaLIFE
chem SCIENCE

Mass Spectrometric optimization

	Q1 mass	Q3 mass	DP	CE	CEP
Enterolactone	297.1	253.1	-140	-26	-21
Enterolactone glucuronide	473.1	297.1	-25	-34	-25
Enterolactone sulfate	377.1	297.1	-55	-32	-13

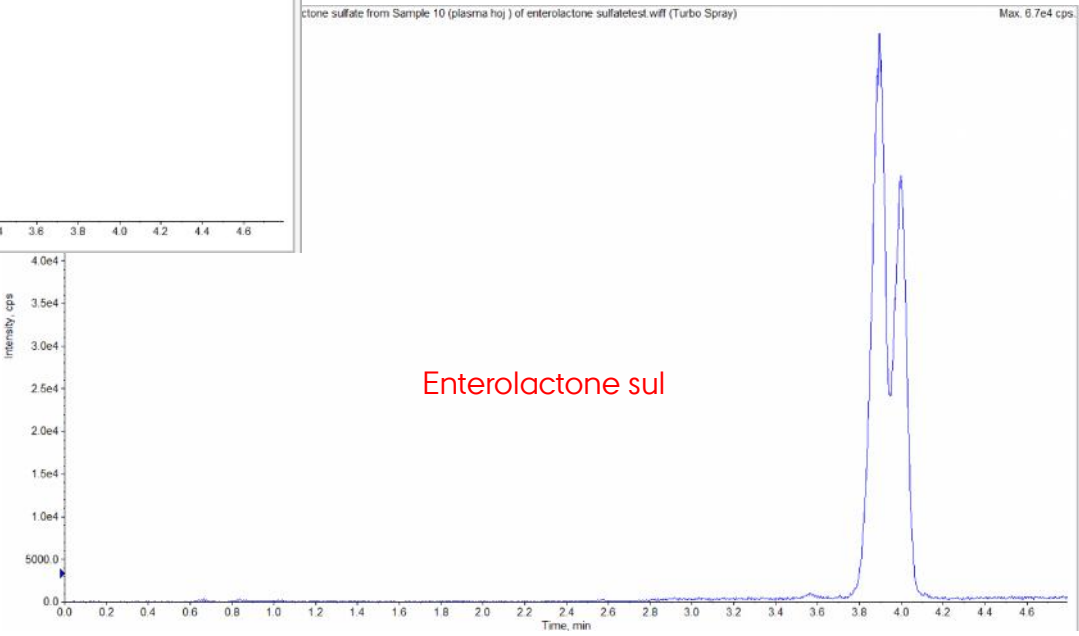
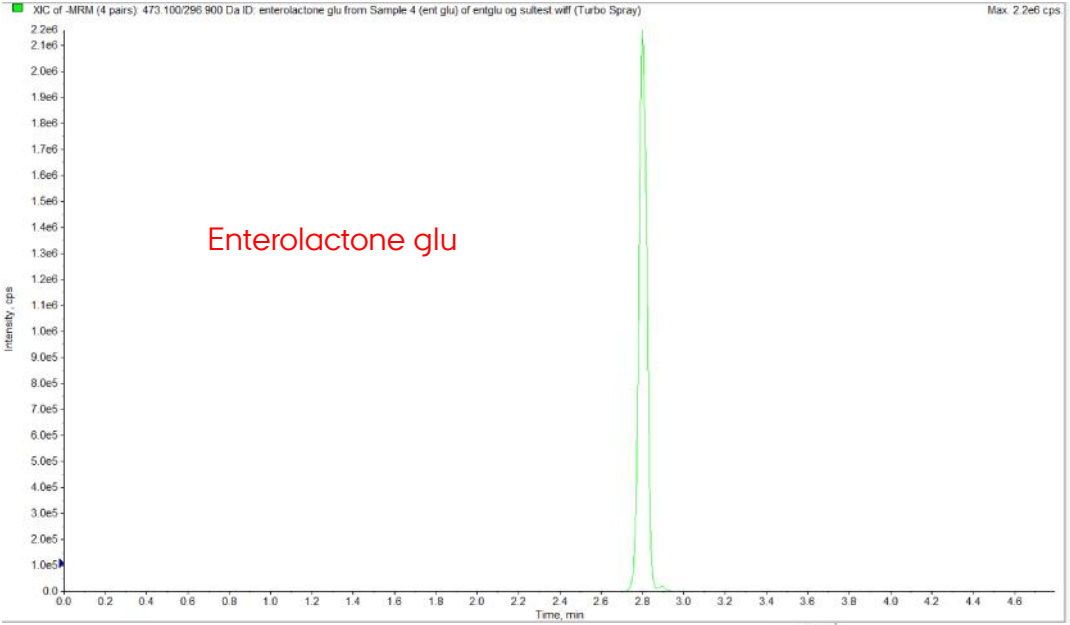
AB SCIEX

