Small flavanoids are responsible for the beer colour instability.

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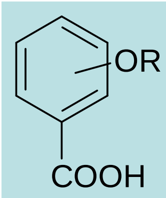
XIIIth J. DE CLERCK CHAIR - session IV -9/09/08

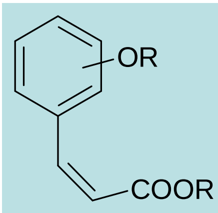
The polyphenols

Phenolic acids

→ **Benzoic acid**



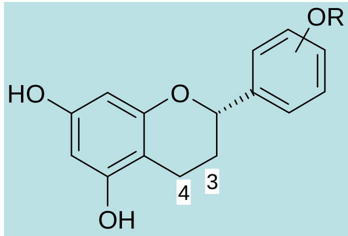
→ **Cinnamic acid**



Antioxidant, aroma precursors

Flavonoids

→ **Flavanoids**



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

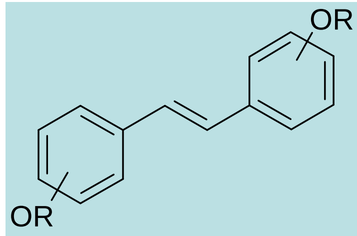
→ **Flavonols**

→ **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.

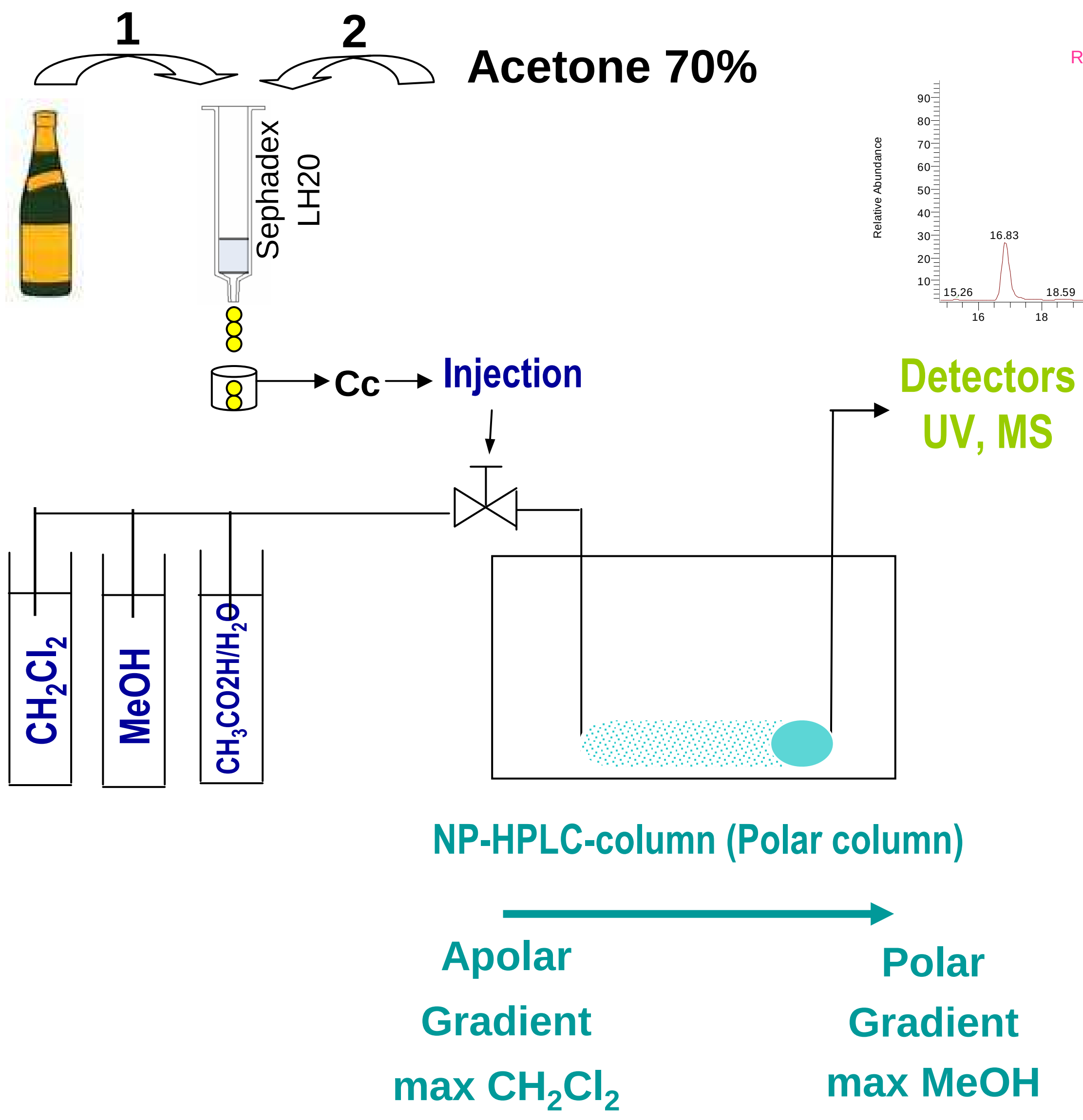
1.1. NP-HPLC-UV and ESI(-)-MS/MS of native small flavanoids.

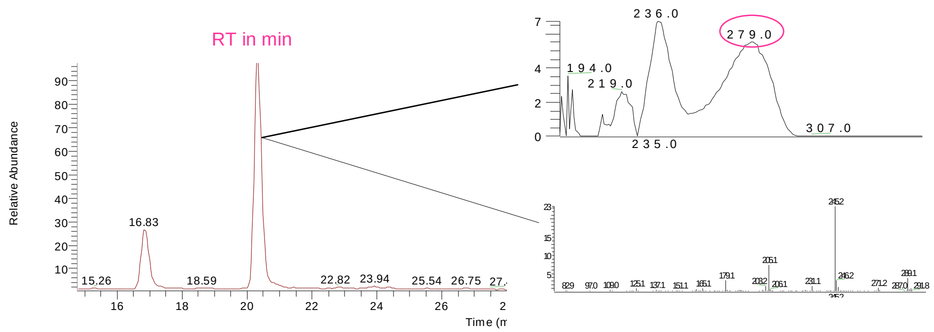
1.2. RP-HPLC-ESI(-)-MS/MS of native small flavanoids.

1.3. Dialysis of higher flavanoids.

1.4. Thiolysis.

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





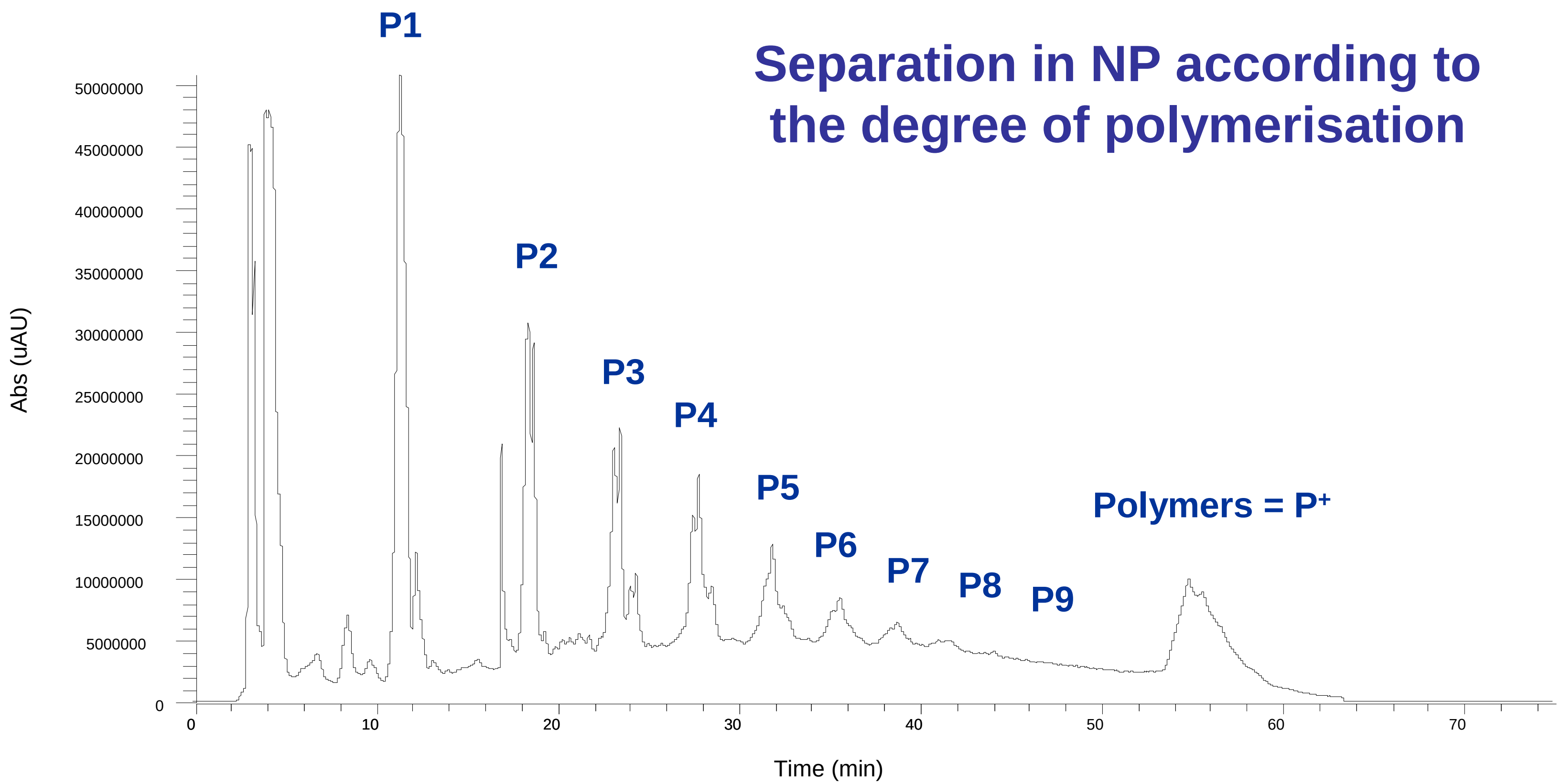
No proanthocyanidin commercial standards

