

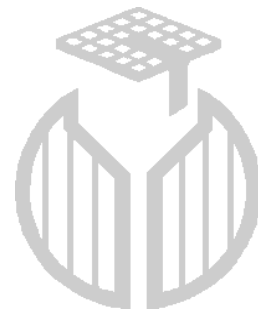
# *Small flavanoids are responsible for the beer colour instability.*

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*Unité de Brasserie et des Industries Alimentaires*



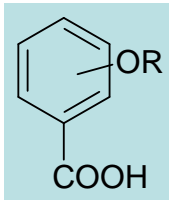
**Fonds  
InBev-Baillet Latour**

*XIIIth J. DE CLERCK CHAIR - session IV - 9/09/08*

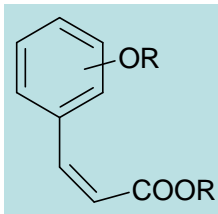
# The polyphenols

## Phenolic acids

→ Benzoic acid



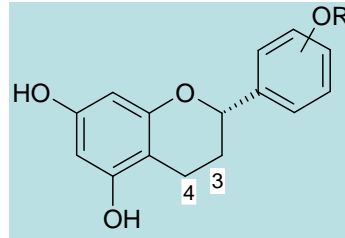
→ Cinnamic acid



Antioxidant, aroma precursors

## Flavonoids

→ Flavanoids



Flavanones  
Flavones  
Neoflavanes  
Flavan-3-ols  
3,4-Flavanediols  
Isoflavones

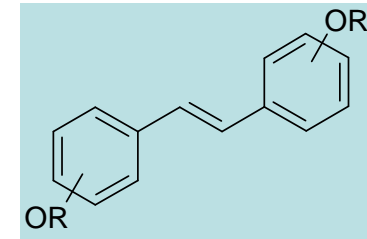
→ Flavonols

→ Prenylchalcones

→ Anthocyanidins

Antioxidant, astringency, color, haze, health benefits (cardioprotector, estrogenic activities,...)

## Stilbenes



Health benefits (cardioprotector, anti-carcinogenic, anti-inflammatory,...)

*In the brewery field, beer polyphenols were mainly studied for their interactions with proteins = colloidal instability*

*Strong impact of:*

*oxygen, temperature, heavy metals, agitation, and light.*



**Colour**



**Astringency**



**Aroma**



**Antioxidant  
activity**

*The goal of this work was to better understand the fate of polyphenols through beer storage.*

*New methodologies were needed to understand what happens to flavanoids in the bottled beer.*



- 1. Development of extraction and detection methods on fresh beer.*
- 2. Which of them are involved in colour instability ?*

*1. Development of extraction and detection methods on fresh beer.*

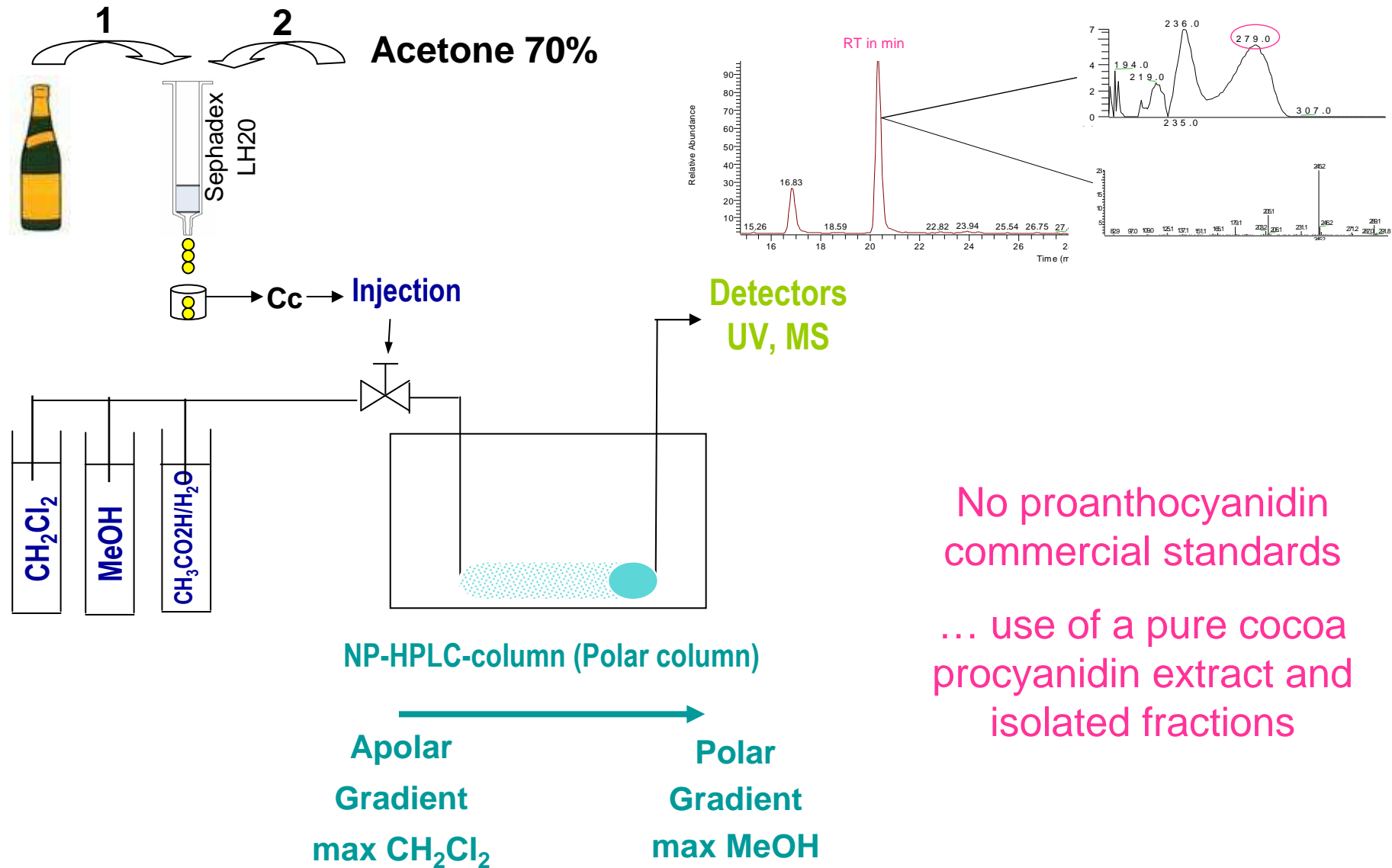
**1.1. NP-HPLC-UV and ESI(-)-MS/MS of native small flavanoids.**