Turbo V™ Source: Turbo Gas Heaters

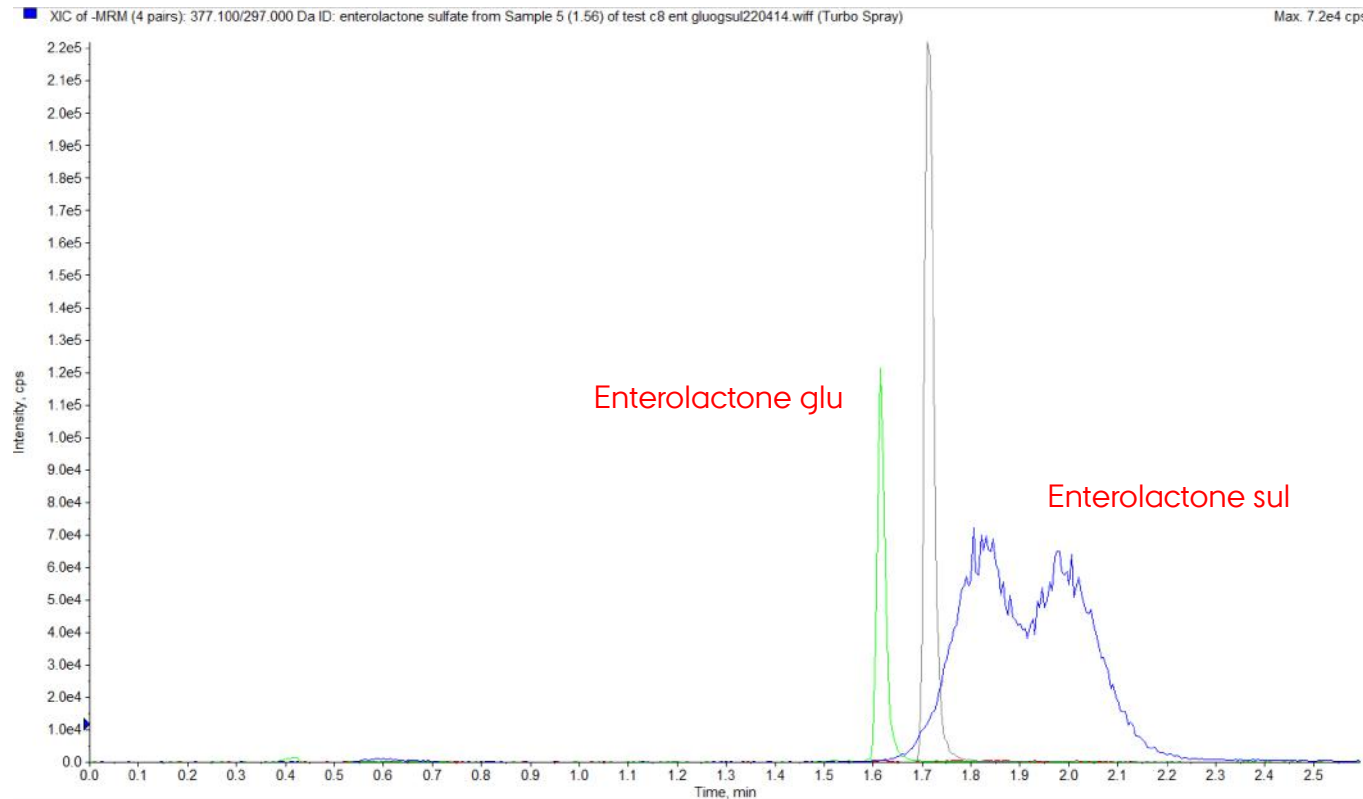


Curtain gas	GS1	GS2	Temperature	Ion Spray Voltage
20	50	20	300	-4500

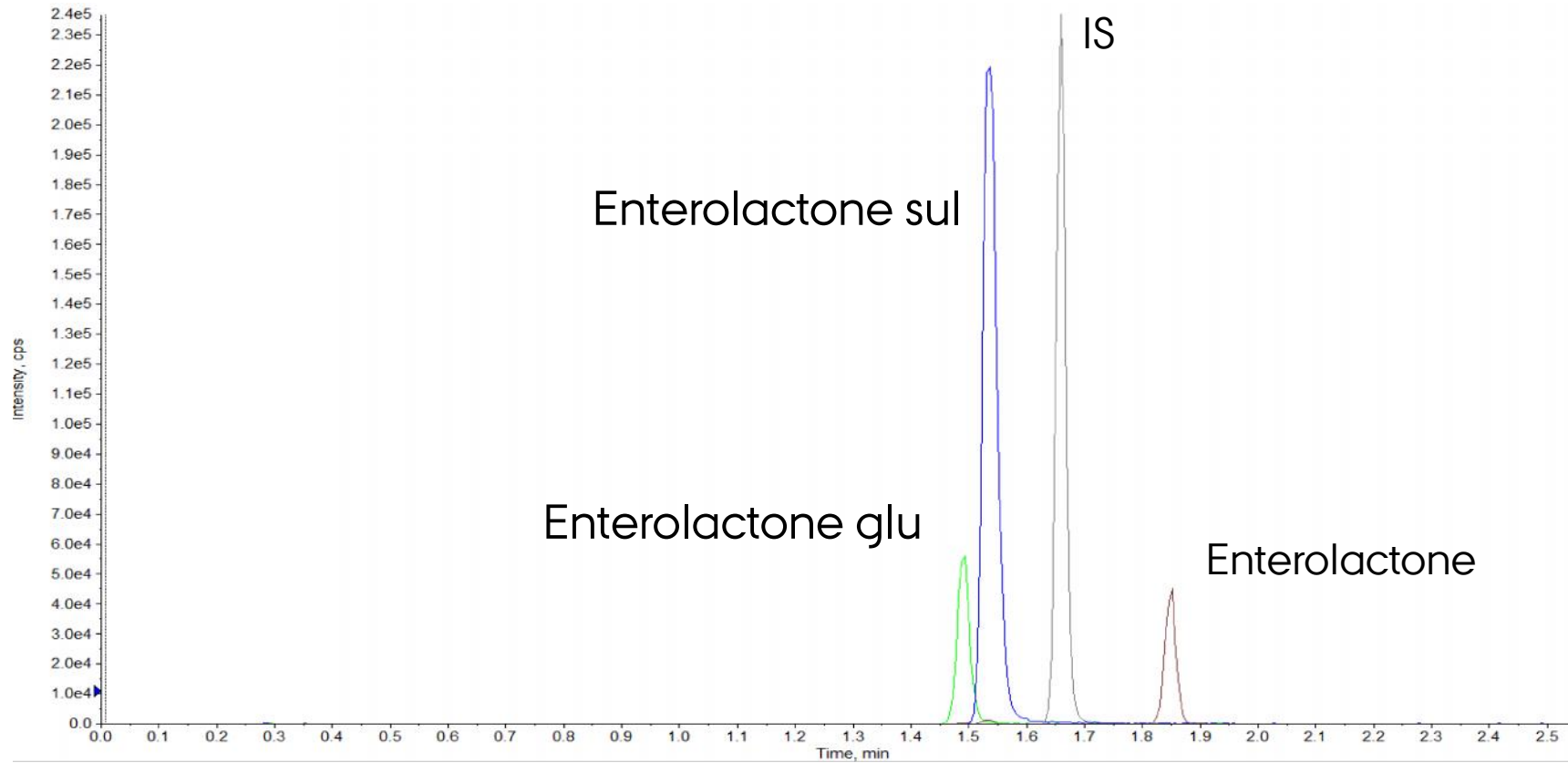
Chromatographic method C 18 column