… use of a pure cocoa procyanidin extract and isolated fractions

UV (λ = 280 nm)

Cocoa procyanidin extract
used as standard

Separation in NP according to the degree of polymerisation



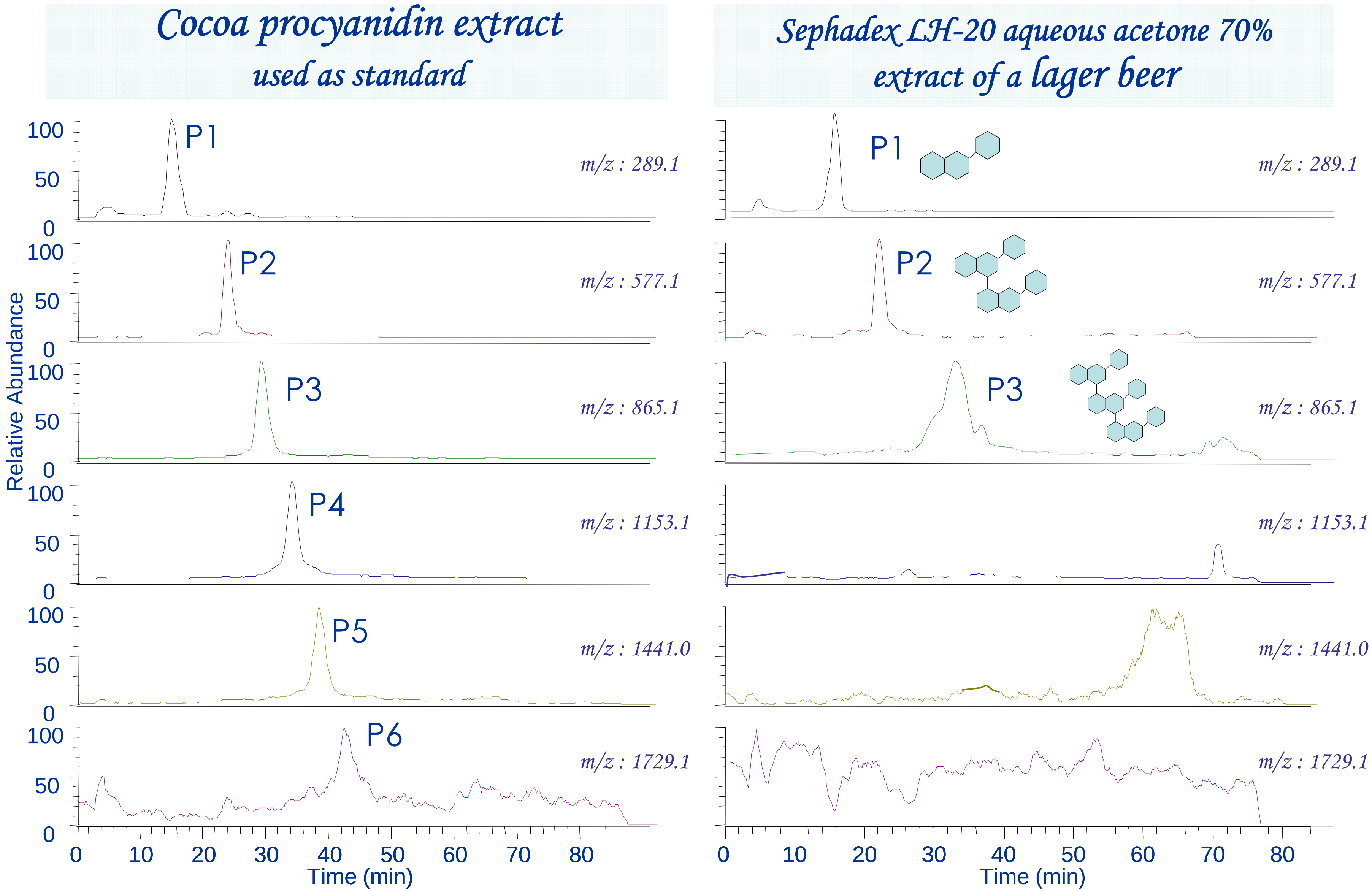
Apolar → **Polar**

Why the use of an MS/MS detector ?

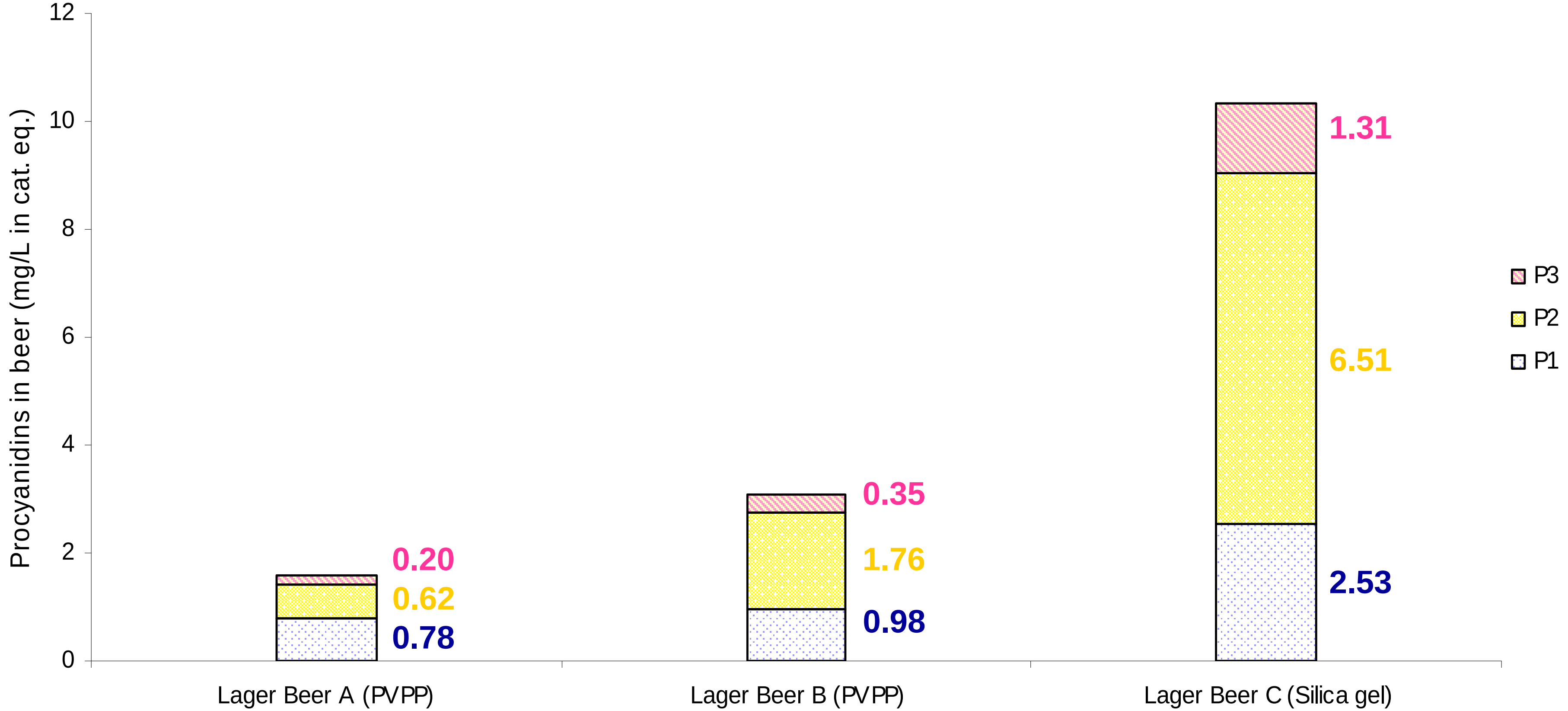
- **Beer**
  - Low concentrations
  - Interfering compounds