**1.2. RP-HPLC-ESI(-)-MS/MS of native small flavanoids.**

**1.3. Dialysis of higher flavanoids.**

**1.4. Thiolyis.**

# 1.1. NP-HPLC-UV and ESI(-)-MS/MS of native small flavanoids (Sephadex LH20 extract)

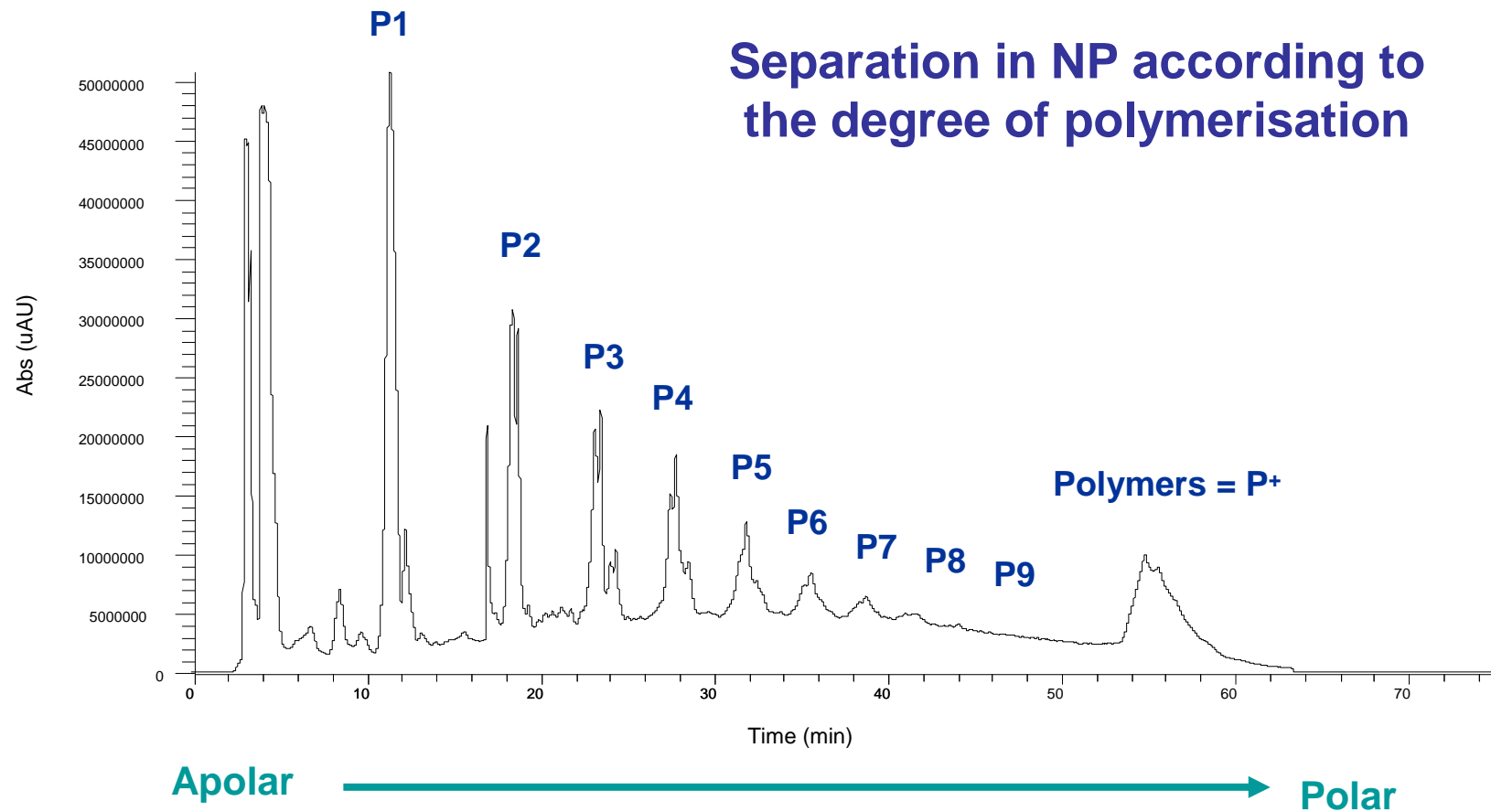


No proanthocyanidin commercial standards

... use of a pure cocoa procyanidin extract and isolated fractions

# UV ( $\lambda = 280 \text{ nm}$ )

*Cocoa procyanidin extract  
used as standard*



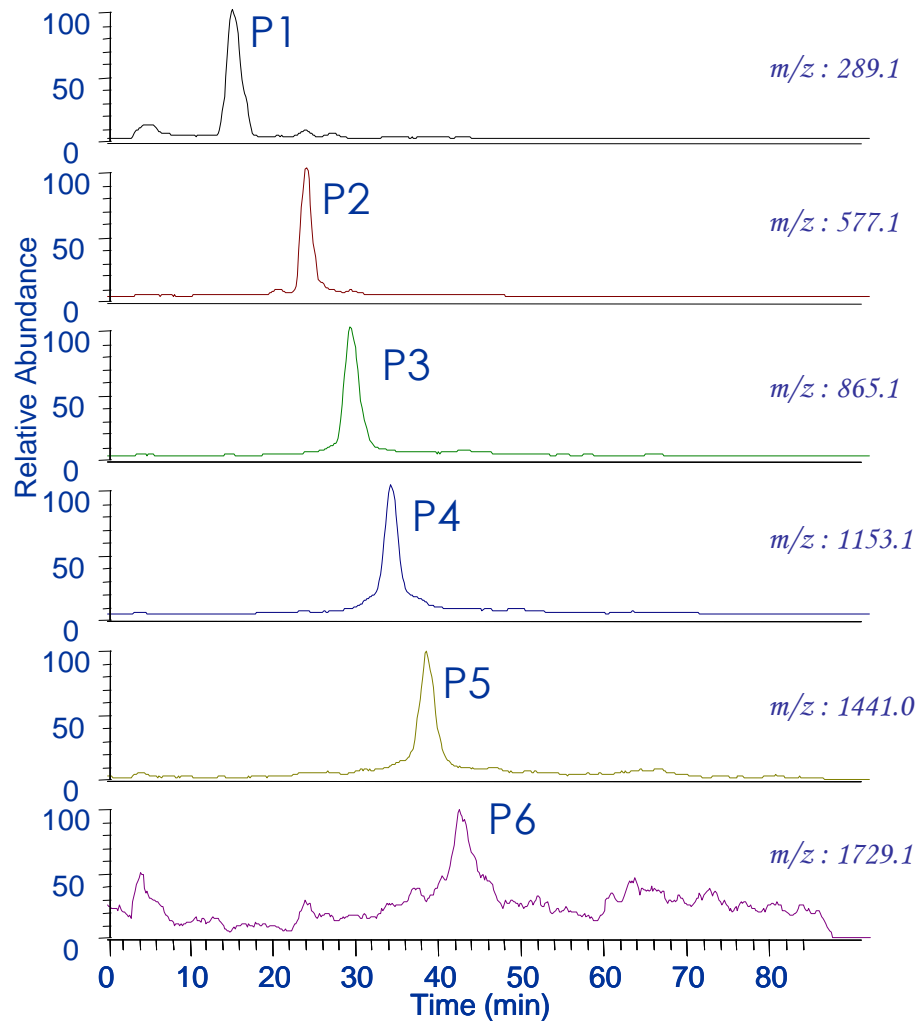
# Why the use of an MS/MS detector ?

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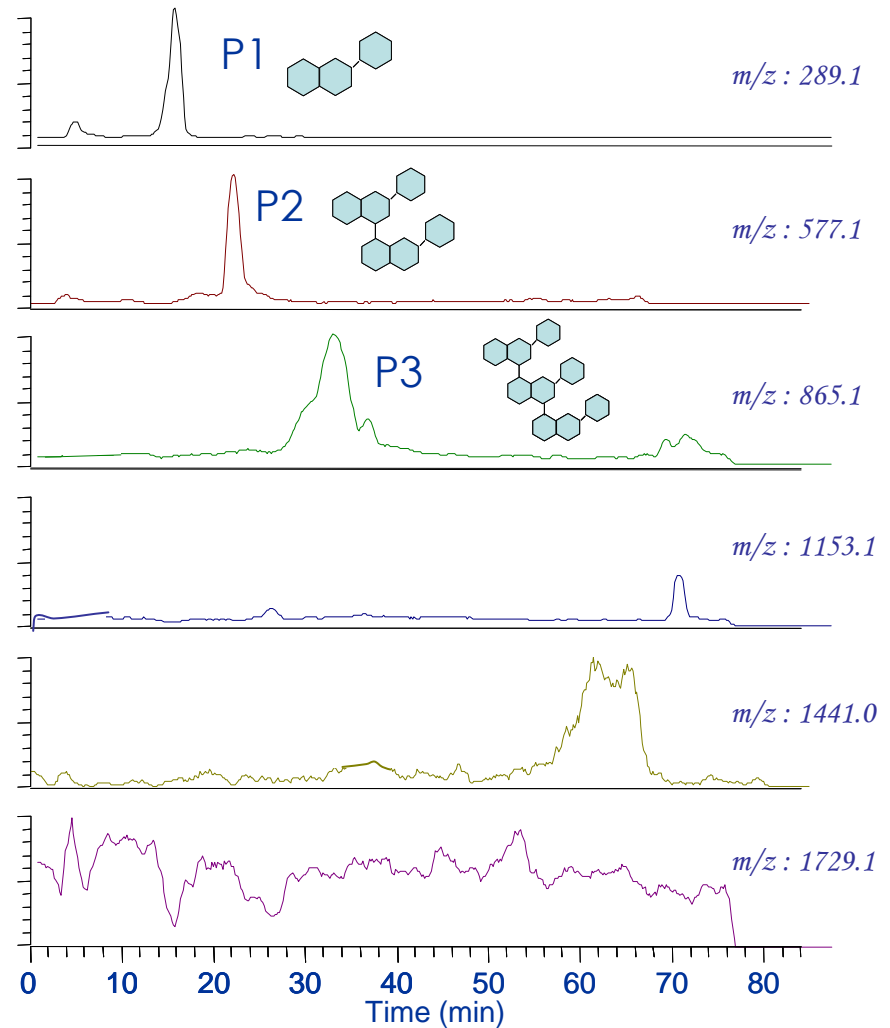
- **Beer**
  - Low concentrations
  - Interfering compounds
  
- **Advantages of the MS**
  - Structural information (MS/MS)
  - More precise quantification (LOD & LOQ  $\searrow$ )

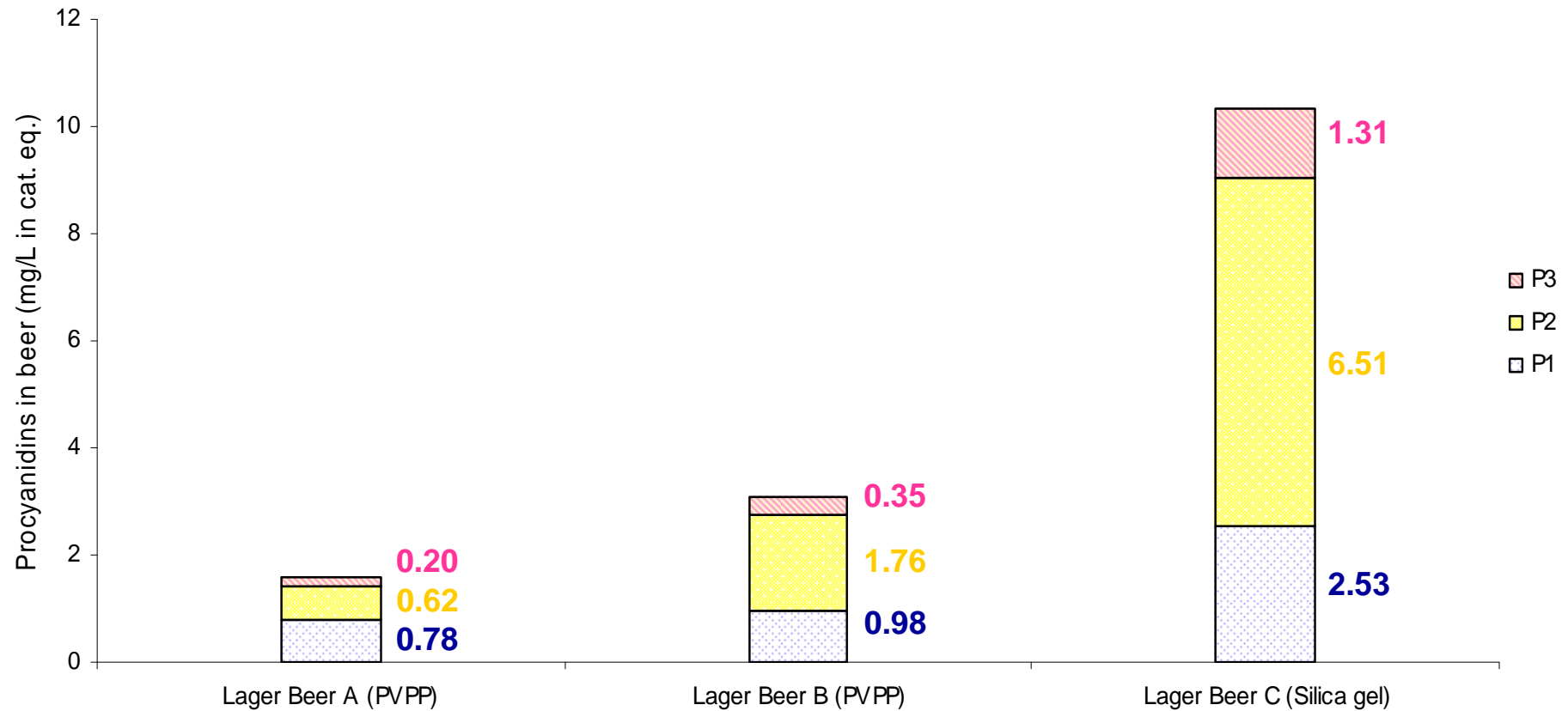
# ESI(-)-MS/MS with NH<sub>4</sub><sup>+</sup>

*Cocoa procyanidin extract  
used as standard*



*Sephadex LH-20 aqueous acetone 70%  
extract of a lager beer*





## PVPP

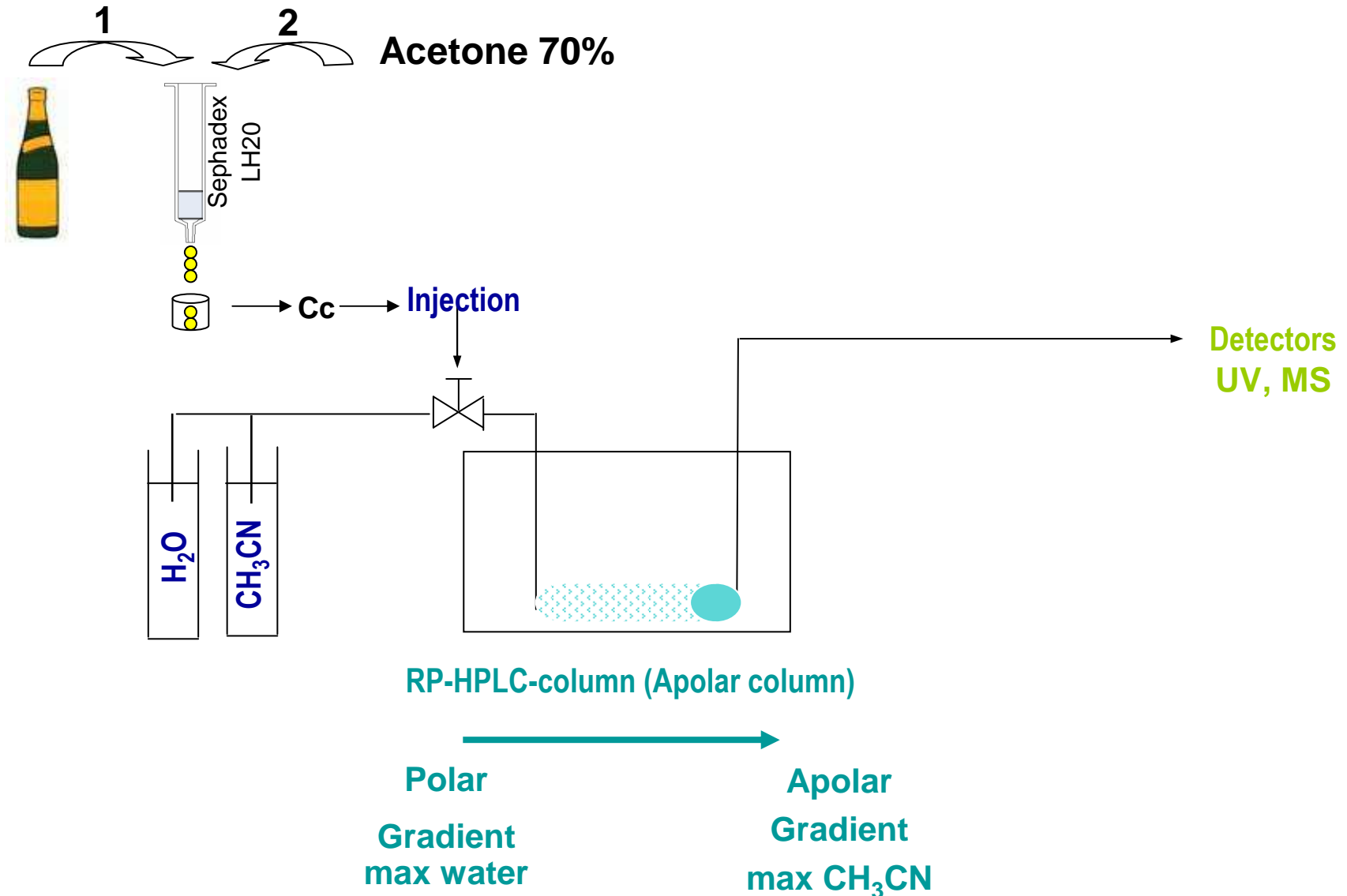
⇒ removes **polyphenols**, especially the most polymerized fractions → P1-P3 < 3 ppm