Chromatographic method C 8 column

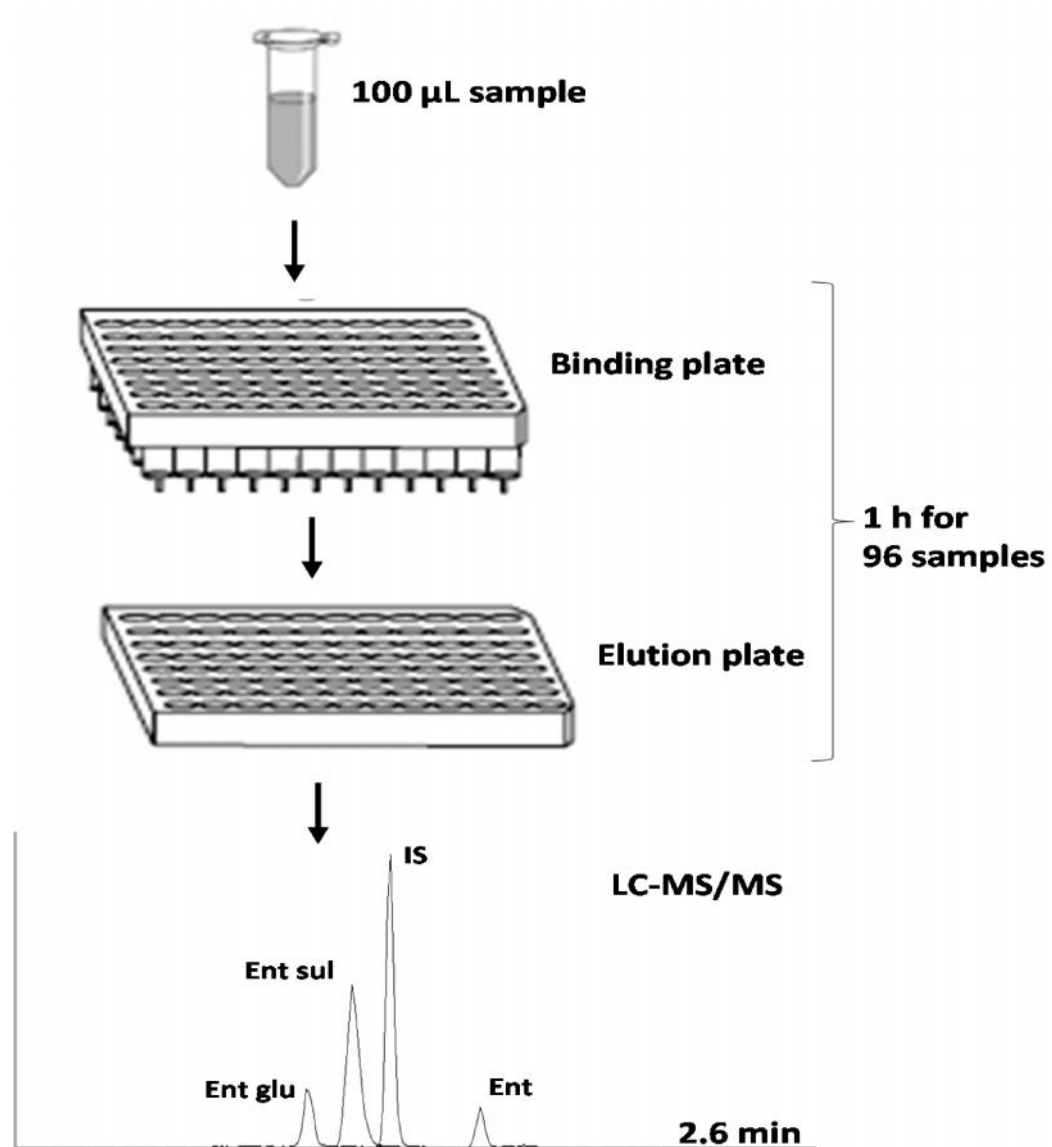


Chromatographic method: Phenyl column



Chromatographic run 2.6 min

Sample preparation (plasma)



Method validation

All the methods were validated according to the guidelines of Food and Drug Administration (FDA)

1. Accuracy
2. Precision
3. Recovery
4. Matrix Effects
5. Linearity
6. Low Limits of Quantification (LLOQ)
7. Selectivity
8. Inter-batch reproducibility
9. Analyte Stability