- **Advantages of the MS**
  - Structural information (MS/MS)
  - More precise quantification (LOD & LOQ ↘)

ESI(-)-MS/MS with NH4⁺



Callemien and Collin, *J. Agric. Food Chem. 2007*, *55, 9066–9073*



PVPP

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

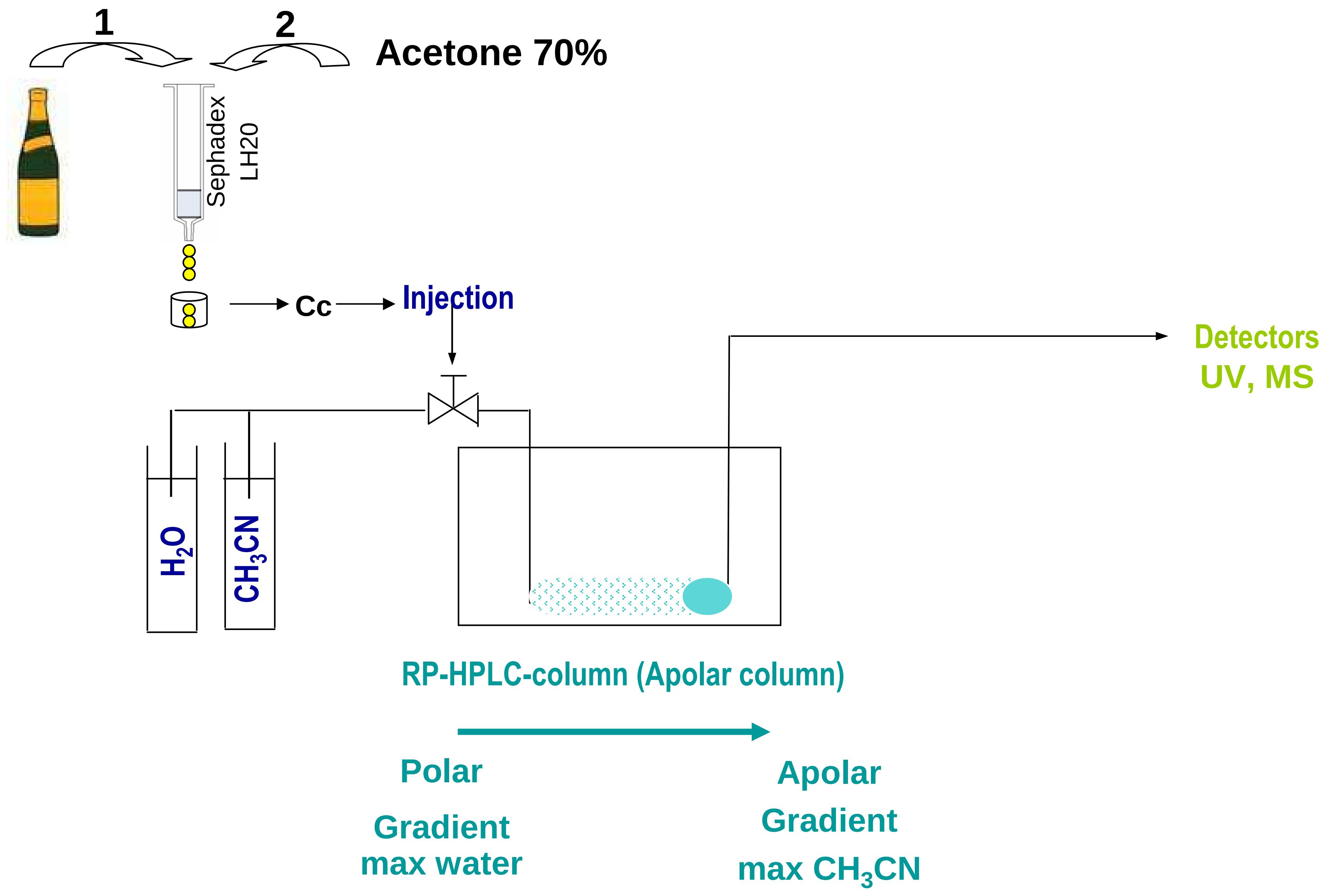
Silicagel

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

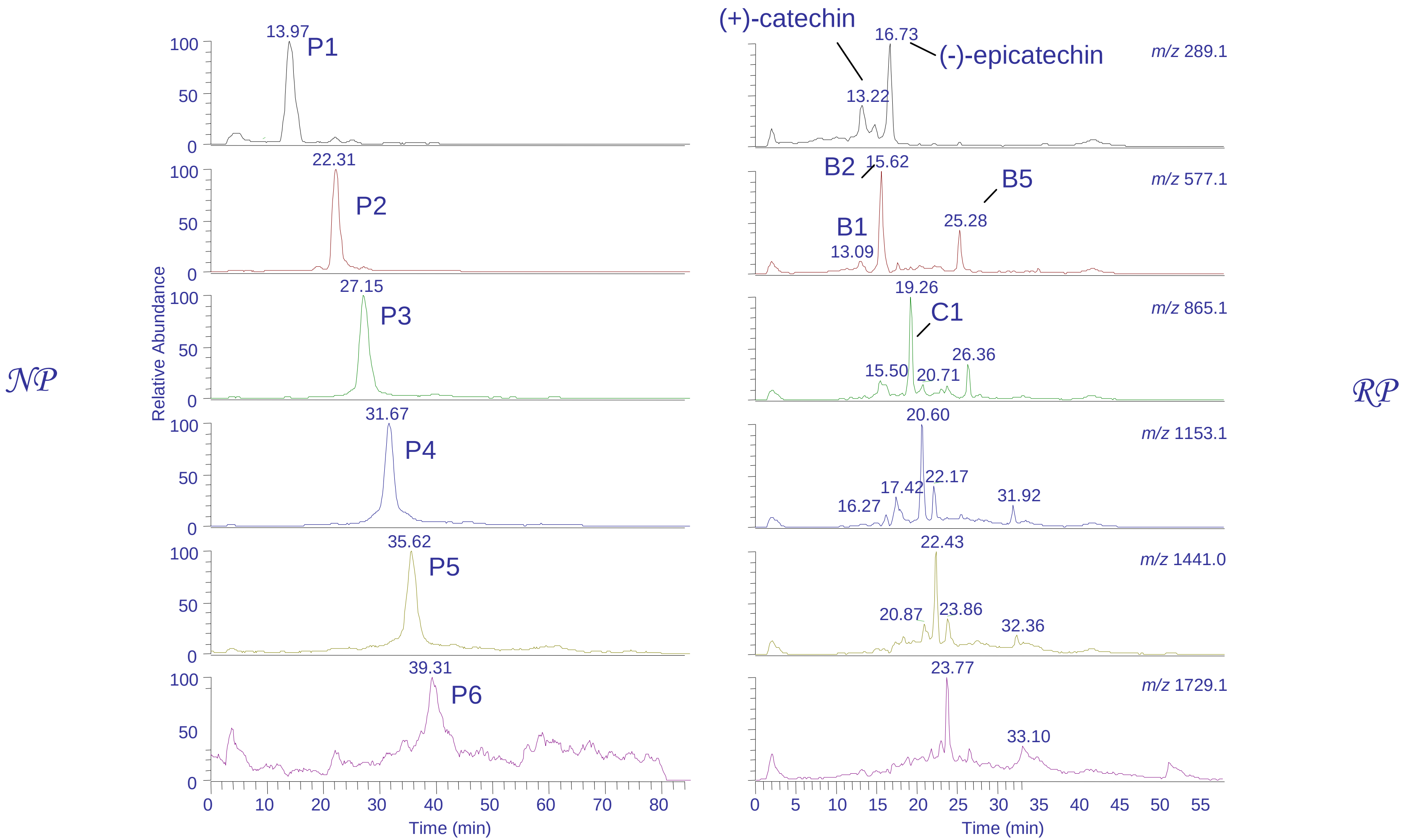
High P2/P1 ratio = 2.6 compared to 0.8

Callemien, Guyot and Collin, *Food Chemistry 110 (2008) 1012–1018*

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**

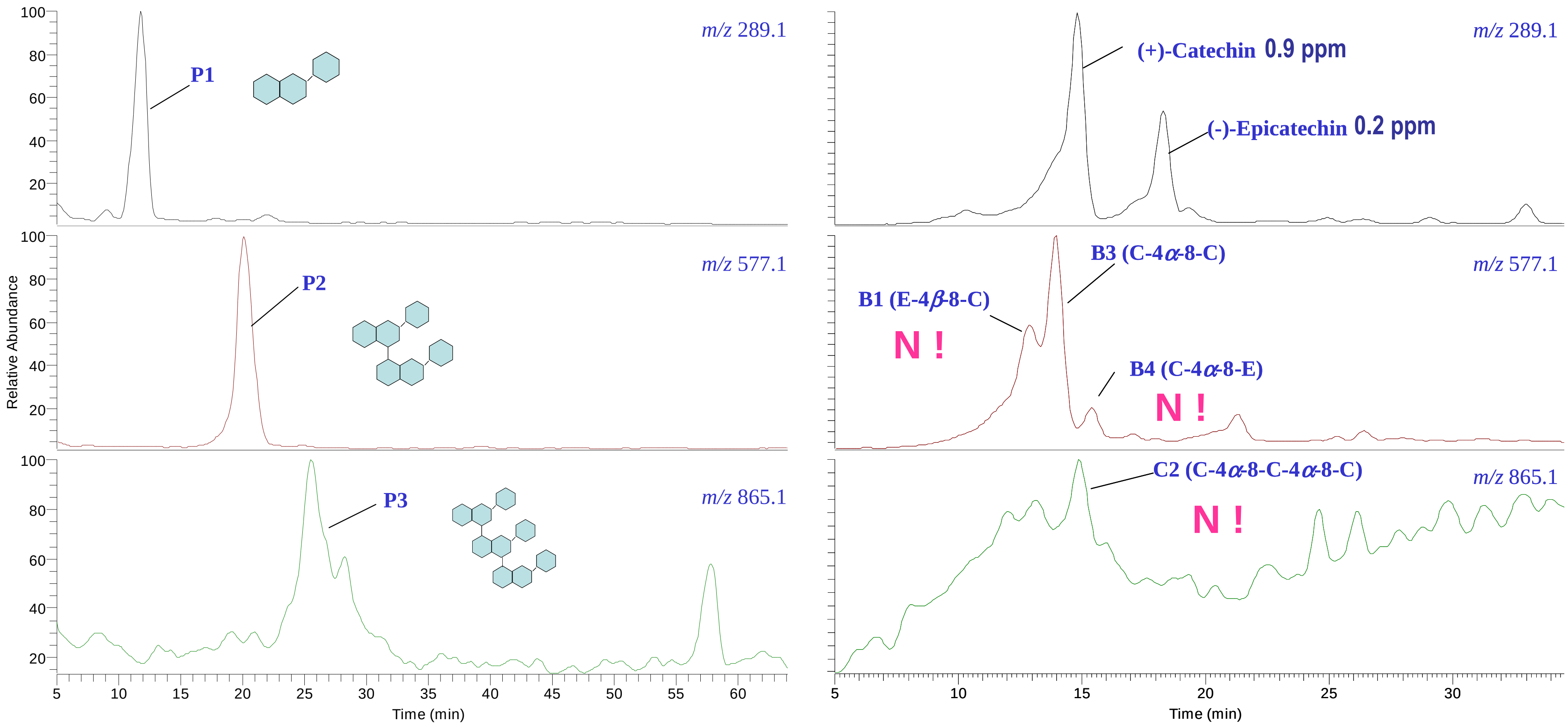


Procyanidins: **B1**: (-) - epicatechin *(4β → 8)* - (+) - catechin; **B2** : (-) - epicatechin *(4β → 8)* - (-)-epicatechin; **B5**: (-) - epicatechin *(4β → 6)* - (-)-epicatechin

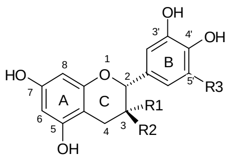
Callemien and Collin, *J. Agric. Food Chem. 2007, 55, 9066–9073*

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

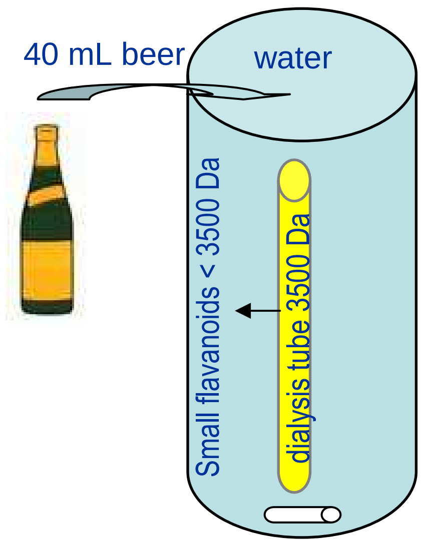
PROCYANIDINS

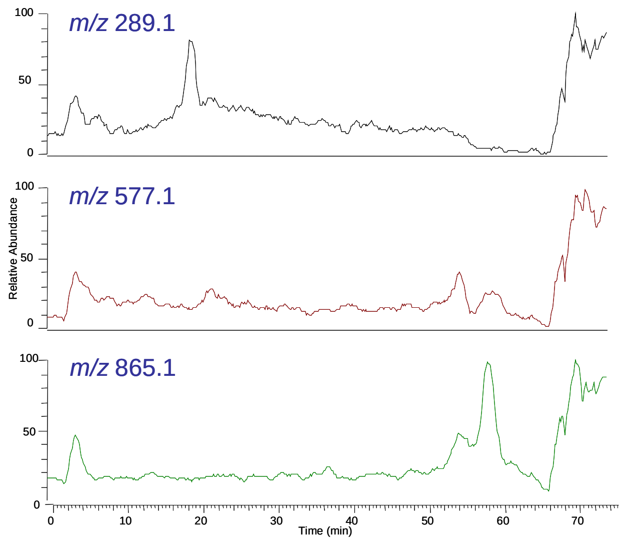


Callemien and Collin, *ASBCJ-2008-0215-01*

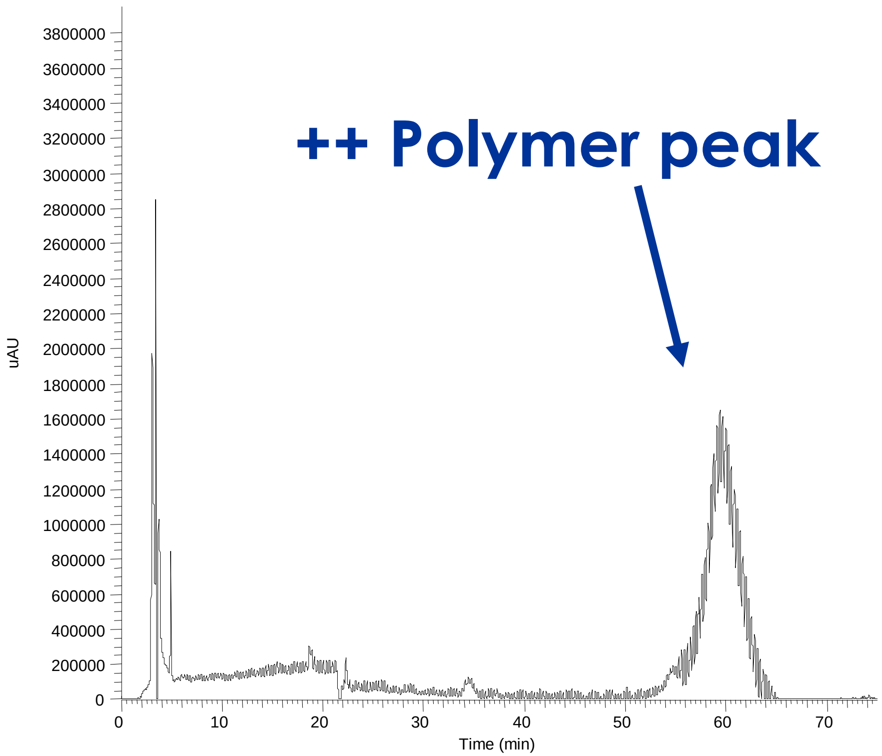
	Flavanoids		Malt	Hop	Fresh beer
Flavan-3-ols	(+)-Catechin	R_1 = H, R_2 = OH, R_3 = H	L	L	L, **D**
	(-)-Epicatechin	R_1 = OH, R_2 = R_3 = H	-	L	L, **D**