## Silicagel

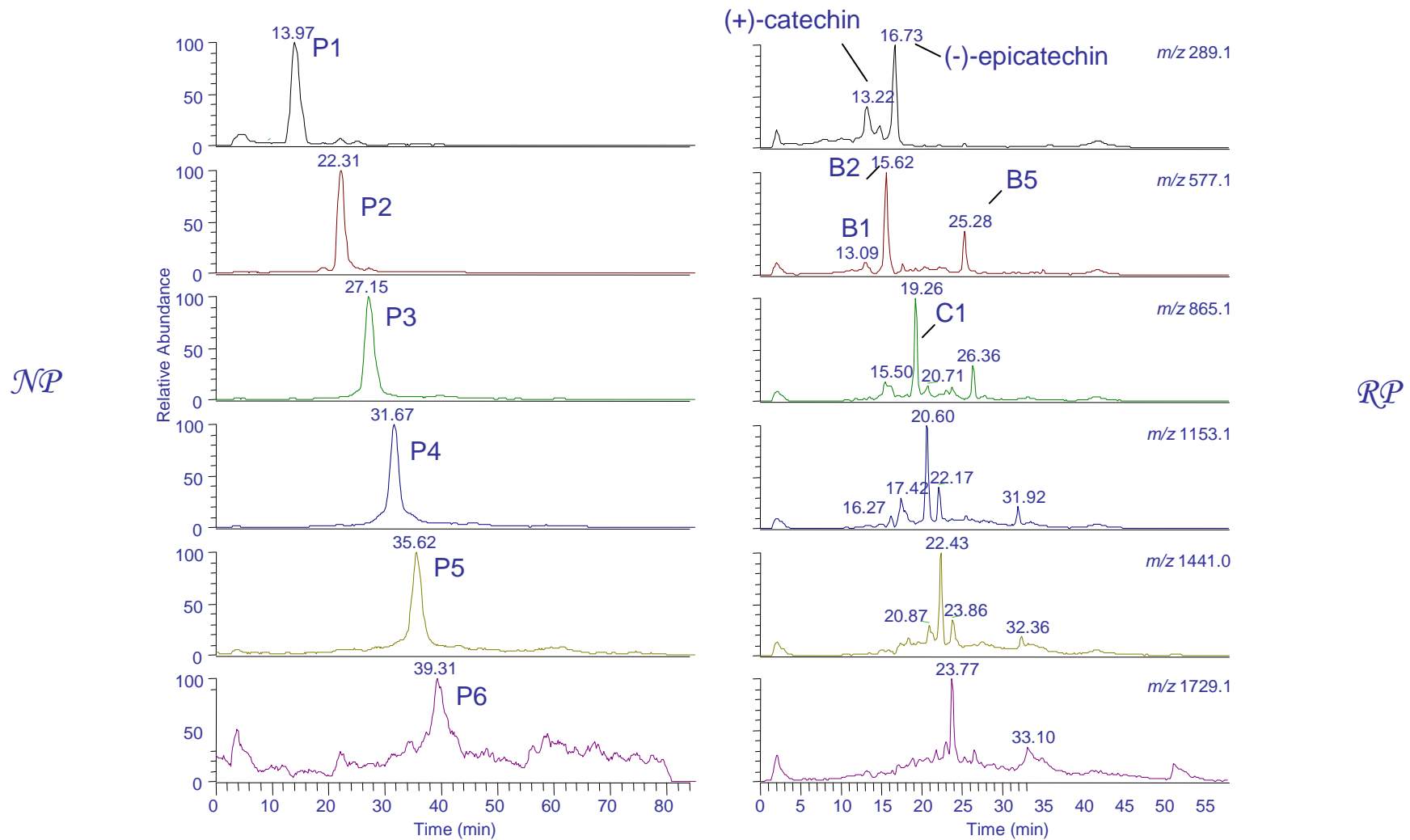
⇒ removes **proteins** (↗ procyanidins : 10 ppm P1 to P3)

High P2/P1 ratio = 2.6 compared to 0.8

## 1.2. RP-HPLC-ESI(-)-MS/MS of native small flavanoids (Sephadex LH20 extract)



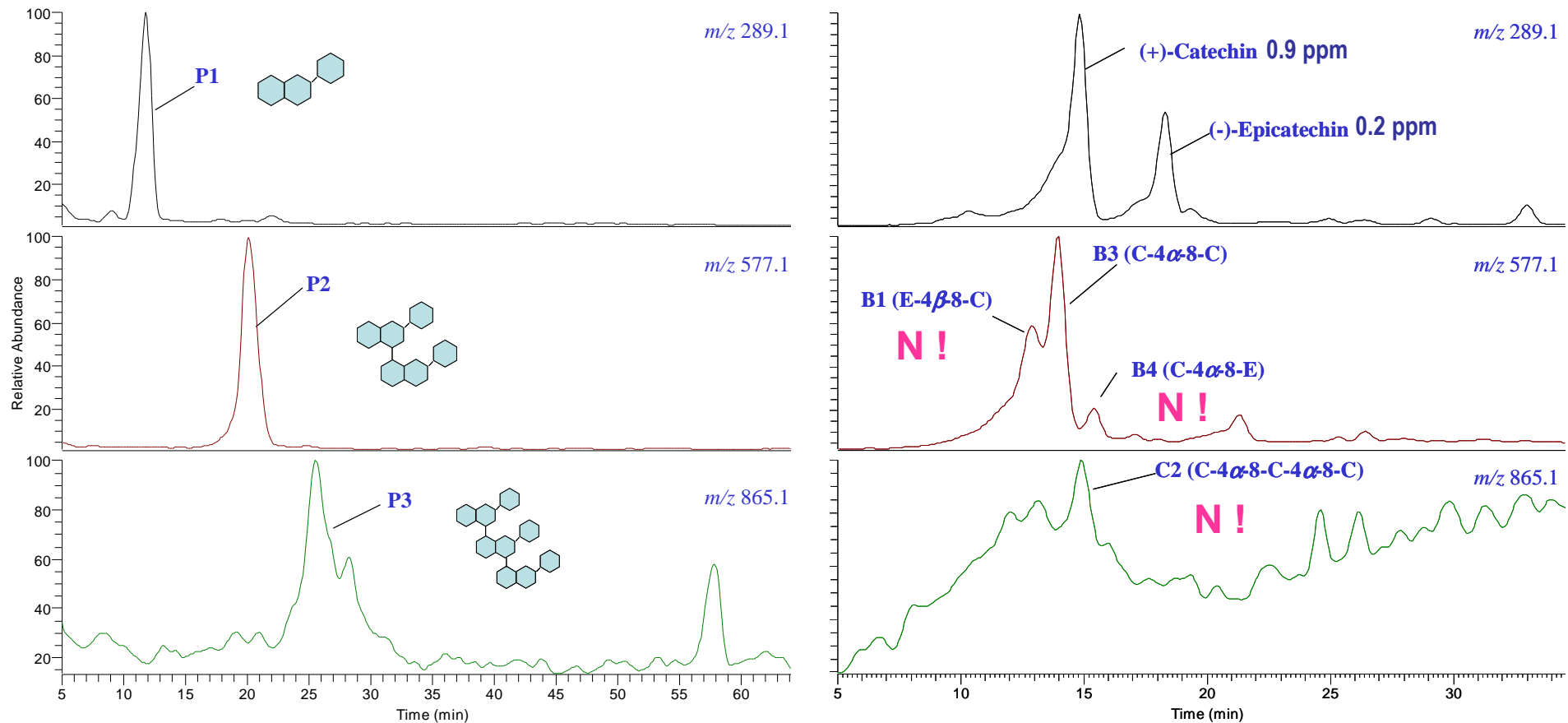
# Comparison between NP and RP-HPLC-ESI(-)-MS/MS separation for P1 to P6 flavanoids of a **cocoa procyanidin extract**

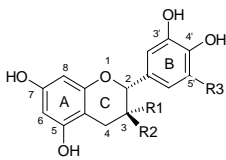


**Procyanidins:** B1: (-) - epicatechin ( $4\beta \rightarrow 8$ ) - (+) - catechin; B2 : (-) - epicatechin ( $4\beta \rightarrow 8$ ) - (-)-epicatechin; B5: (-) - epicatechin ( $4\beta \rightarrow 6$ ) - (-)-epicatechin

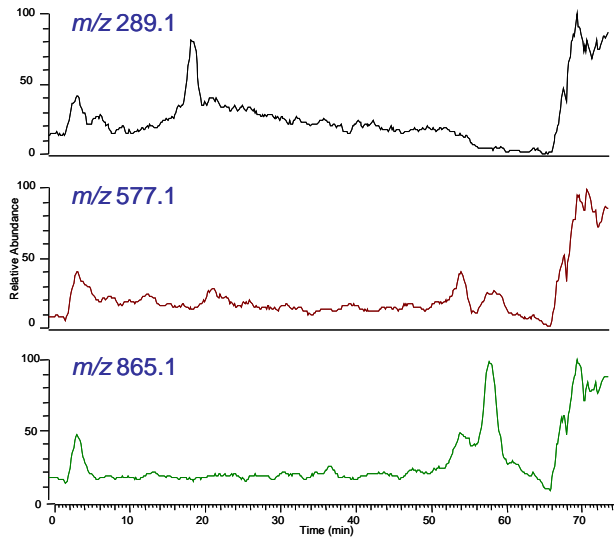
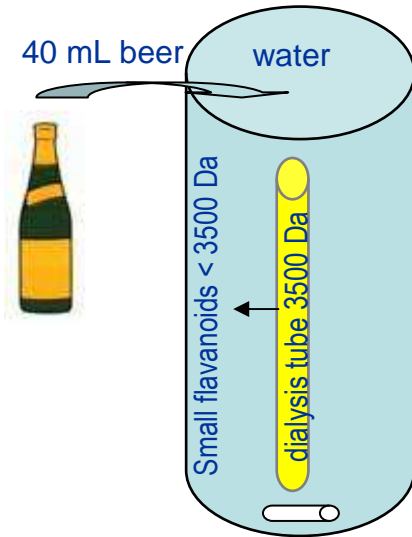
# Comparison between NP and RP-HPLC-ESI(-)- MS/MS separations of a **Sephadex LH20 beer extract**

