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## High-throughput technique –targeted LC-MS/MS method to measure enterolactone “a biomarker of healthy lifestyle” for epidemiological investigations and clinical diagnosis

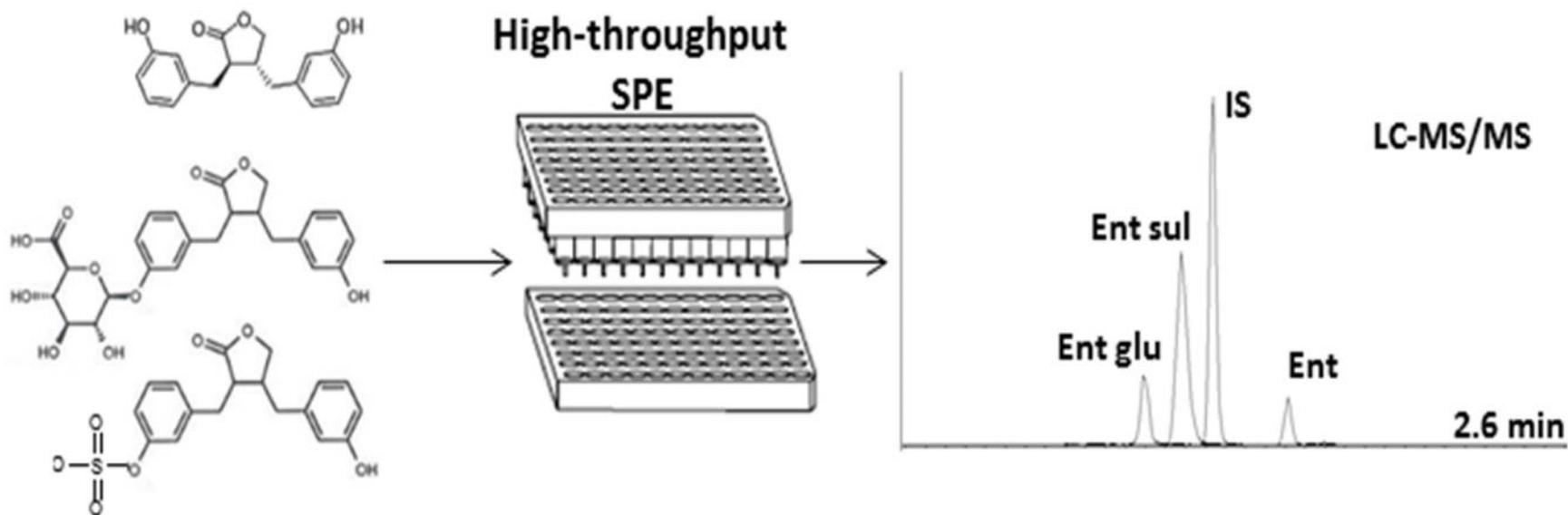
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**High-throughput technique –targeted LC-MS/MS  
method to measure enterolactone “a biomarker of healthy  
lifestyle” for epidemiological investigations and clinical  
diagnosis**



**Abstract:**

Opposite to untargeted metabolomics, targeted metabolomics approach can be applied when the biomarker is known. Enterolactone is a biomarker of healthy lifestyle and therefore used in epidemiological studies in reverse association to lifestyle diseases such as type 2 diabetes, cardiovascular diseases and some forms of cancer. However, the analytical techniques to measure enterolactone in plasma developed so far are based on the hydrolysis of enterolactone with enzymes prior to the measurements and therefore are time consuming. Our purpose was to develop the method that was rapid, reproducible, sensitive and easy to perform. Using the authentic standards of enterolactone, enterolactone glucuronide, and enterolactone sulfate we developed the method that has shown good accuracy and precision at low concentration and high sensitivity, with LLOQ for enterolactone sulfate at 16 pM, enterolactone glucuronide at 26 pM and free enterolactone at 86 pM. The method was applied to 3956 plasma samples from an epidemiological study. The results of PCA indicated that total concentration of enterolactone and concentration of enterolactone glucuronide and sulfate negatively correlated to BMI, age, ratio, cancer type, smoking status and alcohol intake but positively to sport, fruits-, vegetables- and whole-grain intake. We found enterolactone glucuronide to be the major conjugation form and that there was no difference between men and women.