	(-)-Gallocatechin	R_1 = H, R2 = R_3 = OH	-	L	L, **D**
	(-)-Epigallocatechin	R_1 = OH, R_2 = H, R_3 = OH	-	-	L, **D**
Procyanidins B	**B1** (-)-Epicatechin-(4β–8)-(+)-catechin		-	L	-, **N!**
	B2 (-)-Epicatechin-(4β–8)-(-)-epicatechin		-	L	-
	B3 (+)-Catechin-(4α–8)-(+)-catechin		L	L	L, **D**
	B4 (+)-Catechin-(4α–8)-(-)-epicatechin		-	L	-, **N!**
Prodelphinidins B	**B3** (-)-Gallocatechin-(4α-8)-(+)-catechin		L	L	L, **D**
	B9 (-)-Epigallocatechin-(4β-8)-(+)-catechin		-	-	L
	(+)-Catechin-(4α-8)-(-)-gallocatechin		-	L	-
	(+)-Catechin-(4α-6)-(-)-gallocatechin		-	L	-
	(-)-Gallocatechin-(4α-6)-(+)-catechin		-	L	-
Prodelphinidins A	*ent*- (-)-Epigallocatechin-(4α-8, 2α-O-7)-(+)-catechin		-	-	L
	ent- (-)-Epigallocatechin-(4α-6, 2α-O-7)-(+)-catechin		-	-	L
Propelargonidin B	(+)-Afzelechin-(4α–8)-(+)-catechin		-	L	-
Procyanidins C	**C2** (+)-Catechin-(4α-8)-(+)-catechin-(4α– 8)-(+)-catechin		L	L	-, **N!**
	(-)-Epicatechin-(4β–8)-(+)-catechin-(4α– 8)-(+)-catechin		-	L	-
	(-)-Epicatechin-(4β–8)-(-)-epicatechin-(4β– 8)-(+)-catechin		-	L	-
Prodelphinidins C	(-)-Gallocatechin-(4α–8)-(-)-gallocatechin-(4α–8)-(+)-catechin		L	L	-, **N!**
	(-)-Gallocatechin-(4α–8)-(+)-catechin-(4α–8)-(+)-catechin		L	-	-, **N!**
	(+)-Catechin-(4α–8)-(-)-gallocatechin-(4α–8)-(+)-catechin		L	L	-