## PROCYANIDINS

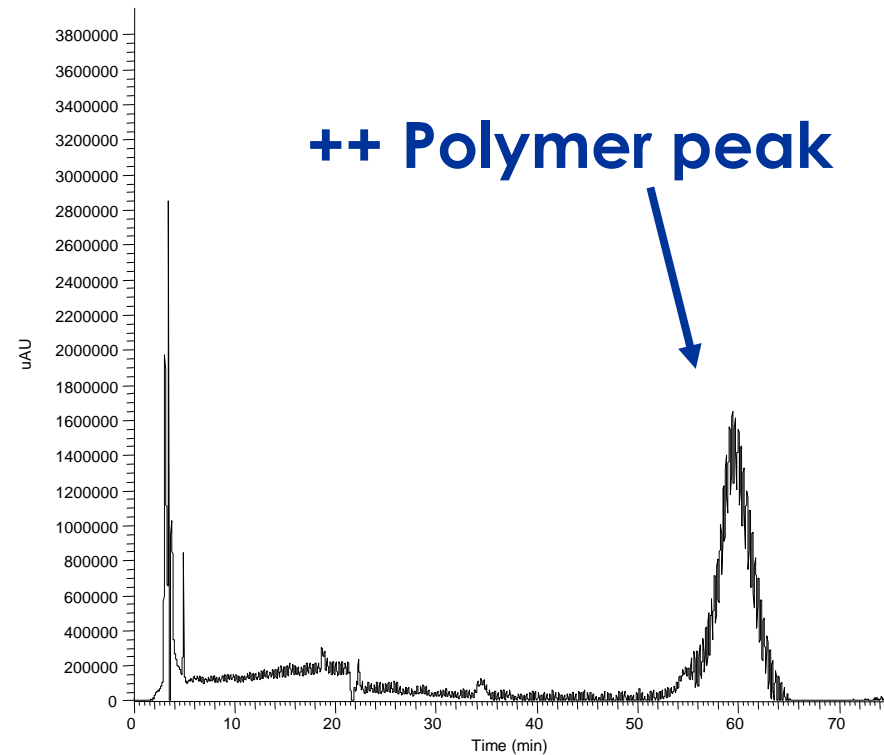


Flavanoids		Malt	Hop	Fresh beer	
	(+)-Catechin	$R_1 = H, R_2 = OH, R_3 = H$	L	L	L, D
	(-)-Epicatechin	$R_1 = OH, R_2 = R_3 = H$	-	L	L, D
	(-)-Gallocatechin	$R_1 = H, R_2 = R_3 = OH$	-	L	L, D
	(-)-Epigallocatechin	$R_1 = OH, R_2 = H, R_3 = OH$	-	-	L, D
<b>Procyanidins B</b>	<b>B1</b> (-)-Epicatechin-(4 $\beta$ -8)-(+)-catechin		-	L	-, <b>N!</b>
	<b>B2</b> (-)-Epicatechin-(4 $\beta$ -8)-(-)-epicatechin		-	L	-
	<b>B3</b> (+)-Catechin-(4 $\alpha$ -8)-(+)-catechin		L	L	L, D
	<b>B4</b> (+)-Catechin-(4 $\alpha$ -8)-(-)-epicatechin		-	L	-, <b>N!</b>
<b>Prodelphinidins B</b>	<b>B3</b> (-)-Gallocatechin-(4 $\alpha$ -8)-(+)-catechin		L	L	L, D
	<b>B9</b> (-)-Epigallocatechin-(4 $\beta$ -8)-(+)-catechin		-	-	L
	(+)-Catechin-(4 $\alpha$ -8)-(-)-gallocatechin		-	L	-
	(+)-Catechin-(4 $\alpha$ -6)-(-)-gallocatechin		-	L	-
	(-)-Gallocatechin-(4 $\alpha$ -6)-(+)-catechin		-	L	-
<b>Prodelphinidins A</b>	<i>ent</i> - (-)-Epigallocatechin-(4 $\alpha$ -8, 2 $\alpha$ -O-7)-(+)-catechin		-	-	L
	<i>ent</i> - (-)-Epigallocatechin-(4 $\alpha$ -6, 2 $\alpha$ -O-7)-(+)-catechin		-	-	L
<b>Propelargonidin B</b>	(+)-Afzelechin-(4 $\alpha$ -8)-(+)-catechin		-	L	-
<b>Procyanidins C</b>	<b>C2</b> (+)-Catechin-(4 $\alpha$ -8)-(+)-catechin-(4 $\alpha$ -8)-(+)-catechin		L	L	-, <b>N!</b>
	(-)-Epicatechin-(4 $\beta$ -8)-(+)-catechin-(4 $\alpha$ -8)-(+)-catechin		-	L	-
	(-)-Epicatechin-(4 $\beta$ -8)-(-)-epicatechin-(4 $\beta$ -8)-(+)-catechin		-	L	-
<b>Prodelphinidins C</b>	(-)-Gallocatechin-(4 $\alpha$ -8)-(-)-gallocatechin-(4 $\alpha$ -8)-(+)-catechin		L	L	-, <b>N!</b>
	(-)-Gallocatechin-(4 $\alpha$ -8)-(+)-catechin-(4 $\alpha$ -8)-(+)-catechin		L	-	-, <b>N!</b>
	(+)-Catechin-(4 $\alpha$ -8)-(-)-gallocatechin-(4 $\alpha$ -8)-(+)-catechin		L	L	-

# 1.3. Dialysis of native higher flavanoids (cut-off 3500 da)



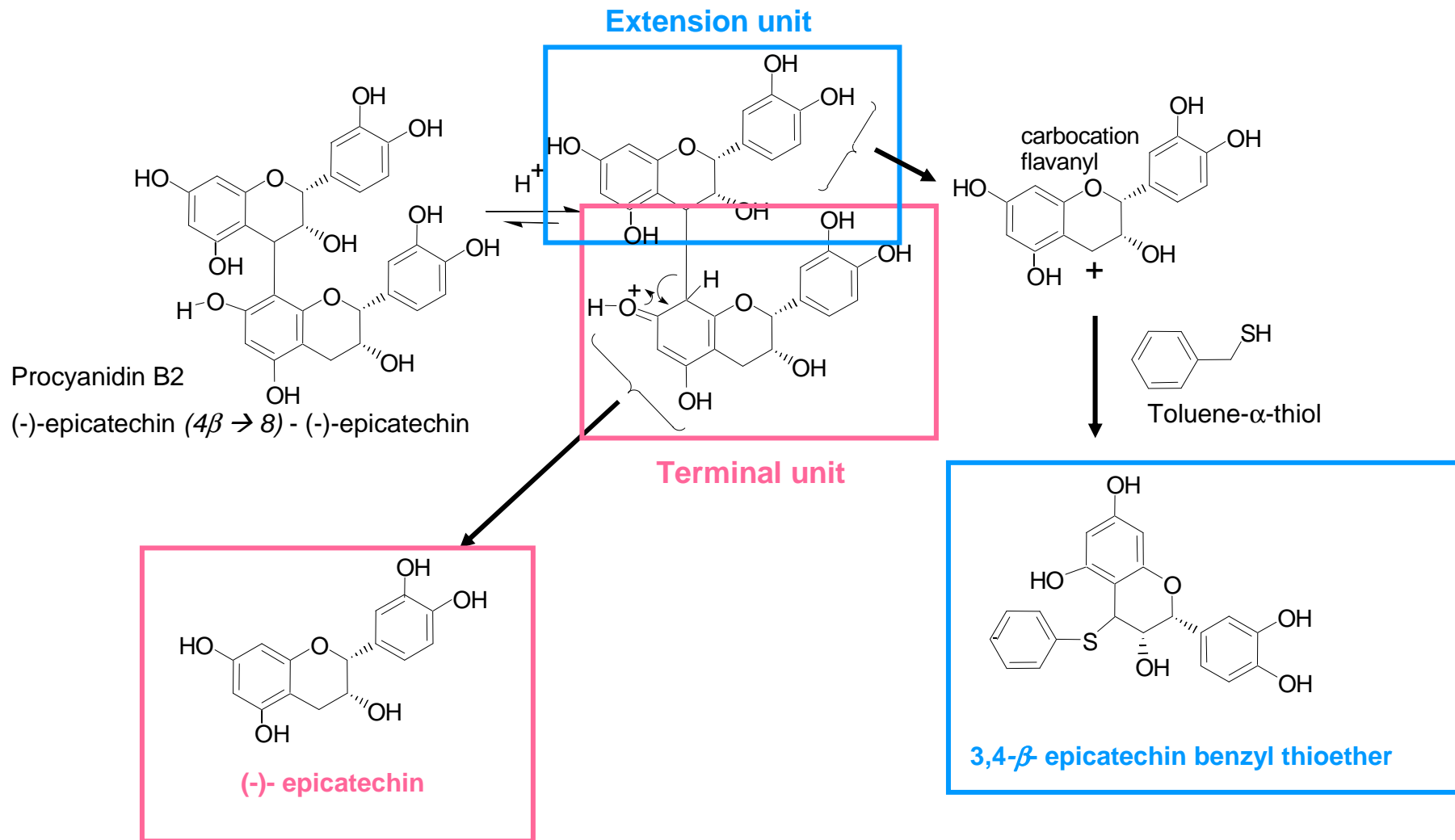
NP-LC-ESI (-)- MS/MS

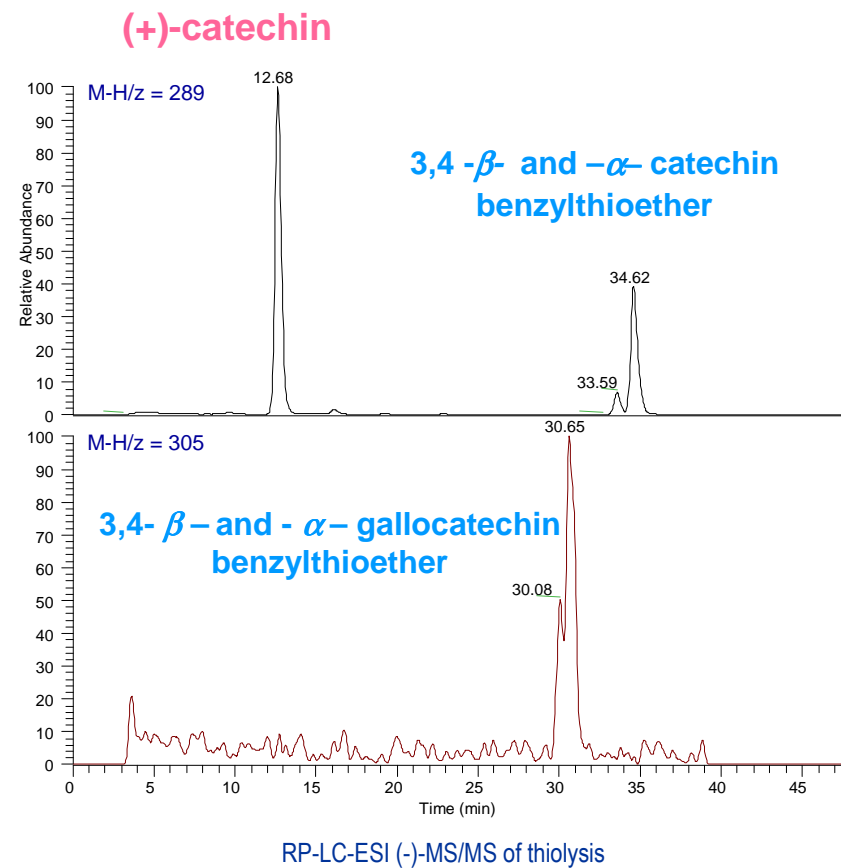
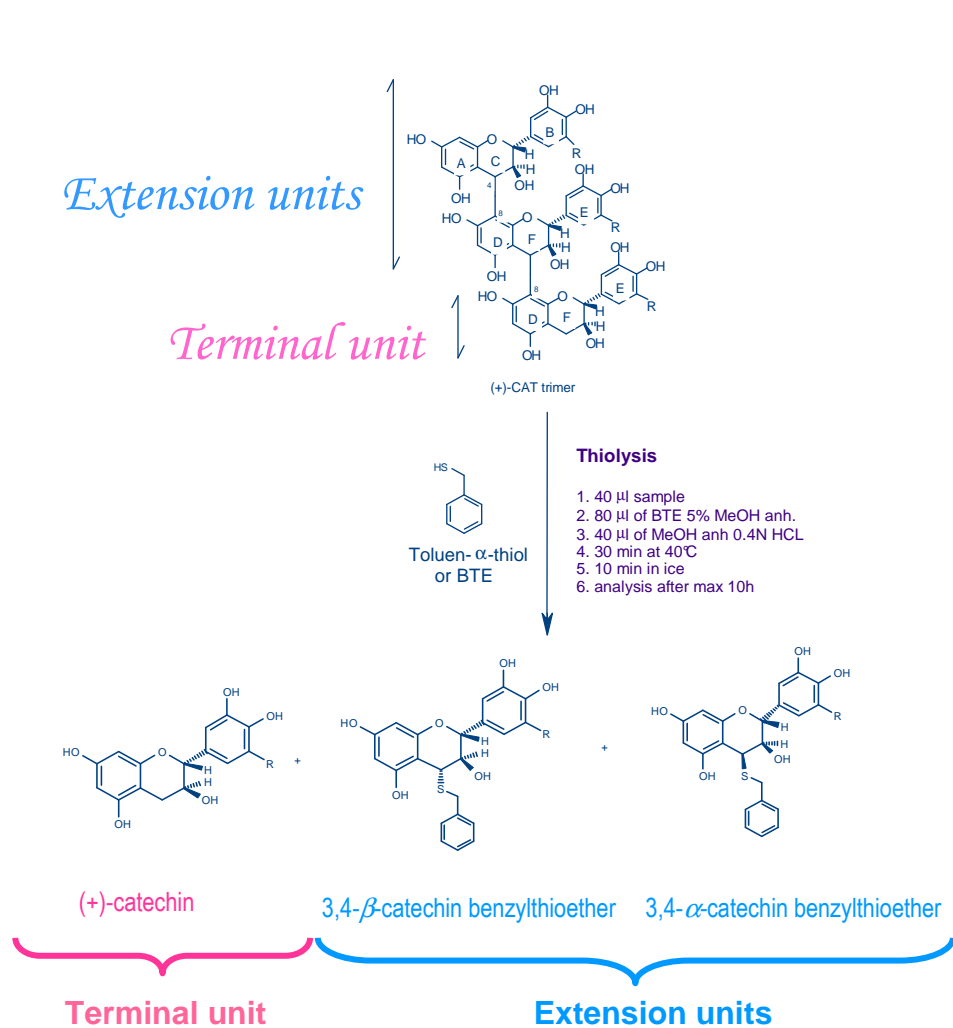