**Keywords:** enterolactone: LC-MS/MS: biomarker: targeted metabolomics



## Introduction

- **Why interesting:**

- Enterolactone was discovered 30 years ago

- Aromatic structure of enterolactone has similarities to steroid metabolites

- **Hypotheses:** enterolactone may protect against hormone-dependent cancers

- Further: research have indicated that enterolactone may have:

antioxidative, estrogenic/antiestrogenic and antiproliferative properties

- **Problem:**

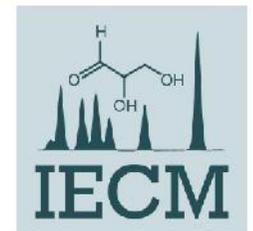
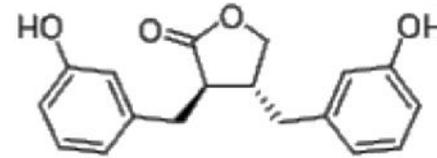
- Methods developed so far are based on hydrolysis of enterolactone with the enzymes  $\beta$ -glucuronidase/sulfatase, time consuming

- Methods measure total concentration of enterolactone

- **Purpose:**

- Quantitative method for direct determination of glucuronidated, sulfated and free enterolactone, without hydrolysis

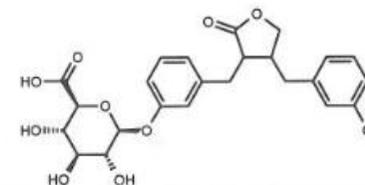
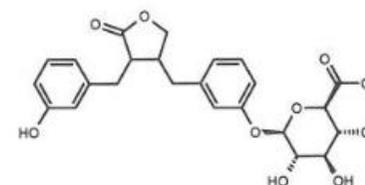
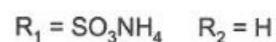
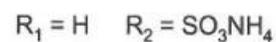
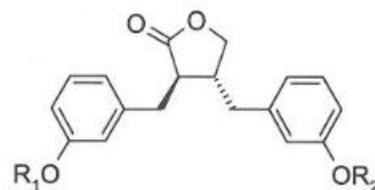
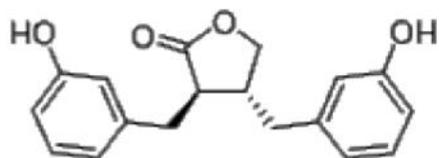
- Sensitive, reproducible and high throughput method with short analytical time



# Results and discussion

## Method development

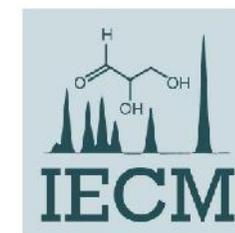
- Authentic standards of enterolactone glucuronide, enterolactone sulfate and enterolactone



## MS/MS optimization

- Manual tuning and Flow Injektion Analysis (FIA) were used to optimize the MS instrument
- Curtain gas 20 psig, Gas 1 50, Gas 2 20, Temperature 300 °C and ion spray 4500 eV