Method validation

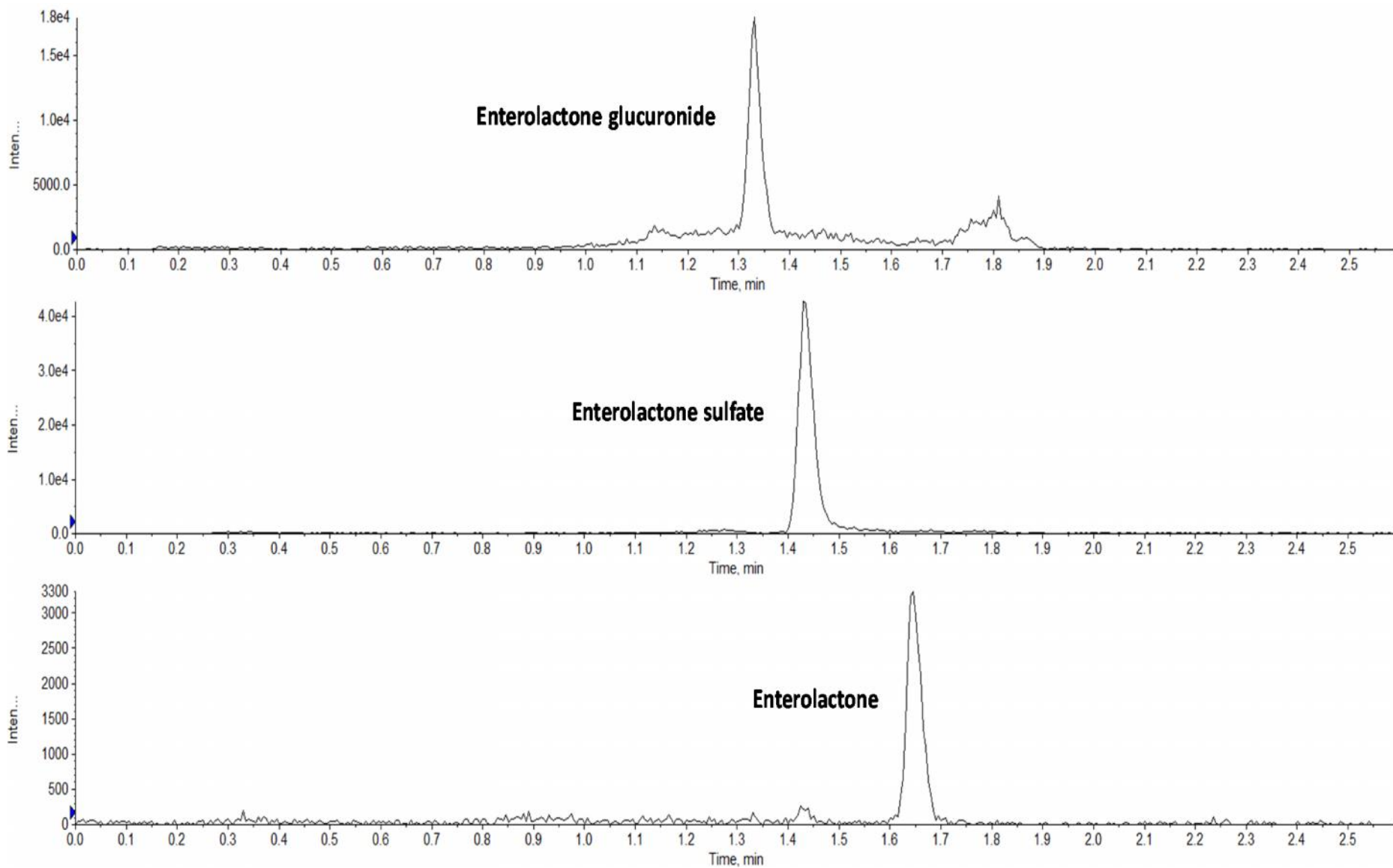
1. QC samples spiked with low (0.0977 ng/mL), medium (1.56 ng/mL) and high (6.25 ng/mL) concentration of standards
2. QC samples represented the matrix validated with the lowest possible concentration of lignans
3. IS was isotope-labeled glycocholic acid and was used to validate matrix effects and overall instrument performances