NP-LC-UV ( $\lambda = 280$  nm)

**++ No P1 to P3**

# 1.4. Optimization of a methodology for precise determination of proanthocyanidin structures : thiolysis- RP-HPLC-ESI(-)-MS/MS





✓ DP = (ext + ter)/ter

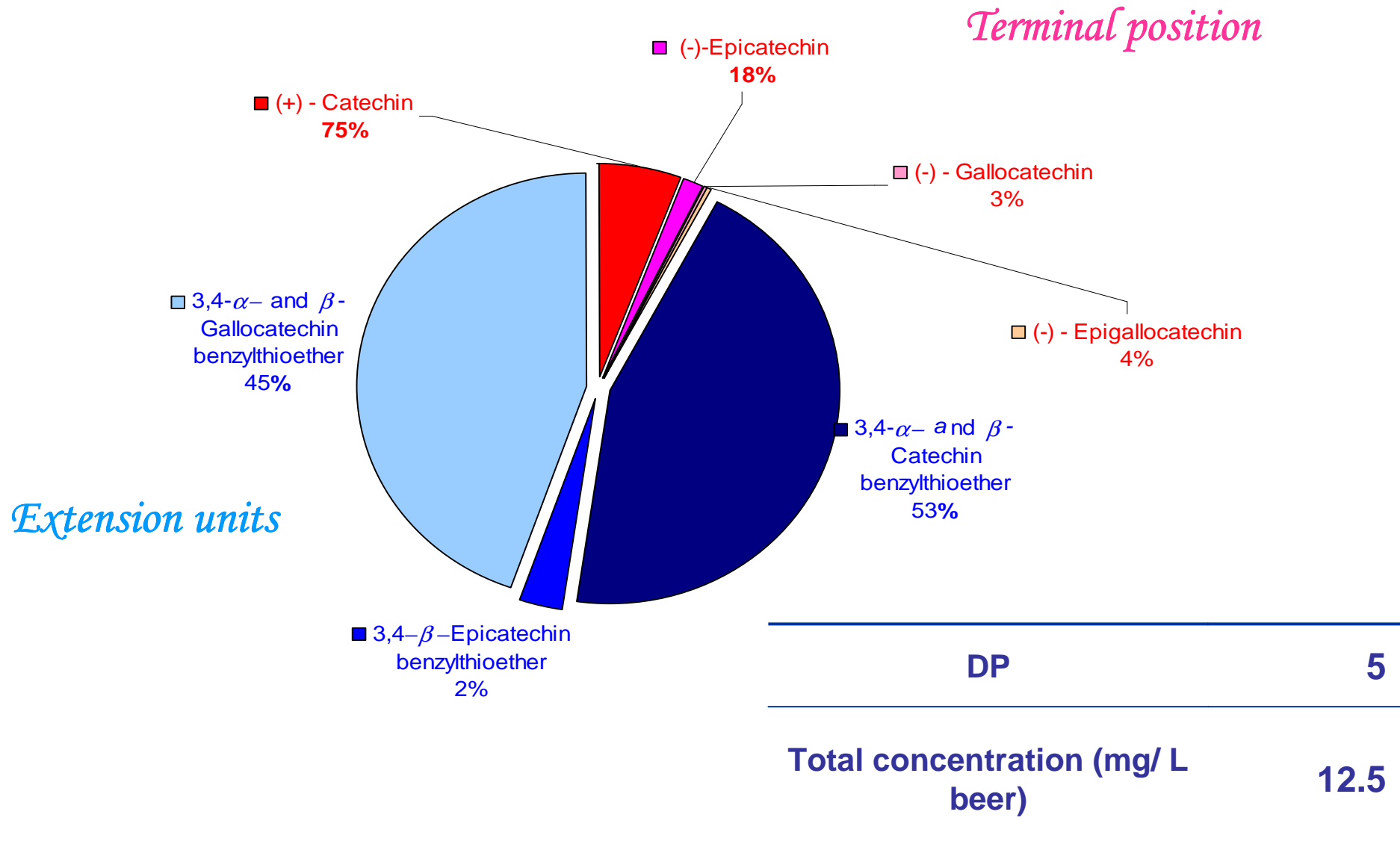
✓ Proanthocyanidin concentrations

✓ Identification of the **terminal units**

✓ Identification of the **extension units**

# Small oligomers

Thiolysis on the LH20 extract of a fresh PVPP beer



# Small oligomers

Thiolysis of pure beer fractions isolated by NP-HPLC-UV from the Sephadex LH20 extract

	P1 (%)	P2 (%)	P3 (%)
(+)-Catechin	97	92	85
(-)-Epicatechin	3	8	8
(-)-Gallocatechin	-	-	6
(-)-Epigallocatechin	-	-	1
3,4- $\alpha$ - and $\beta$ -Catechin benzylthioether	-	89	25
3,4- $\beta$ -Epicatechin benzylthioether	-	5	2
3,4- $\alpha$ - and $\beta$ - Gallocatechin benzylthioether	-	6	73

**More catechin in the monomers**

**More Catechin in the terminal units of the dimers extension**

**More Catechin in the terminal units of the trimers**  
**Gallocatechin in the extension**

Procyanidin B3

Prodelphinidin C2

	Malt		Aromatic hop		Bitter hop	
	Native (%)	(%)	Native (%)	(%)	Native (%)	(%)
(+)-Catechin	91	90	82	78	62	54
(-)-Epicatechin	5	2	14	14	38	41
(-)-Gallocatechin	4	5	4	6	-	2
(-)-Epigallocatechin	-	3	-	2	-	2
3,4- $\alpha$ and $\beta$ -Catechin benzylthioether	-	36	-	41	-	48
3,4- $\beta$ -Epicatechin benzylthioether	-	1	-	15	-	13
3,4- $\alpha$ and $\beta$ - Gallocatechin benzylthioether	-	63	-	14	-	11
3,4- $\beta$ -Epigallocatechin benzylthioether	-	-	-	30	-	28
mDP	10.6		7.5		7.0	
Proanthocyanidins (g/kG)	12.0(12.7*)		43.4 (45.0*)		27.2 (28.0*)	

\*Including native flavan-3-ol monomers

➔ Catechin : Malt and Hop

➔ Epicatechin : Hop

➔ Catechin : Malt and Hop

➔ Gallocatechin : Malt

➔ Epigallocatechin : Hop

mDP Malt > mDP Hop > mDP Beer

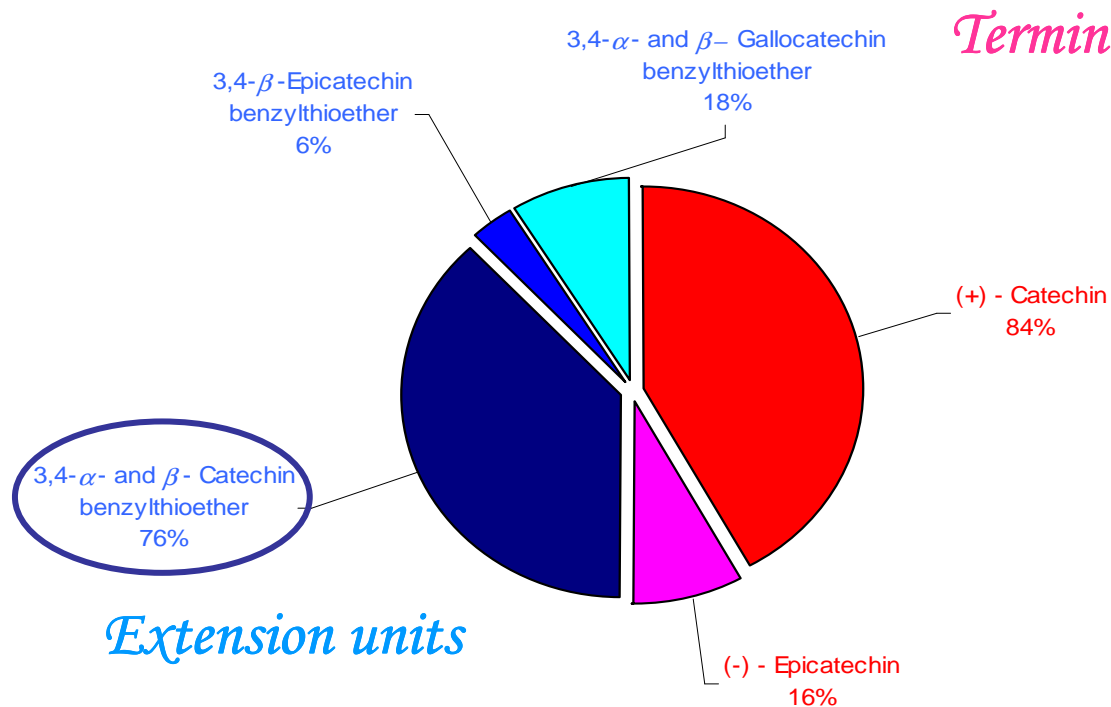
Depolymerization not reversible during the process

[Hop] > [Malt]

Aromatic Hop > Bitter Hop

# Higher oligomers

Thiolysis of a dialysis freeze-dried extract



Higher mDP values expected

5 vs DP > 12 (3500 Da)