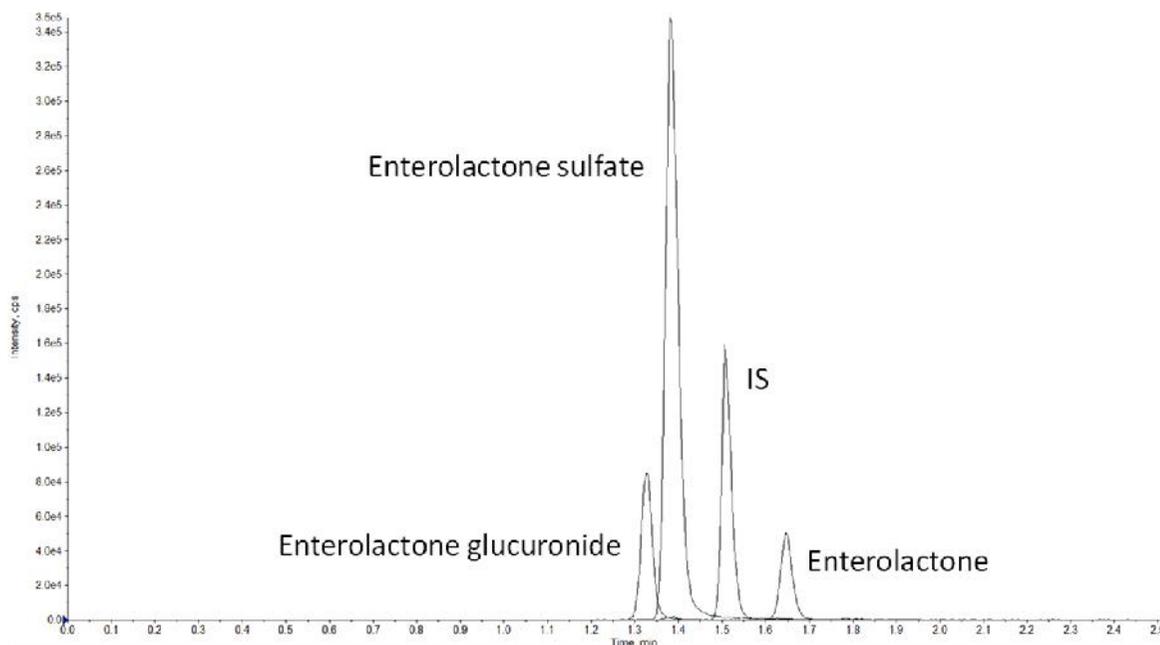
	Q1 mass	Q3 mass	DP	EP	CE	CEP
Enterolactone	297.1	253.1	-140	-10	-26	-21
Enterolactone glucuronide	473.1	297.1	-25	-10	-34	-25
Enterolactone sulfate	377.1	297.1	-55	-10	-32	-13



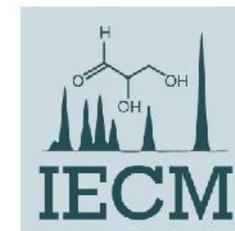
## Results and discussion

### LC optimization

- Phenyl sorbent gave the best resolution, symmetrical peak-shape and no isomer separation compared to C18



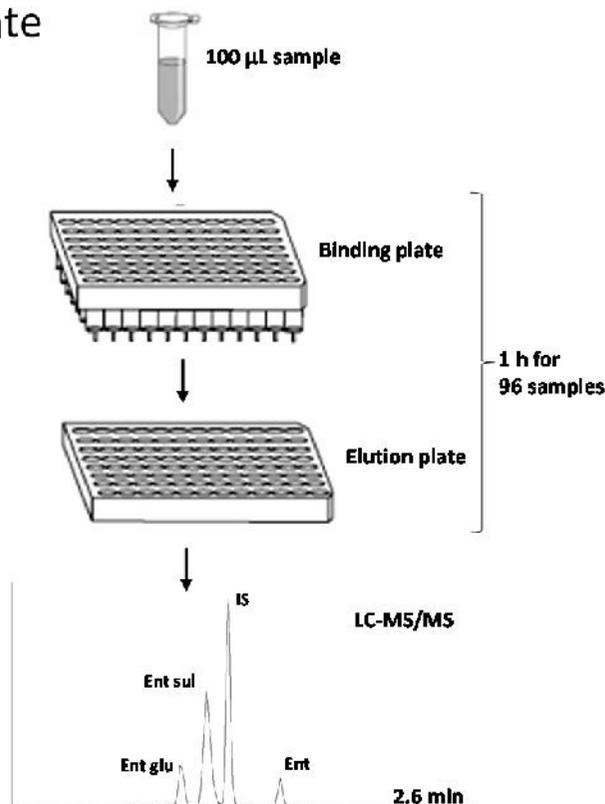
- MRM chromatogram of authentic standards in concentration 1.56 ng/mL
- Enterolactone glucuronide RT (1.33), Enterolactone sulfate RT (1.38), Internal standard (IS) RT (1.51), and Enterolactone RT (1.65)
- 2.6 min for separation and 2 min for equilibration