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





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

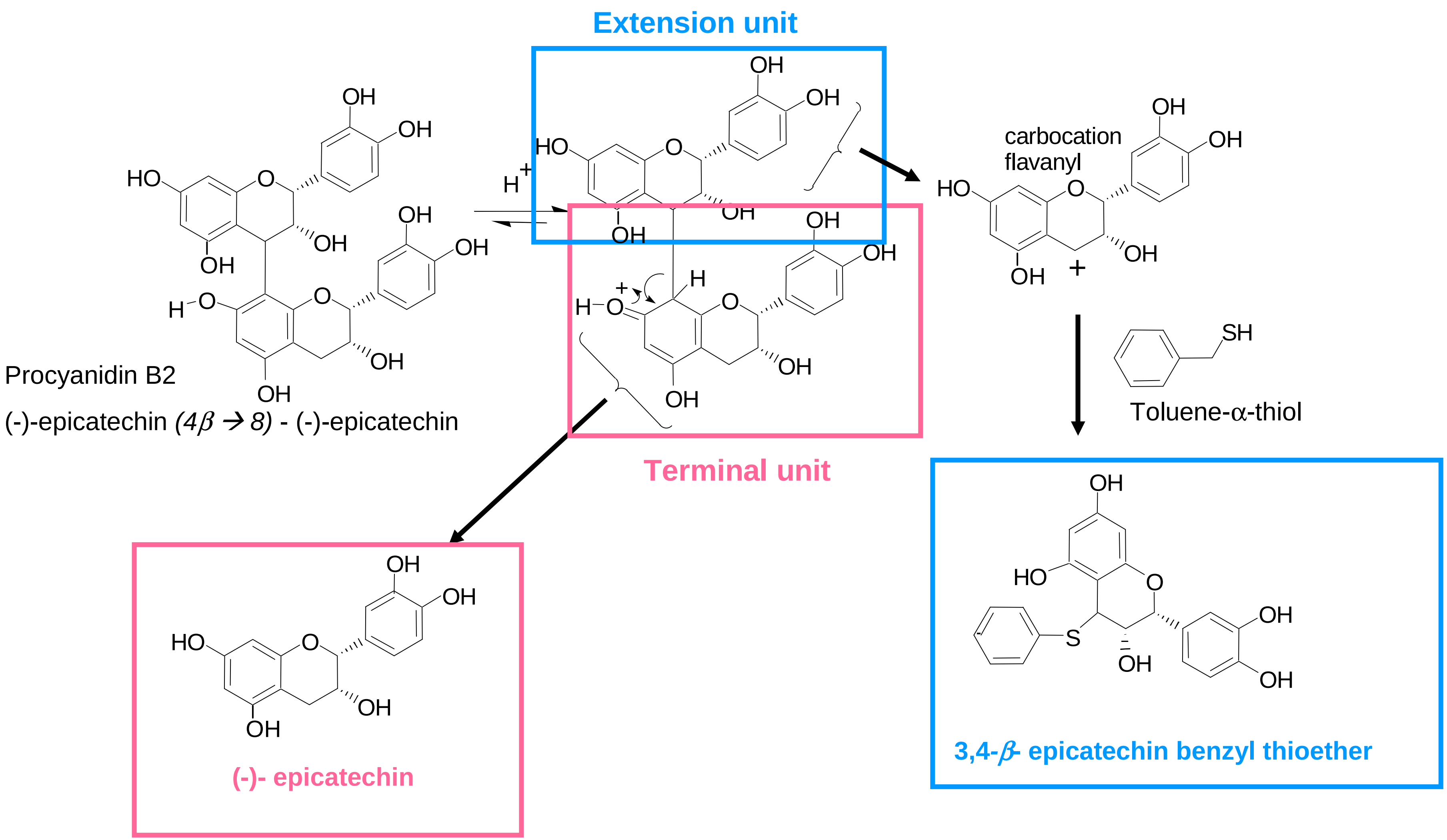


NP-LC-UV (λ = 280 nm)