? Presence in beer of more complex polyphenolic structures not completely degraded by

thiolysis

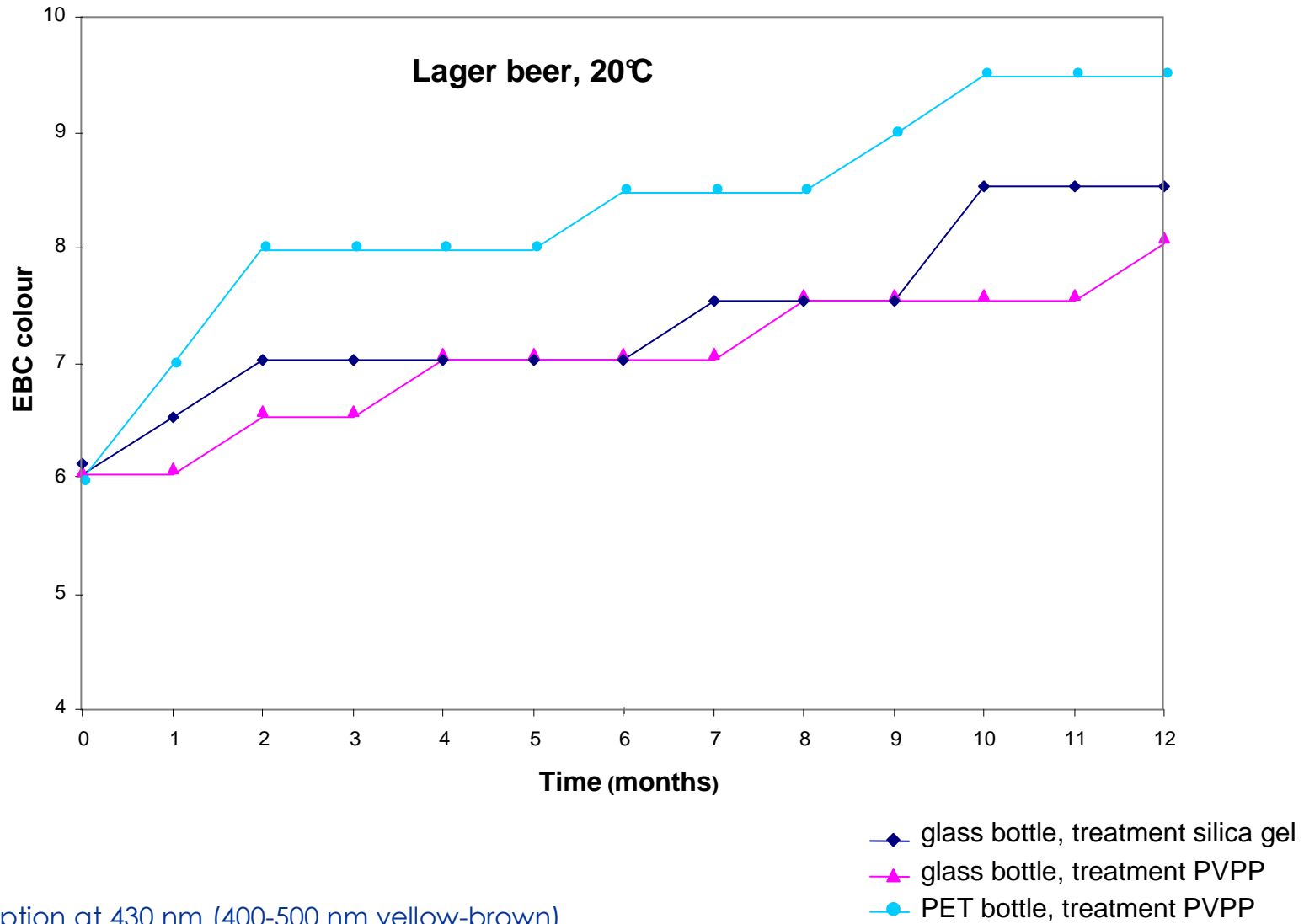
**Natural oligomers exhibit a relatively low DP whilst transformed oligomers account for most of the heavy flavanoid content**

*2. The most promising methods were then applied on aged beer samples in order to investigate the chemical changes happening to flavanoids through storage.*

*Which of them are involved in colour ?*

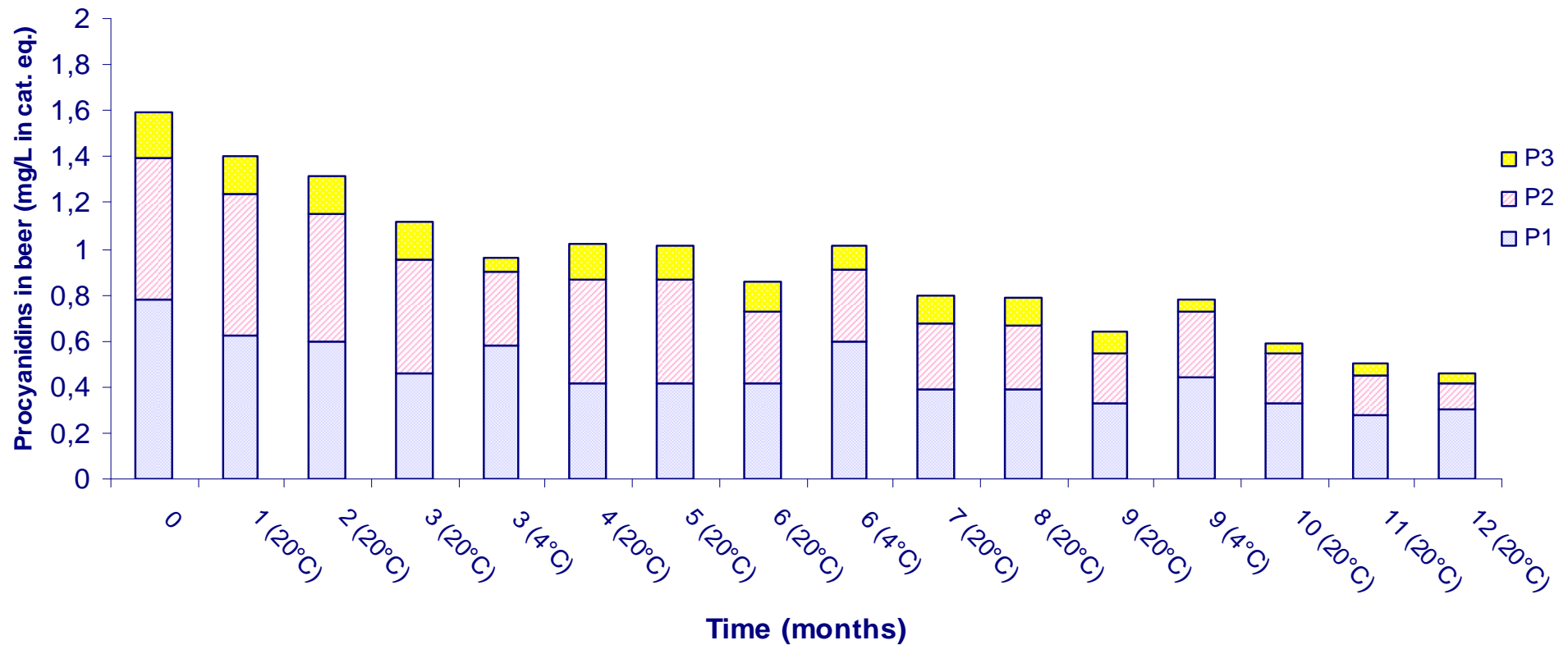
*Macroscopic data ⇔ Molecular data*

# Beer yellow-brown colour increases through storage



EBC = Absorption at 430 nm (400-500 nm yellow-brown)

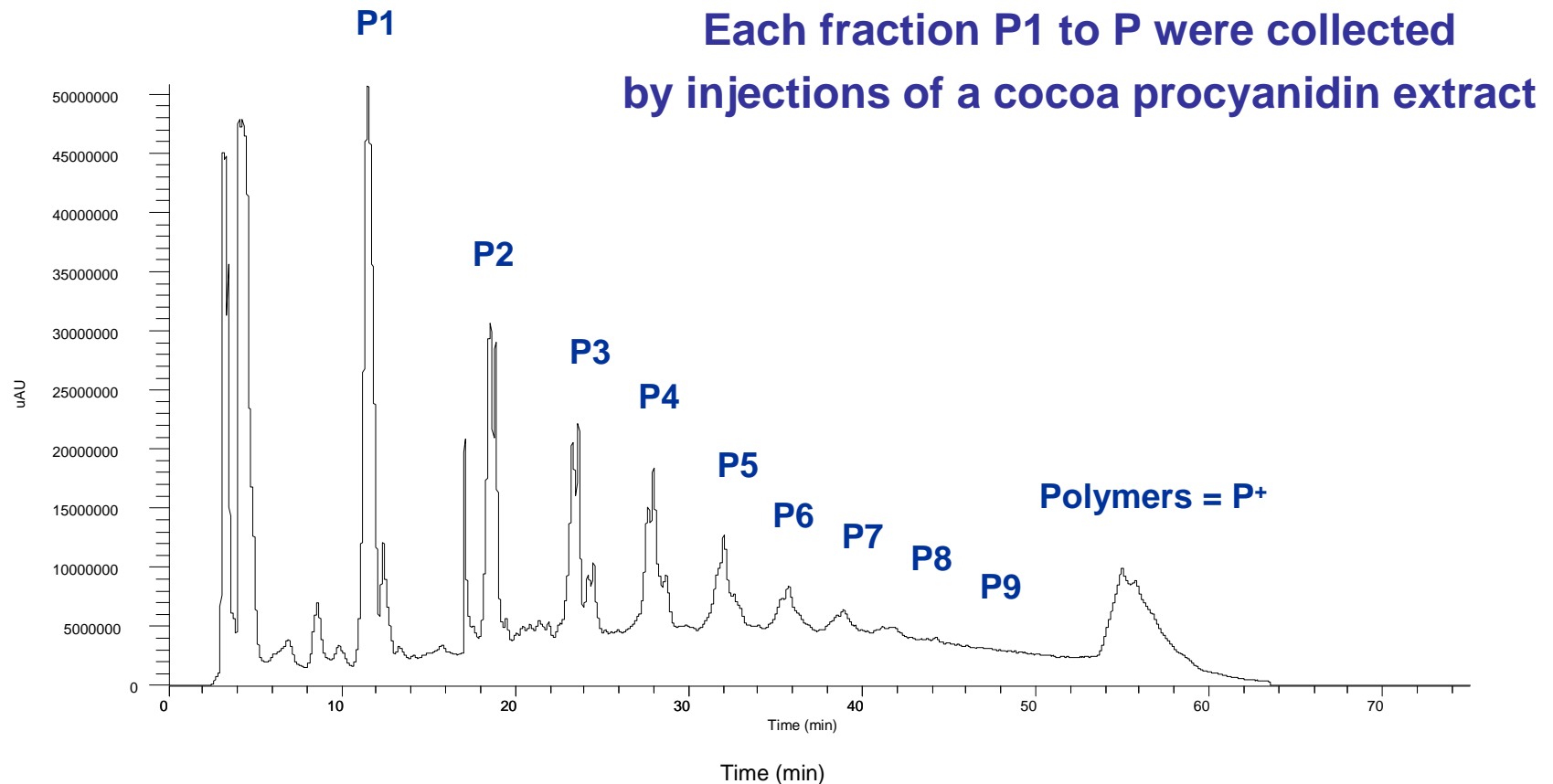
# Small flavanoids are very unstable in the bottle



Beer with PVPP treatment-NP-HPLC-ESI(-)-MS/MS quantification

# Which procyanidins fractions are responsible of the colour changes ?

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**Fresh solution not coloured**

**Follow-up of the colour of each fraction at 4°C and 20°C with and without light**

Colour of the different procyanidin fractions after 60 days  
Yellow-brown colour from (+)-catechin & (-)-epicatechin

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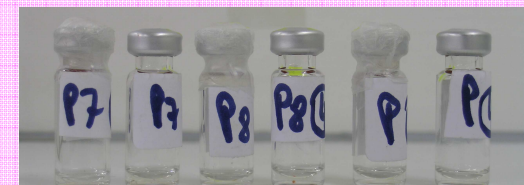
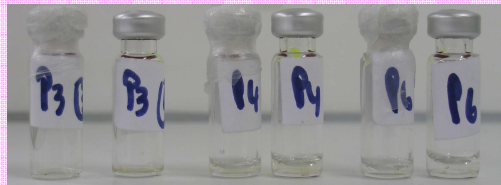
*At 4°C in a dark room*