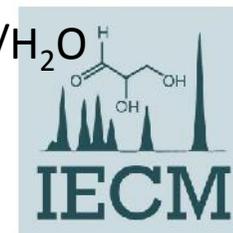
## Results and discussion

### SPE clean up optimization

- Samples were cleaned up using SPE Oasis HLB 96-well plates 30  $\mu\text{m}$  (10 mg) and a vacuum manifold with constant flowrate



- The elution of the analytes was performed with 300  $\mu\text{L}$  50/40/10 % ACN/MeOH/ $\text{H}_2\text{O}$  and collected on the elution plate for direct quantification on the LC-MS/MS instrument

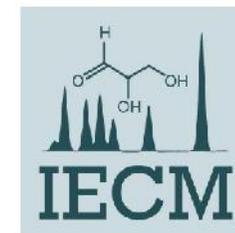


## Results and discussion

### Calibration curves

- Calibration curves were prepared from the working solution in the range of 0.0061 – 12.5 ng/mL in 50/40/10 % ACN/MeOH/H<sub>2</sub>O
- The test of calibration curves in both pure solvent and test plasma showed that the different matrices had no discernible effect on the calibration slope or intercept
- Since the concentration of enterolactone and its conjugates is known to vary in humans, it was desirable to prepare the calibration curves with wide concentration range (10-12 points)
- Low limit of quantification (LLOQ) was down to 26 pM (12.2 pg/mL) for enterolactone glucuronide and 16 pM (6.1 pg/mL) for enterolactone sulfate and slightly higher for enterolactone with LLOQ of 86 pM (24.4 pg/mL)

	LLOQ nM (ng/mL)	ULOQ nM (ng/mL)	r
Enterolactone	0.086 (0.0244)	41.9 (12.5)	0.9987
Enterolactone glucuronide	0.026 (0.0122)	26.3 (12.5)	0.9996
Enterolactone sulfate	0.016 (0.0061)	33.0 (12.5)	0.9992



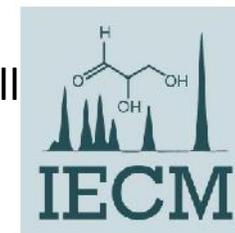
## Results and discussion

### Method validation

- Validation of the method was performed with the blank test plasma to demonstrate the accuracy, precision, and recovery of the measurements at three concentrations, low (0.0977 ng/mL), medium (1.56 ng/mL) and high (6.25 ng/mL) using five measurements per concentration

	Intra-batch									Inter-batch			
	Accuracy (RE %)			Precision ( $\pm$ RSD %)			Recovery %			Precision ( $\pm$ RSD %)		Recovery %	
	L	M	H	L	M	H	L	M	H	L	H	L	H
<b>Enterolactone</b>	10.7	10.1	6.4	4.2	5.7	0.5	110.5	99.3	102.3	4.1	3.1	100.5	99.6
							$\pm$ 15.4	$\pm$ 7.4	$\pm$ 3.1			$\pm$ 10.3	$\pm$ 3.6
<b>Enterolactone glucuronide</b>	6.4	7.8	0.6	7.7	4.9	4.9	87.5	99.9	94.5	4.9	6.0	86.0	91.7
							$\pm$ 13.4	$\pm$ 9.6	$\pm$ 6.0			$\pm$ 14.6	$\pm$ 6.2
<b>Enterolactone sulfate</b>	6.7	12.9	5.8	2.4	3.1	2.9	100.7	67.2	68.5	7.6	8.9	101.9	65.5
							$\pm$ 10.3	$\pm$ 4.9	$\pm$ 6.5			$\pm$ 11.5	$\pm$ 2.2