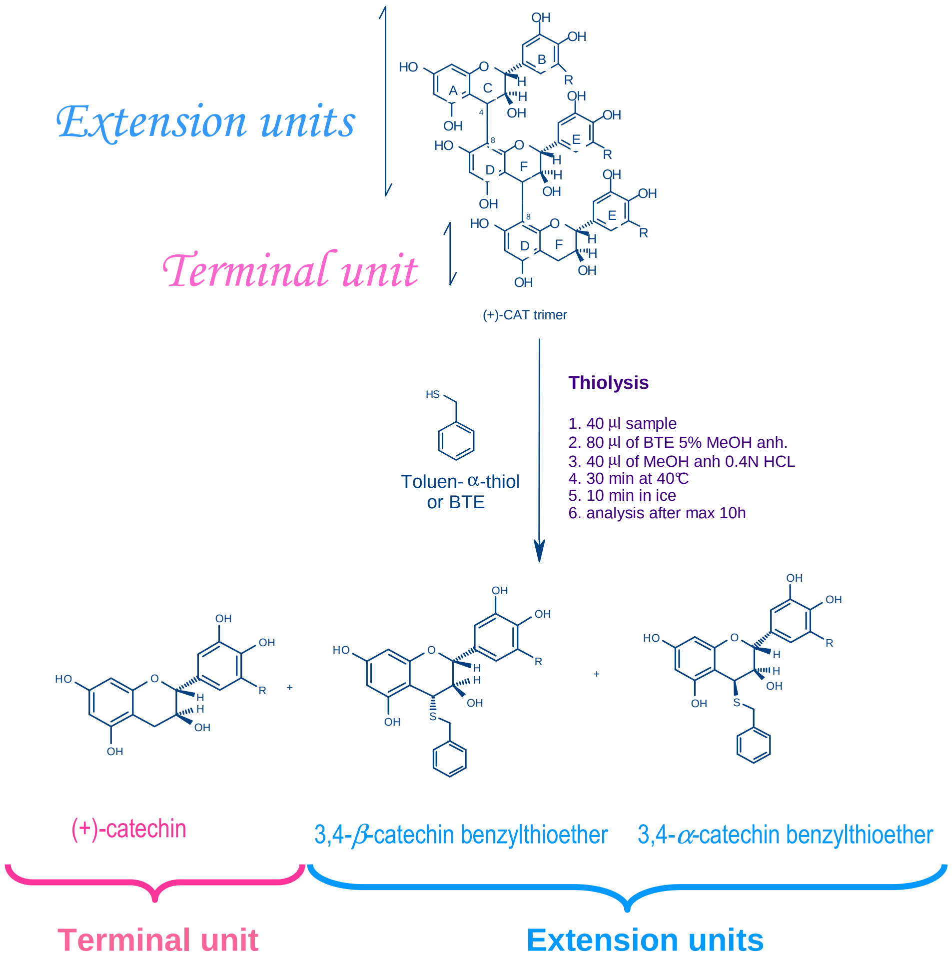
++ No P1 to P3

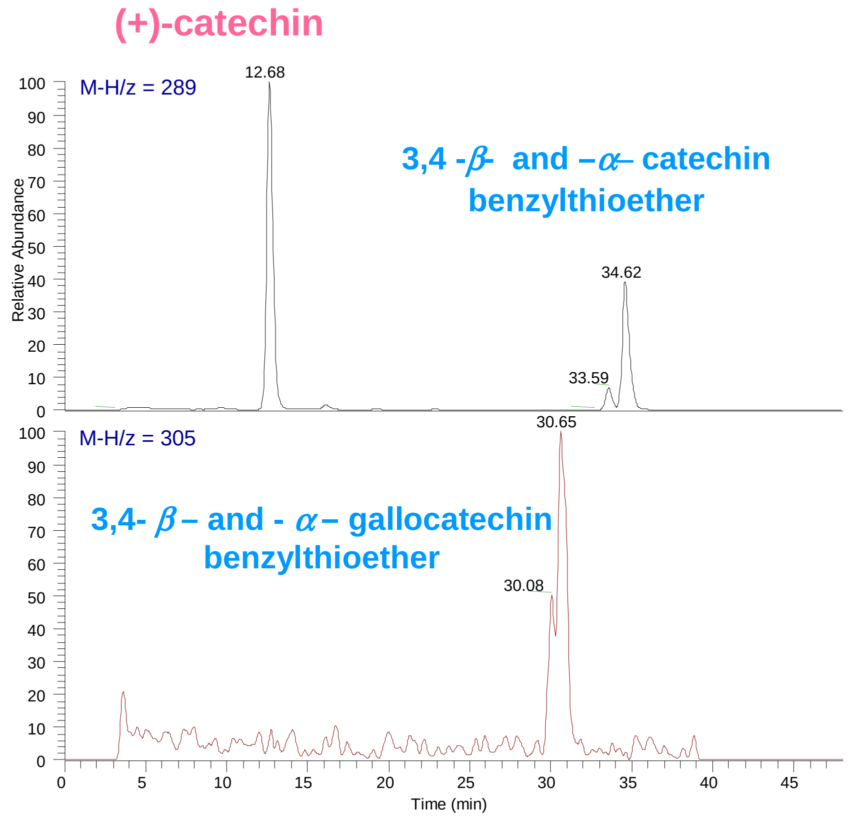
Callemien and Collin, *Proc. EBC 2007, 69, 1-8*

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



Callemien, Guyot and Collin, *Food Chemistry 110 (2008) 1012–1018*



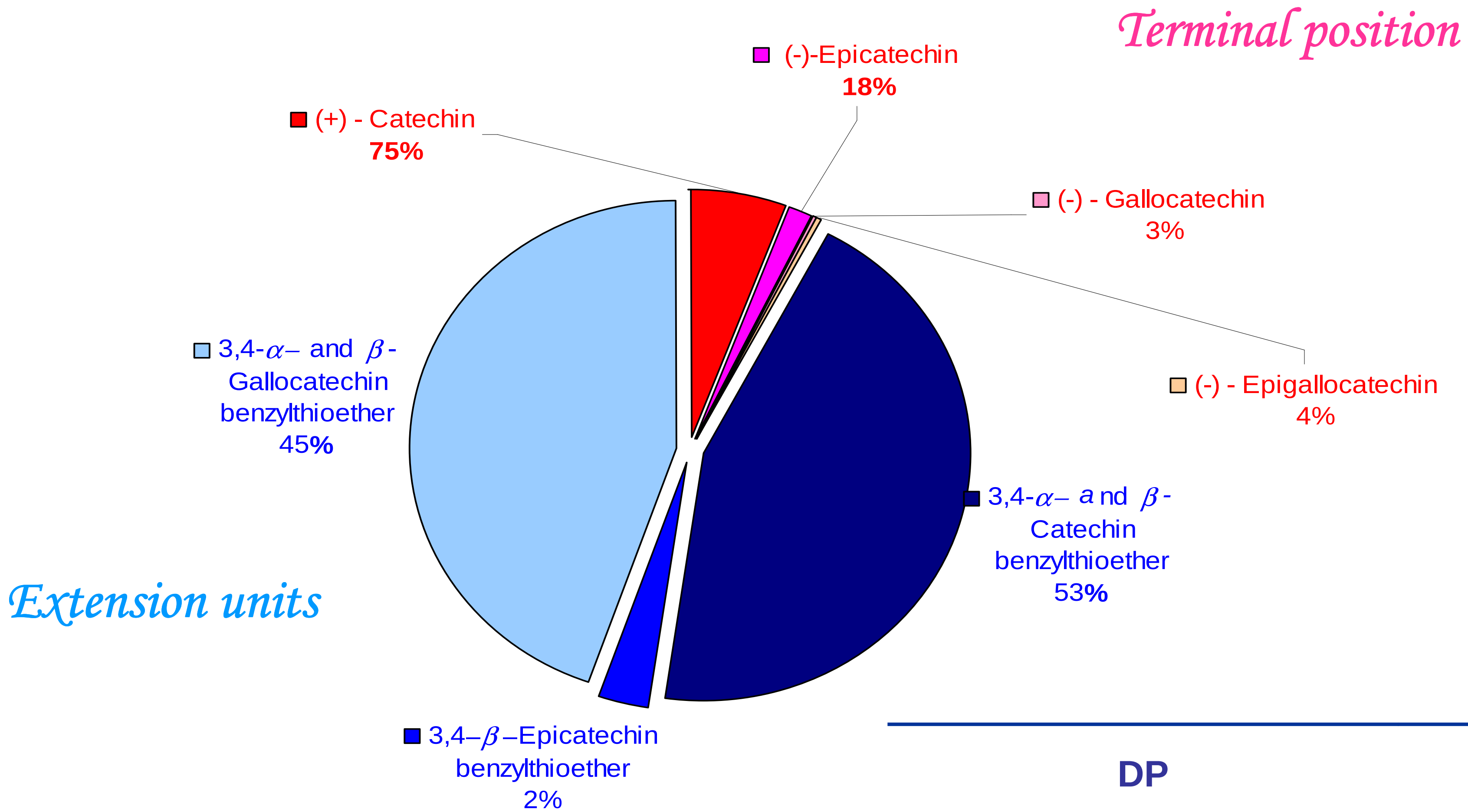


RP-LC-ESI (-)-MS/MS of thiolysis

- ✓ 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



DP	**5**
Total concentration (mg/ L beer)	**12.5**

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-α- and β-Catechin benzylthioether	-	89	25
3,4-β-Epicatechin benzylthioether	-	5	2
3,4-α- and β- Gallocatechin benzylthioether	-	6	73

More catechin in the monomers

More Catechin in the terminal units of the dimers
extension

Procyanidin B3

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

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-α- and β-Catechin benzylthioether	-	**36**	-	**41**	-	**48**
3,4-β-Epicatechin benzylthioether	-	1	-	15	-	13
3,4-α- and β- Gallocatechin benzylthioether	-	**63**	-	14	-	11
3,4-β-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

mDP Malt > mDP Hop > mDP Beer

Depolymerization not reversible during the process

→ Catechin : Malt and Hop

→ Gallocatechin : Malt

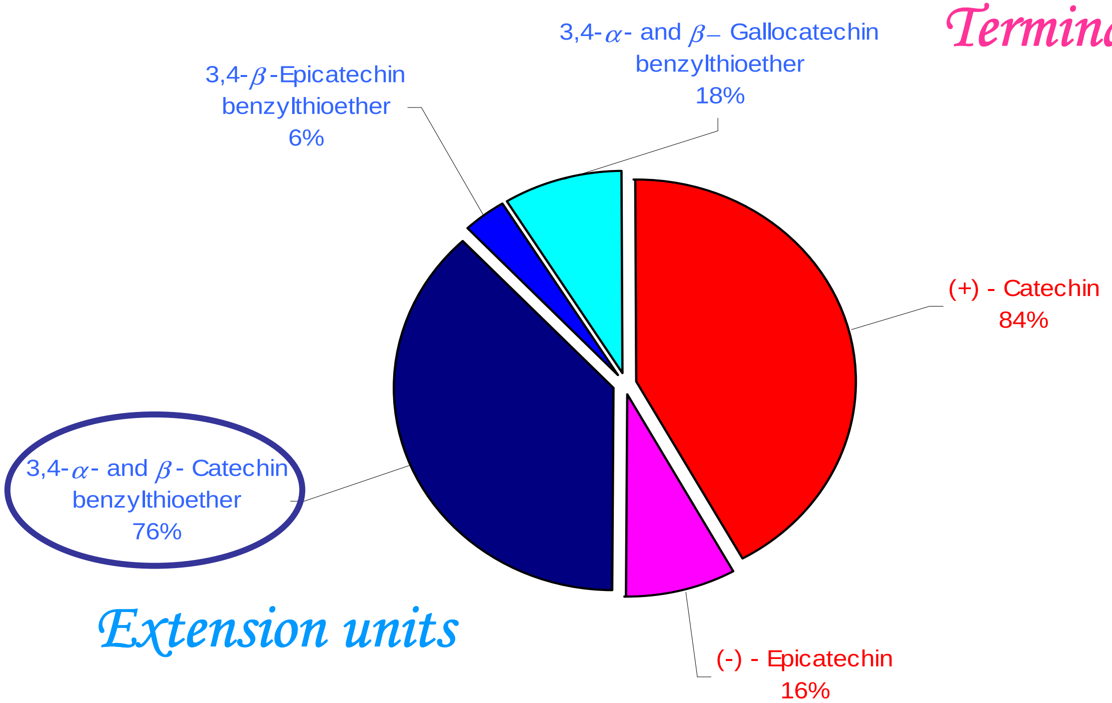
→ Epigallocatechin : Hop

[Hop] > [Malt]