*At 20°C continuously exposed to light*

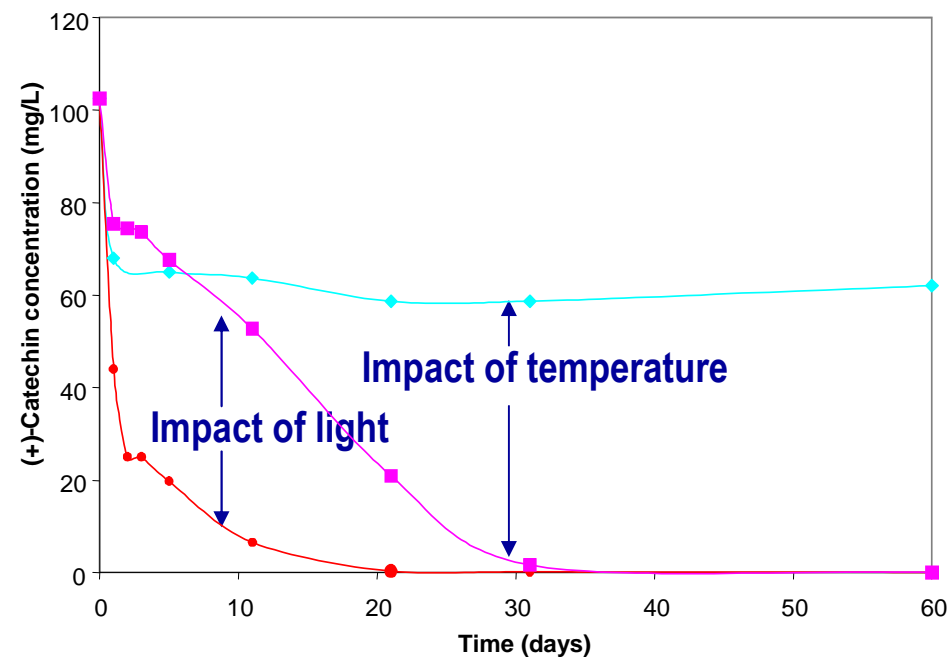
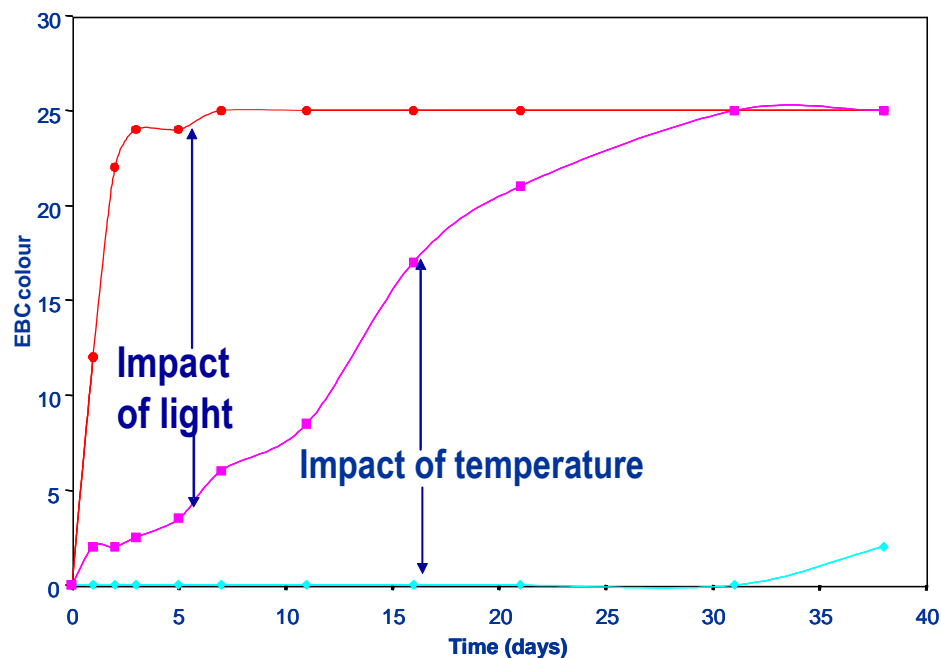


*At 20°C in a dark room*



(+)- catechin at 4°C, 20°C with and without light

Yellow-brown colour ↗ : impact of temperature and light!

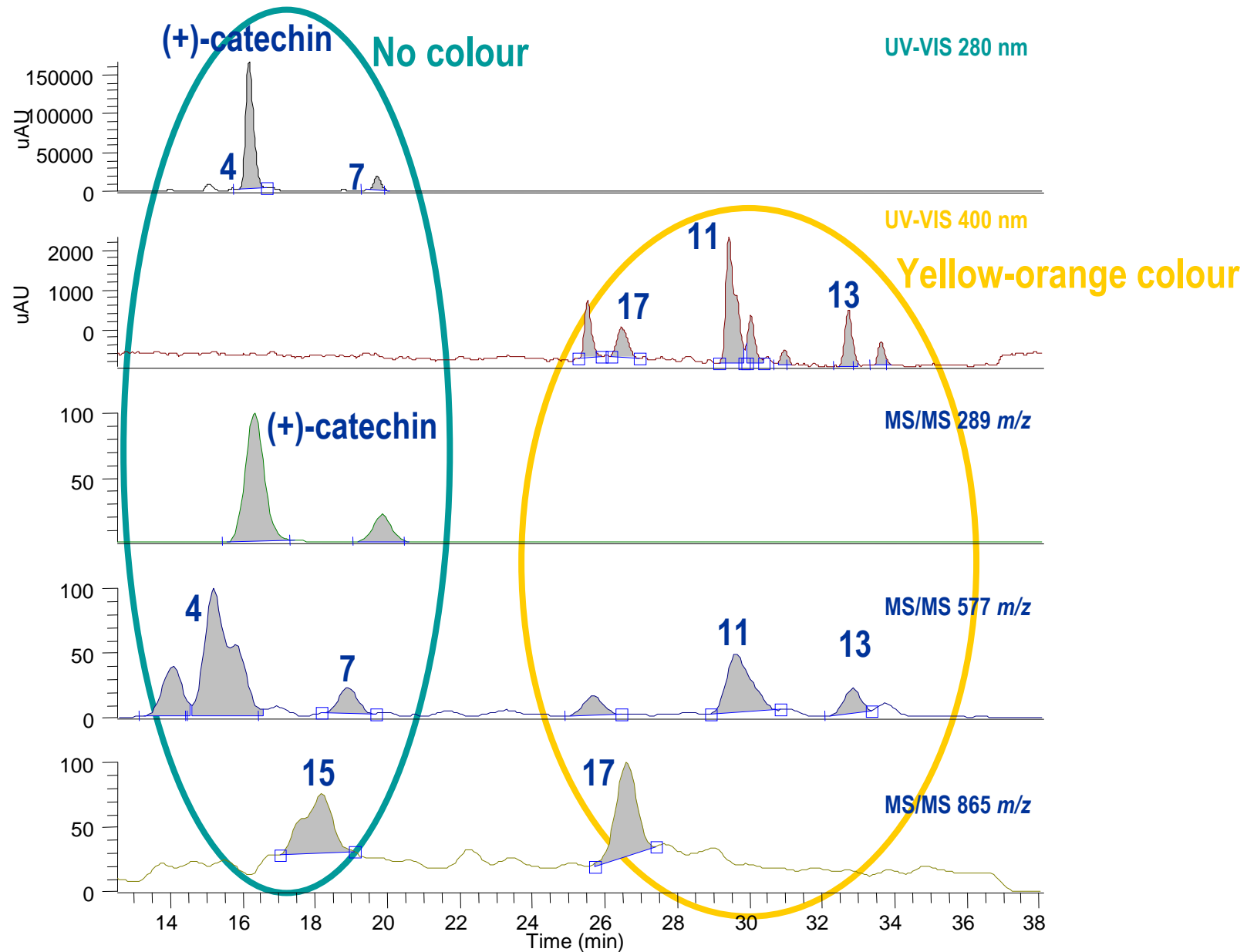


- 20°C, L
- 20°C, D
- ◆ 4°C, D

EBC = Absorption at 430 nm (400-500 nm yellow-brown)

# Identification of the coloured compounds

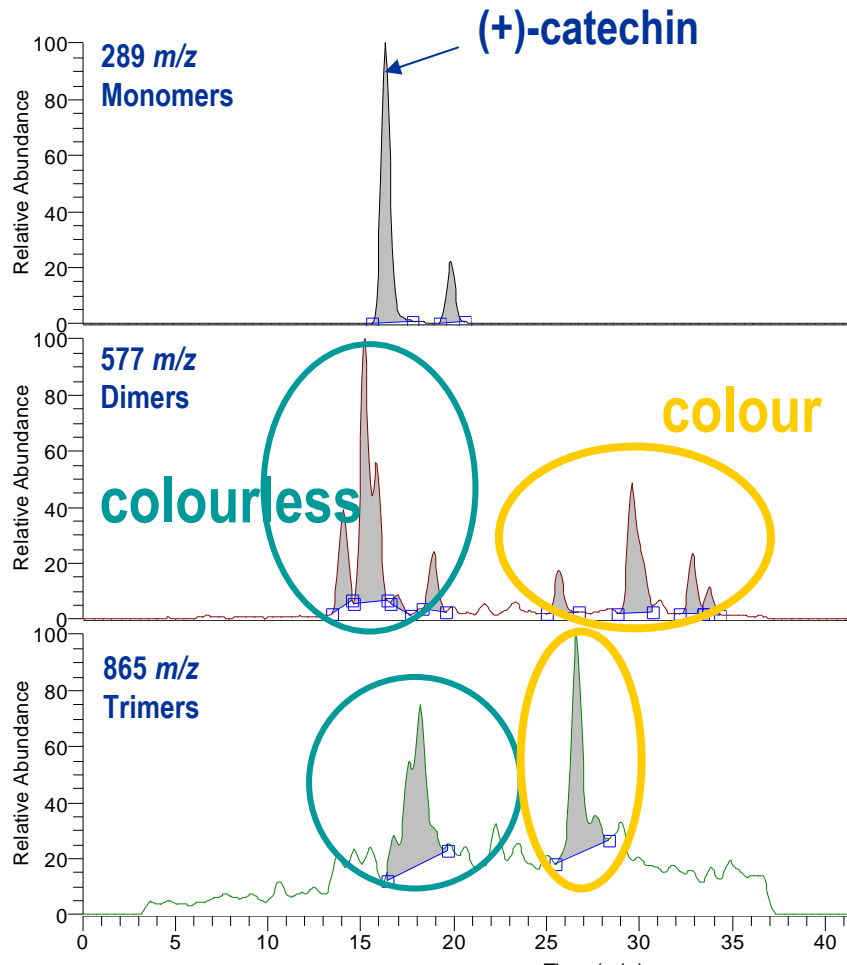
## Colourless and yellow-brown new structures