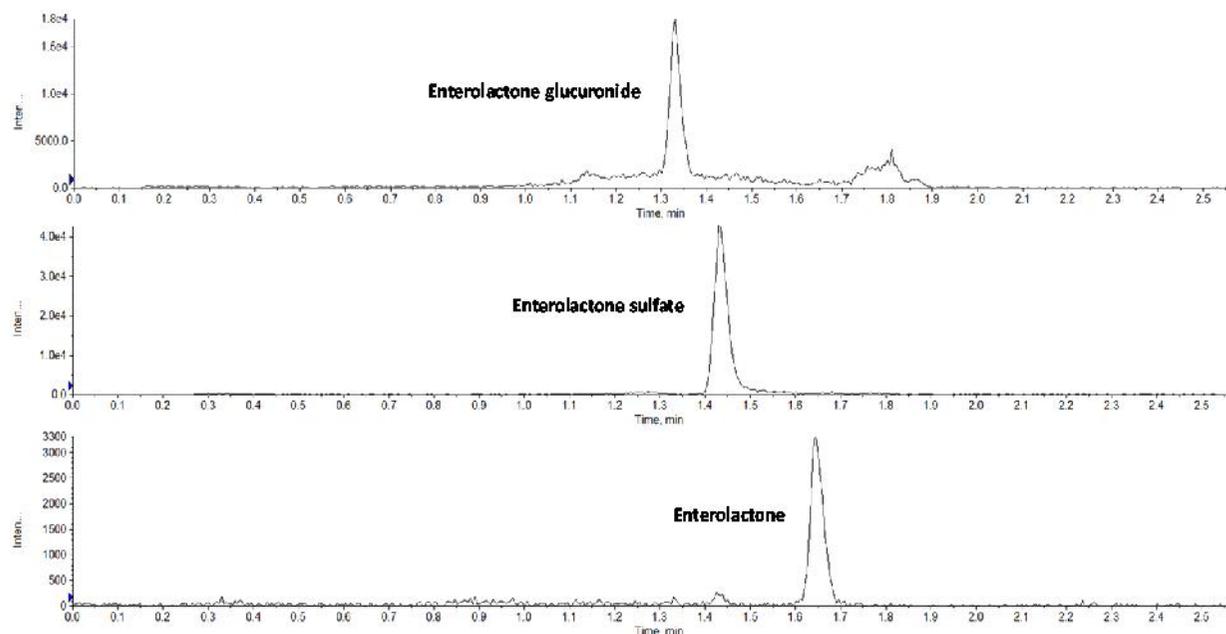
- **High recoveries** with exception for enterolactone sulfate, which showed lower recovery at medium and high concentrations, indicates that enterolactone sulfate has lower affinity towards the SPE column
- The intra-batch accuracy and precision were below the set criterion of 15 % at all three concentrations



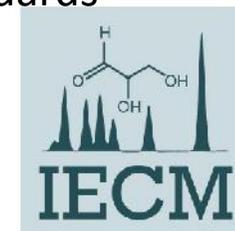
# Results and discussion

## Method validation

- High sensitivity of the method correlated well with high selectivity, so that low background noise and no interfering peaks were observed



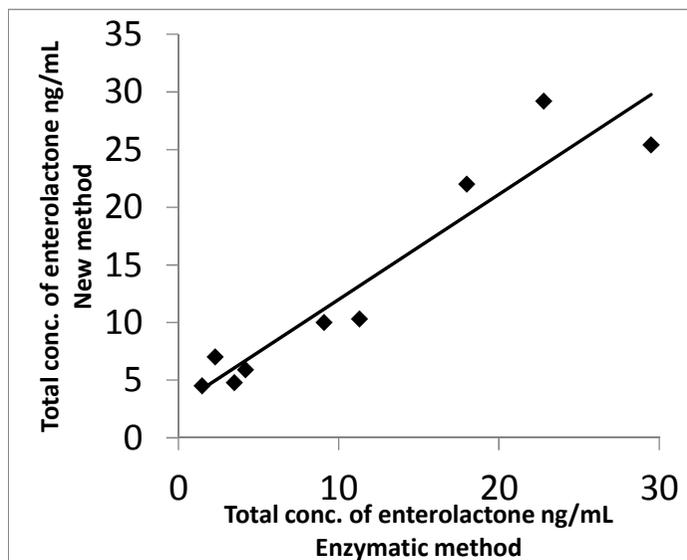
- Extracted ion chromatogram of enterolactone glucuronide, enterolactone sulfate and enterolactone in the test plasma spiked with 0.0977 ng/mL low concentration standards