Aromatic Hop > Bitter Hop

Higher oligomers

Thiolysis of a dialysis freeze-dried extract



Terminal position

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

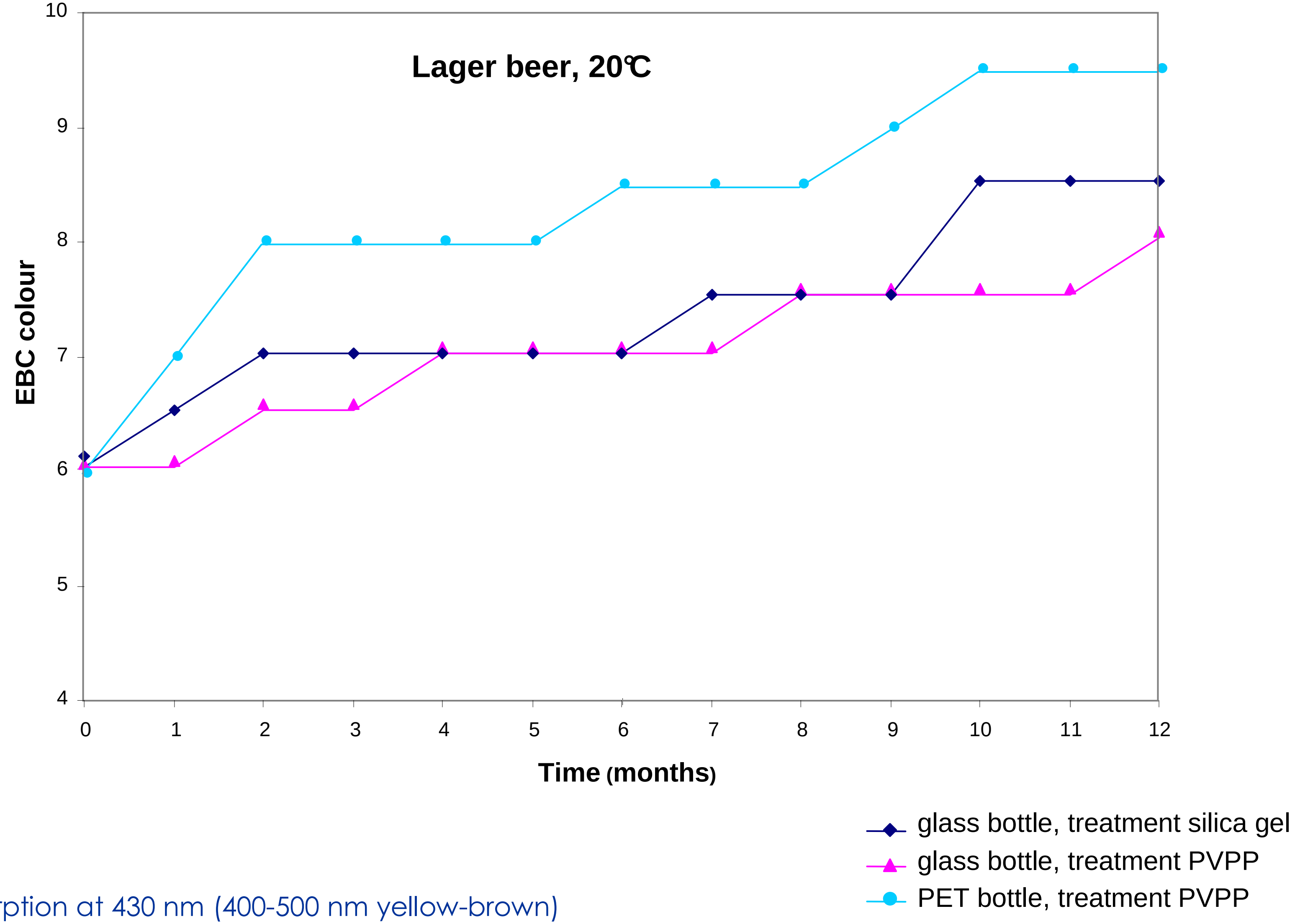
Callemien and Collin, *Proc. EBC 2007, 69, 1-8*

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

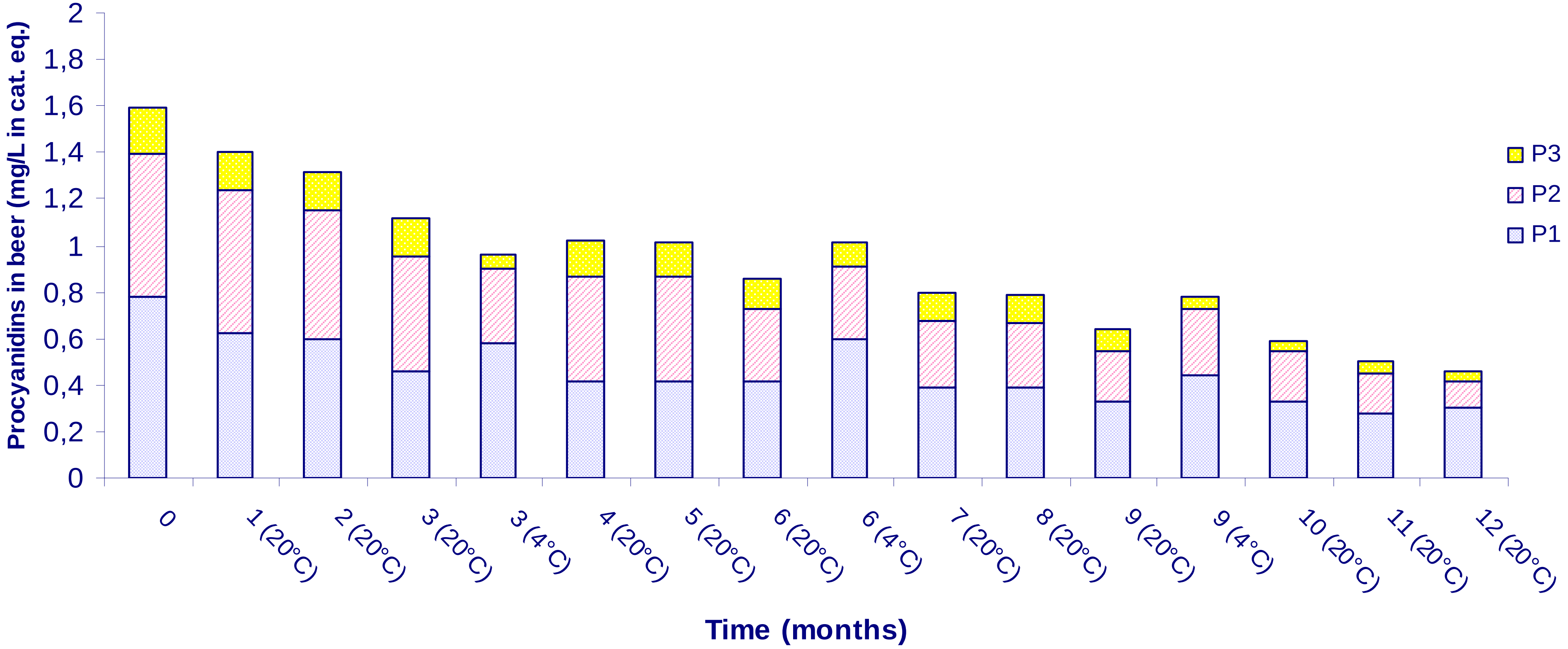
Beer yellow-brown colour increases through storage