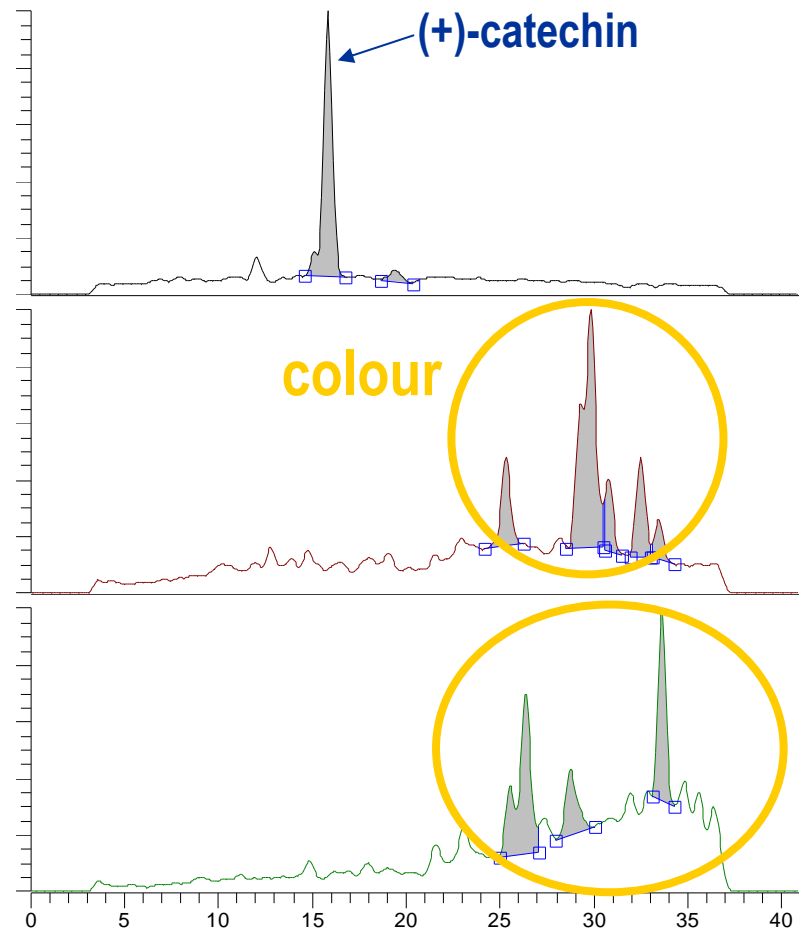
# Degradation of (+) catechin

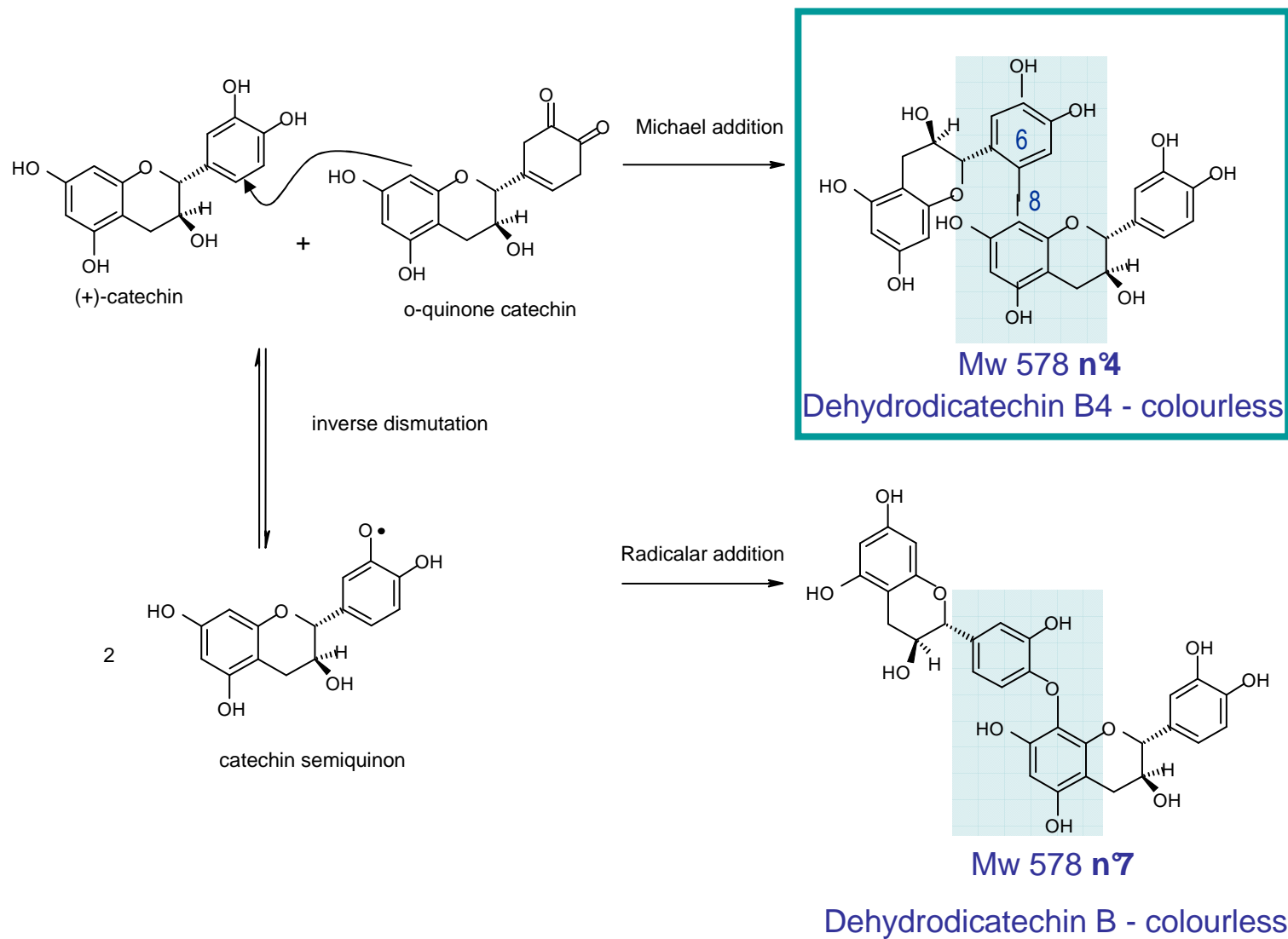
## Formation of **colourless** and **colour** dimers and trimers

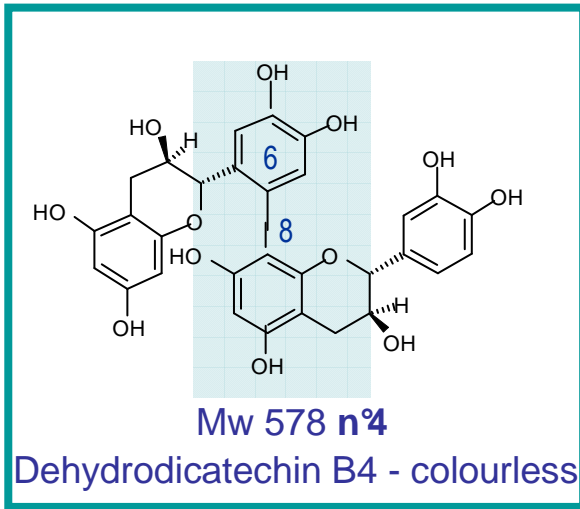
*5 days at 20°C darkness*



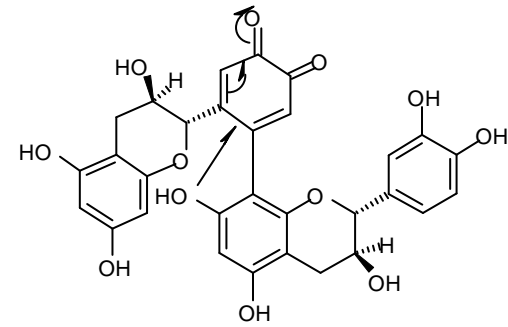
*31 days at 20°C darkness*



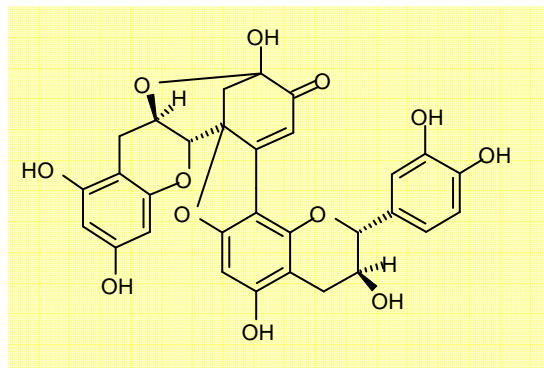




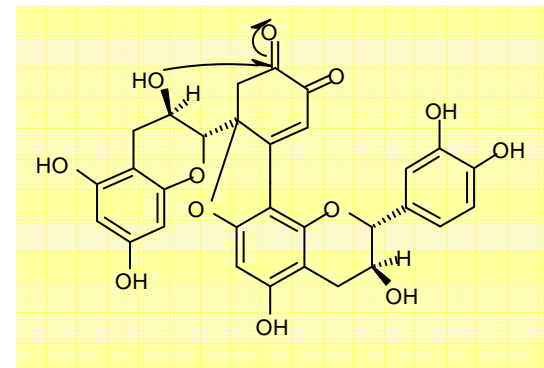
Oxidation



Radicalar addition

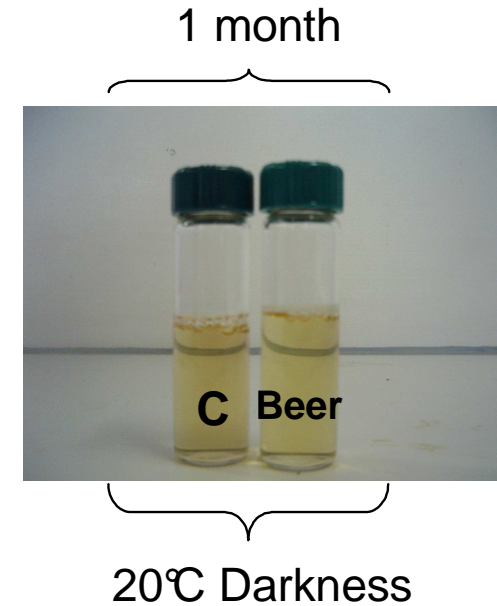
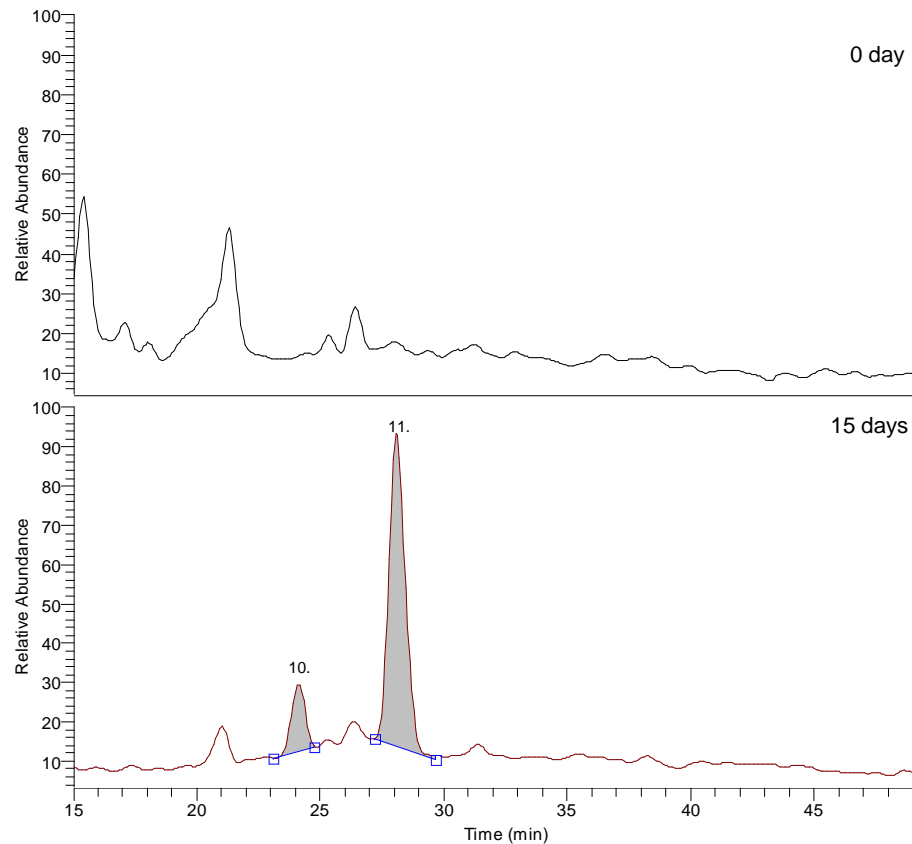


Radicalar addition





# Similar reactions in beer !



*In beer, (+)-catechin is the main precursor of colour. MS/MS enabled us to identify them as issued from oxidation and intramolecular radical additions of dehydrodiccatechin B4.*

# Conclusions on fresh beer

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## Small flavanoids (Sephadex LH20 extract)

→ ~ 10 ppm P1, P2, P3 (procyanidins + prodelphinidins)

( 3x ↗ if silicagel filtered beer instead of PVPP and much more dimers)

→ P1: (+)-catechin > (-)-epicatechin > (-)-gallocatechin and (-)-epigallocatechin Hop /Malt

P2: B3 (C-4 $\alpha$ -8-C) > B1<sup>N</sup> (E-4 $\alpha$ -8-C) > B4<sup>N</sup> (C-4 $\alpha$ -8-E) & B3 (GC-4 $\alpha$ -8-C)

P3: C2<sup>N</sup> (C-4 $\alpha$ -8-C-4 $\alpha$ -8-C) & GC-4 $\alpha$ -8-C-4 $\alpha$ -8-C<sup>N</sup> & GC-4 $\alpha$ -8-GC-4 $\alpha$ -8-C<sup>N</sup>

## Higher oligomers (Dialysis)

→ Transformed oligomers account for most of the heavy flavanoid content (apparent DP = 5 << 12)

→ Catechin (+ epi + gallocat) = extension unit

Catechin (+ epi) = terminal unit