## Results and discussion

### Applicability of the method

- The applicability of the developed LC-MS/MS method was verified by measuring nine test plasma samples, using two methods: the method in which the total concentration of enterolactone was determined by enzymatic hydrolysis and the new method



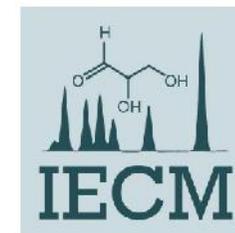
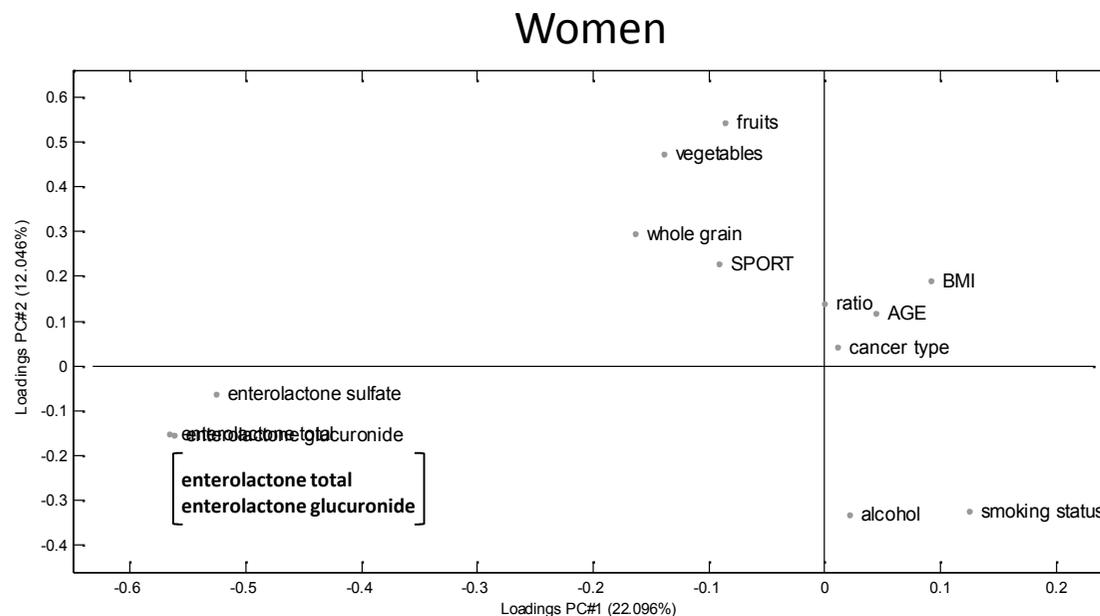
- The results showed high correlation between the enzymatic method and the new method with a correlation coefficient of 0.949