Results: accuracy, precision, recovery, inter-batch, LLOQ

	Accuracy (RE %)	Precision (RSD %)	Recovery %	Inter-batch (RSD %)	LLOQ pg/mL
Enterolactone	< 11	< 8	110 - 102	< 5	24.4
Enterolactone glu	< 8	< 8	88 - 100	< 7	12.2
Enterolactone sul	< 13	< 4	67 - 100	< 9	6.1

Concentrations spiked: LOW, MEDIUM and HIGH

Selectivity (spiked 97.7 pg/mL): plasma



High-Throughput LC–MS/MS Method for Direct Quantification of Glucuronidated, Sulfated, and Free Enterolactone in Human Plasma

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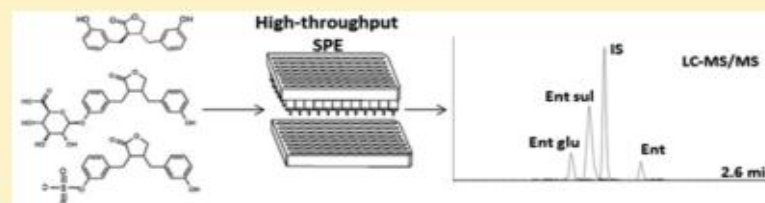
[‡]Danish Cancer Society Research Center, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

Supporting Information

ABSTRACT: Sulfation and glucuronidation constitute a major pathway in humans and may play an important role in biological activity of metabolites including the enterolignan, enterolactone. Because the aromatic structure of enterolactone has similarities to steroid metabolites, it was hypothesized that enterolactone may protect against hormone-dependent cancers. This led to numerous epidemiological studies. In

this context, there has been a demand for rapid, sensitive, high-throughput methods to measure enterolactone in biofluids. Different methods have been developed using GC–MS, HPLC, LC–MS/MS and a fluoroimmunoassay; however, most of these methods measure the total concentration of enterolactone, without any specification of its conjugation pattern. Here for the first time we present a high-throughput LC–MS/MS method to quantify enterolactone in its intact form as glucuronide, sulfate, and free enterolactone. The method has shown good accuracy and precision at low concentration and very high sensitivity, with LLOQ for enterolactone sulfate at 16 pM, enterolactone glucuronide at 26 pM, and free enterolactone at 86 pM. The short run time of 2.6 min combined with simple sample clean up and high sensitivity make this method attractive for the high-throughput of samples needed for epidemiological studies. Finally, we have adapted the new method to quantify enterolactone and its conjugates in 3956 plasma samples from an epidemiological study. We found enterolactone glucuronide to be the major conjugation form and that conjugation pattern was similar between men and women.

KEYWORDS: enterolactone glucuronide, enterolactone sulfate, free enterolactone, LC–MS/MS, human plasma, method



Further development and improvements of the method

1. Enterodiol (free, glucuronide and sulfate)
2. Internal standards
3. Encrease the volume of sample due to low concentration of the free enterolactone in the plasma
4. Possibility to measure other compounds as glucuronides and sulfates for eksempel steroid hormones