# Results and discussion

## Applicability of the method (PCA)

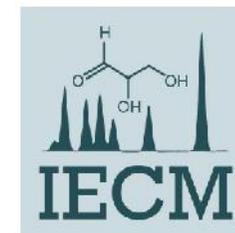
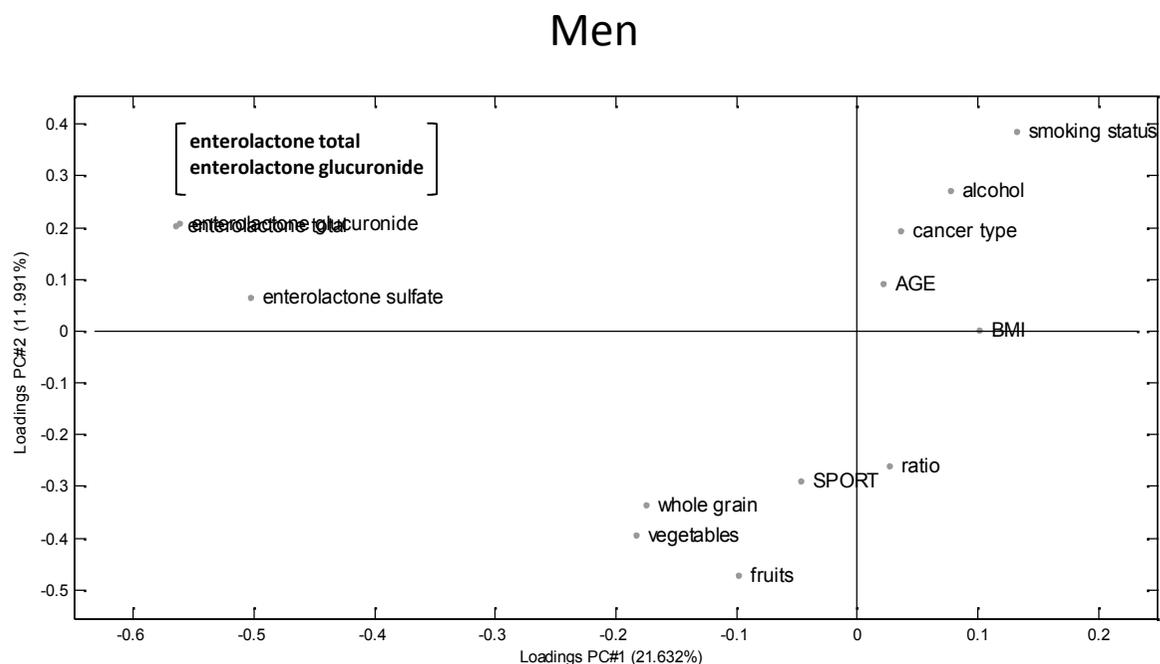
- Using this novel LC-MS/MS method, we have successfully measured 3956 plasma samples from 3913 participants (men=1961 and women=1952) of the Diet, Cancer and Health cohort



# Results and discussion

## Applicability of the method (PCA)

- Using this novel LC-MS/MS method, we have successfully measured 3956 plasma samples from 3913 participants (men=1961 and women=1952) of the Diet, Cancer and Health cohort



# Results and discussion

## Results of PCA

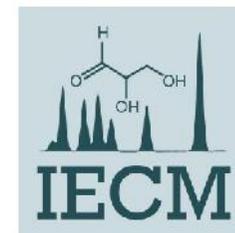
- To study the interrelationship between total concentration of enterolactone and its conjugates and dietary and lifestyle factors
- PCA loading plot of PC1 versus PC2 indicated that the total concentration and the concentration of enterolactone glucuronide/sulfate negatively correlated to BMI, age, ratio, cancer type, smoking status and alcohol intake but positively to sport, fruits-, vegetables- and whole-grain intake – this was **expected as enterolactone is a biomarker of healthy lifestyle**
- In general PCA loading plots for women and men were similar
- Small difference - in “ratio” (ratio is calculated as enterolactone sulfate / (enterolactone sulfate + enterolactone glucuronide)) of enterolactone sulfate indicated that ratio may be influenced by the age, BMI and to smaller extend cancer type diagnosed in case of women, but does not have any correlation to any of variable in PCA in case of men



## Results and discussion

### General results

- Our results showed that by far the most abundant form of enterolactone is glucuronidated
- Wide concentration range of enterolactone glucuronide among the human samples, from 0.2 to 650 nM and mean concentration of 28.5 nM
- Concentration of enterolactone sulfate varied from 0 to 30 nM with mean concentration of 1.3 nM
- Concentration of free enterolactone was quantifiable in only few samples
- Our results are therefore in good agreement with the first studies performed on enterolactone in the 1980s, in which it was concluded that enterolactone was almost exclusively conjugated with glucuronic acid



# Results and discussion

## General results

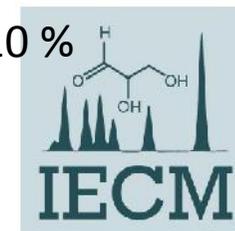
- Our further calculations on the mean percentage distribution of enterolactone glucuronide and sulfate in plasma for men and women

	men		women	
	% Distribution ±SD	Concentration (nM) ±SD	% Distribution ±SD	Concentration (nM) ±SD
<b>Enterolactone glucuronide</b>	95.3 ±3.0	27.8 ±35.0	94.8 ±5.2	29.3 ±37.9
<b>Enterolactone sulfate</b>	4.7 ±3.0	1.2 ±1.5	5.2 ±5.2	1.4 ±2.1

	men			women		
	% Enterolactone sulfate <sup>1</sup>			% Enterolactone sulfate <sup>1</sup>		
	0-10	10-25	>25	0-10	10-25	>25
<b>n</b>	1846 (94.1 %)	113 (5.8 %)	2 (0.1 %)	1815 (93.0 %)	128 (6.6 %)	9 (0.4 %)

<sup>1</sup>Calculated as enterolactone sulfate / (enterolactone sulfate + enterolactone glucuronide)

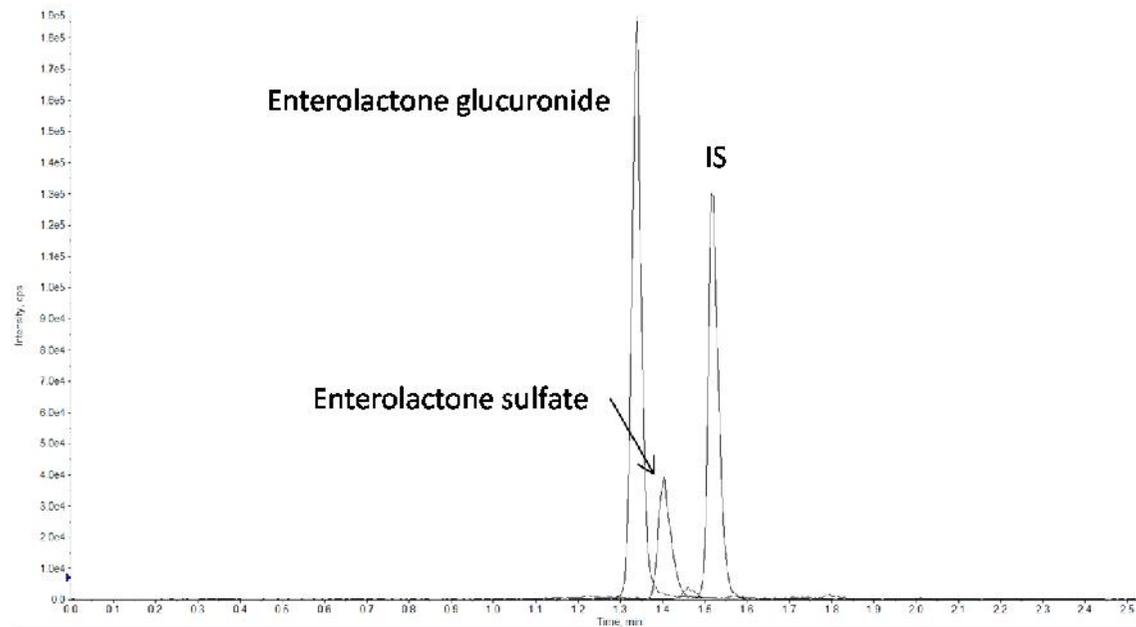
- Percentage distribution for men and women had similar pattern
- Percentage distribution of enterolactone sulfate have shown that very few people have enterolactone sulfate higher than 25 %, 0.1 % for men and 0.4 % for women
- The majority of both men and women had enterolactone sulfate not higher that 10 %



# Results and discussion

## General results

- MRM chromatogram of enterolactone glucuronide and enterolactone sulfate in one of the plasma samples from Cancer and Health cohort



## Conclusions

- Over the past 35 years many good methods have been developed using GC-MS, HPLC, LC-MS/MS and a fluoroimmunoassay
- However, the quantification of enterolactone has always been based on its total concentration and not on its intact forms
- **Here we have presented a rapid, reproducible and sensitive LC-MS/MS method developed to quantify three intact forms of enterolactone in plasma: enterolactone glucuronide, enterolactone sulfate and free enterolactone**
- The prospect of directly measuring the enterolactone and its conjugates in plasma may therefore offer a new perspective on the role of lignans in human health



## Acknowledgments

We thank Innovation Fund Denmark for financing the project “The effects of enterolignans in chronic diseases - ELIN” (0603-00580B). The authors have no conflict of interest.

We also thank ReseaChem GmbH for producing enterolactone glucuronide and Enterolactone sulfate