Danish study

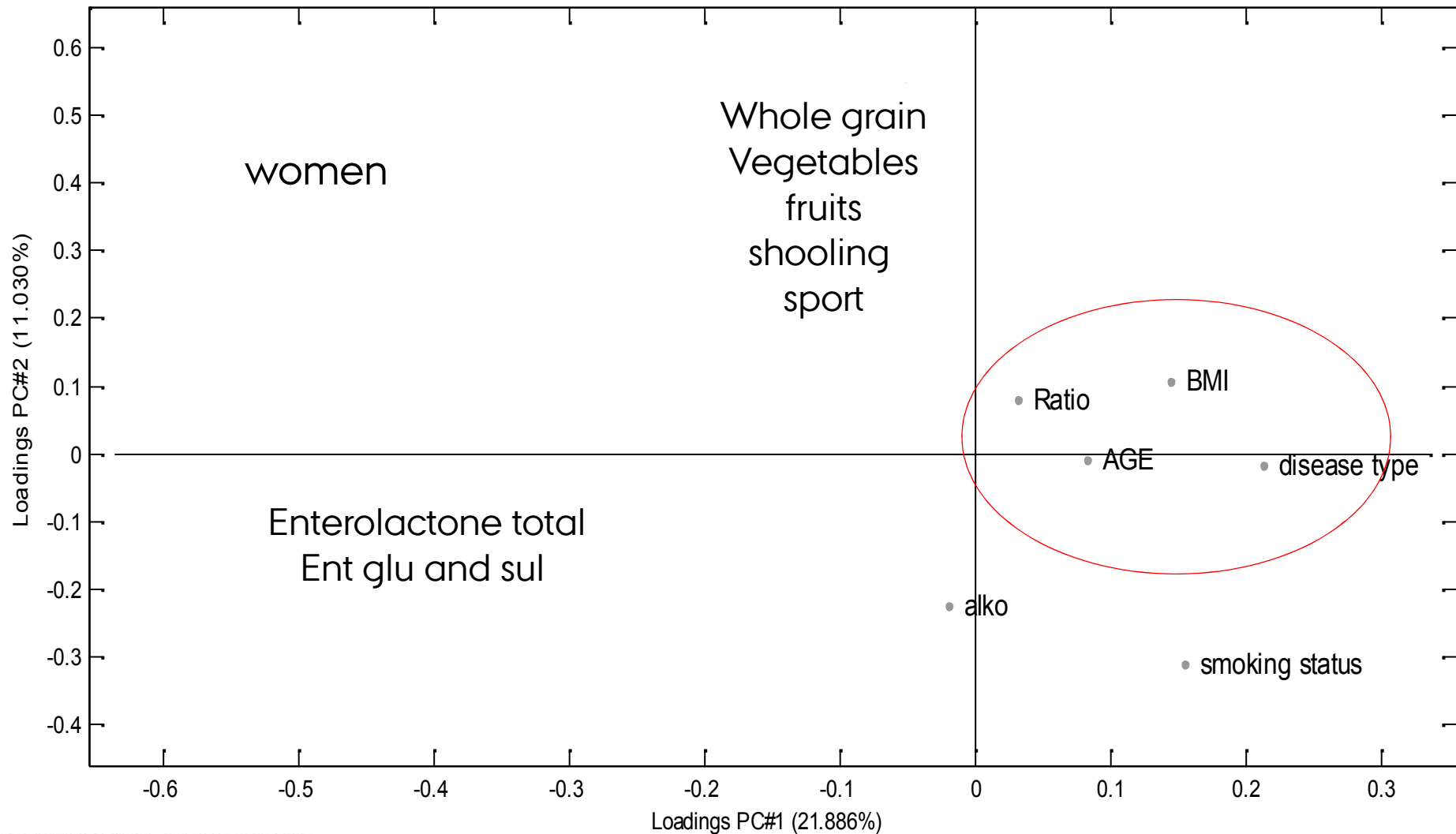
Diet Cancer and Health Cohort

Danish Cancer Society Research Center

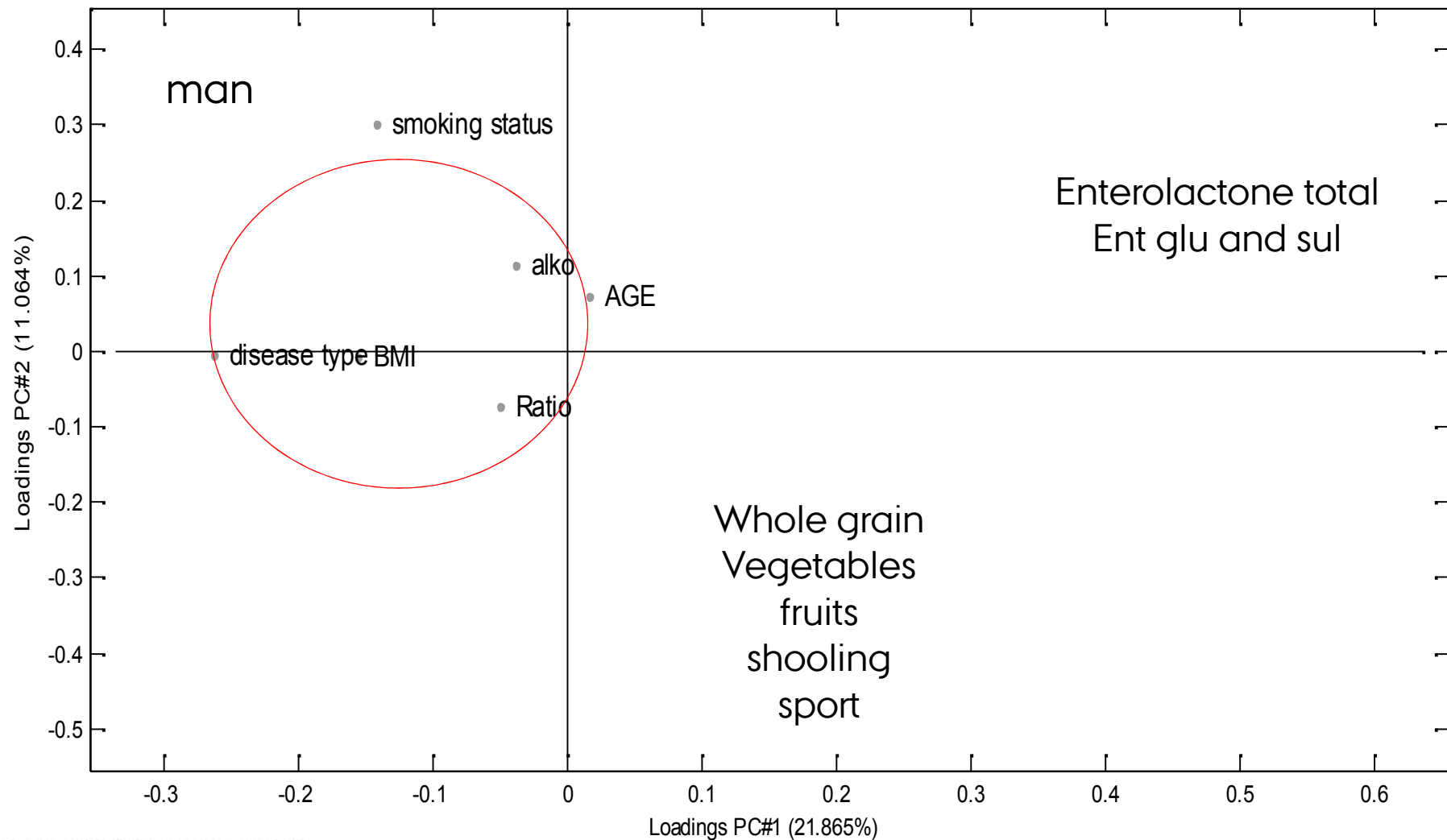
6000 participants

1. BMI and Age
2. alcohol use (g/day) **lifestyle and dietary information**
3. smoking status (former, current, never)
4. sport (yes/no) **Principal Component Analysis**
5. schooling (low, middle, high)
6. whole-grain intake (g/day)
7. intake of fruits and vegetables (g/day)
8. Diagnosis: cancer, CVD and type 2 diabetes
9. Total concentration of enterolactone
10. Concentration of ent glu and ent sul
11. Ration (ent sulfat/ent total) conjugation pattern

Results → Epidemiological study Danish Diet Cancer and Health Cohort



Results → Epidemiological study Danish Diet Cancer and Health Cohort



Conclusion

Application of LC-MS/MS was very useful → possible to measure enterolactone as free and conjugated → **novel** information on conjugation pattern of enterolactone and correlation between conjugation pattern of enterolactone and metabolic state of the body

1. We hope that measuring enterolactone in its intact forms will contribute with new knowledge on the role of enterolactone in human health
2. Since the method is rapid and easy to perform it can be used for high-throughput of samples and therefore will be the method of choice for future epidemiological studies and clinical diagnosis

PhD Course (October/November)

PhD course at Department of Animal Science 30 October – 3 November 2017

Hands-on targeted and untargeted LC-MS metabolomics with emphasis on measuring phytochemicals in plasma and urin

Level of course: Master, PhD and Postdoc



Aknowledgement



The effects of enterolignans in chronic disease



Innovation Fund Denmark

RESEARCH TECHNOLOGY & GROWTH

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Knud Erik Bach Knudsen