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

Callemien and Collin, *J. Agric. Food Chem. 2007, 55, 9066–9073*

Small flavanoids are very unstable in the bottle

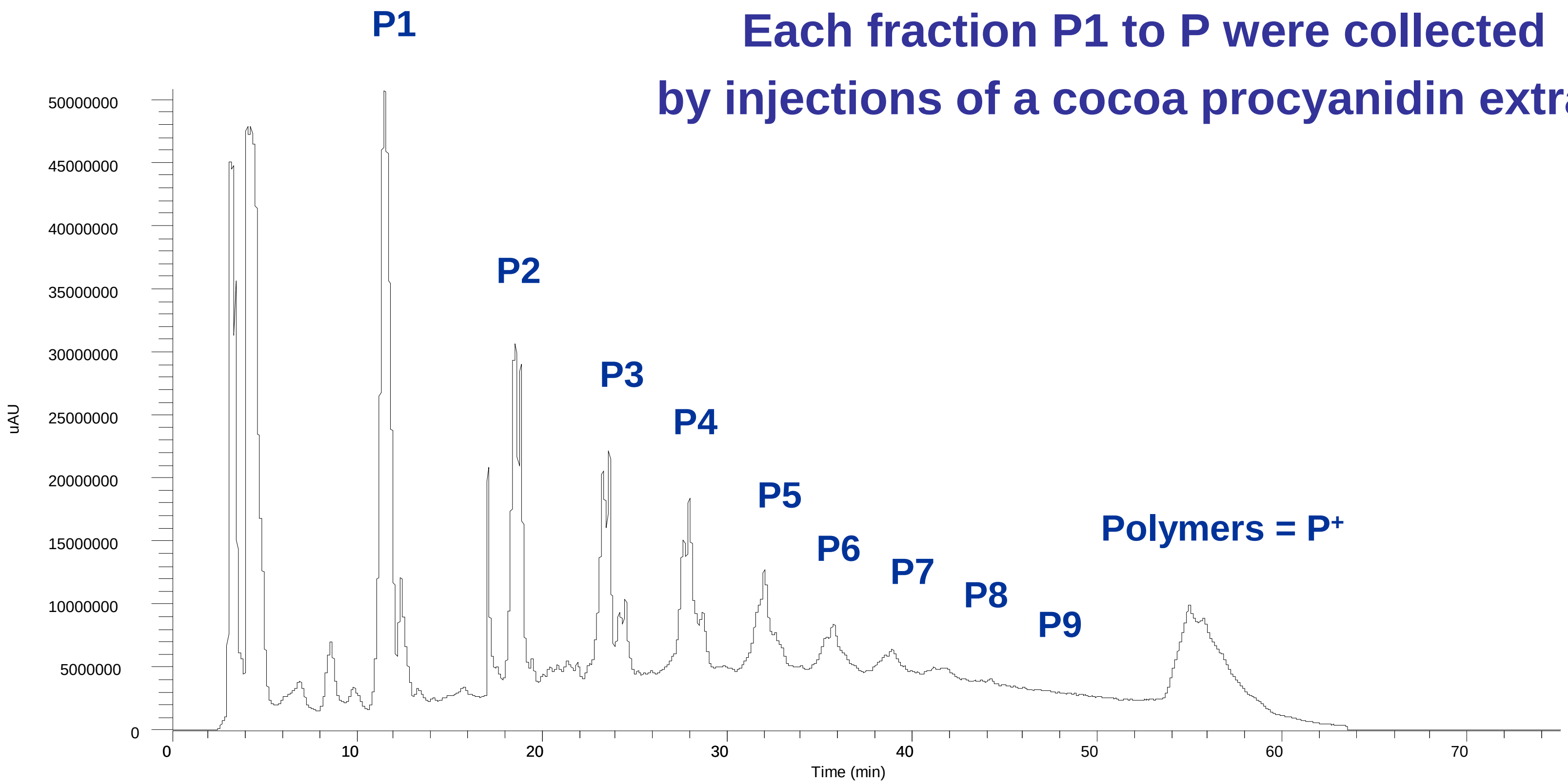


Beer with PVPP treatment-NP-HPLC-ESI(-)-MS/MS quantification

Callemien and Collin, *J. Agric. Food Chem. 2007*, *55, 9066–9073*

Which procyanidins fractions are responsible of the colour changes ?

Each fraction P1 to P were collected by injections of a cocoa procyanidin extract



Time (min)

Fresh solution not coloured

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

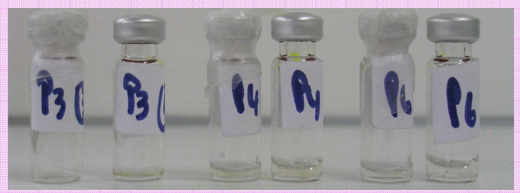
Colour of the different procyanidin fractions after 60 days

Yellow-brown colour from (+)-catechin & (-)-epicatechin

At 4°C in a dark room

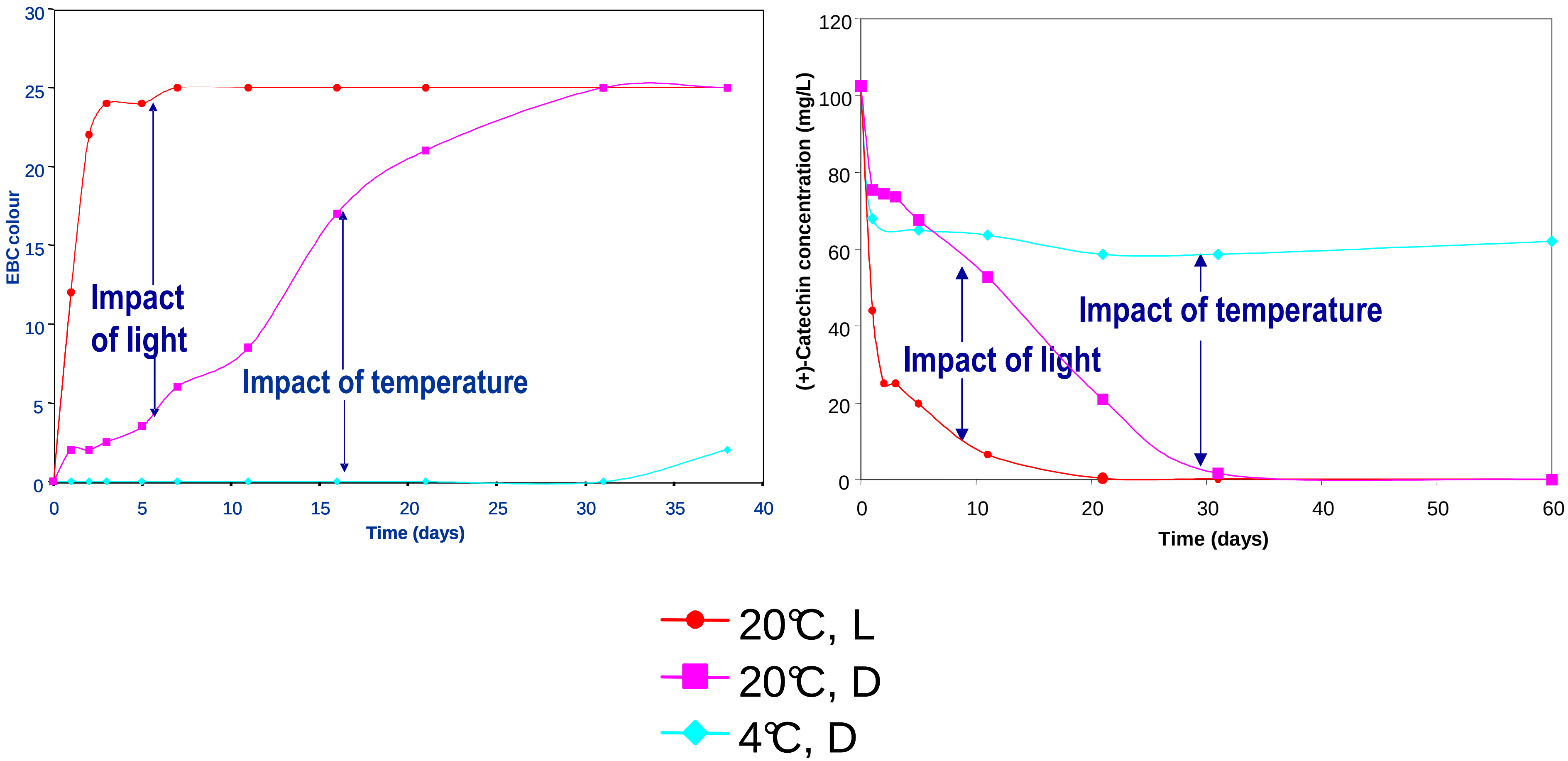
At 20°C continuously exposed to light

At 20°C in a dark room



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

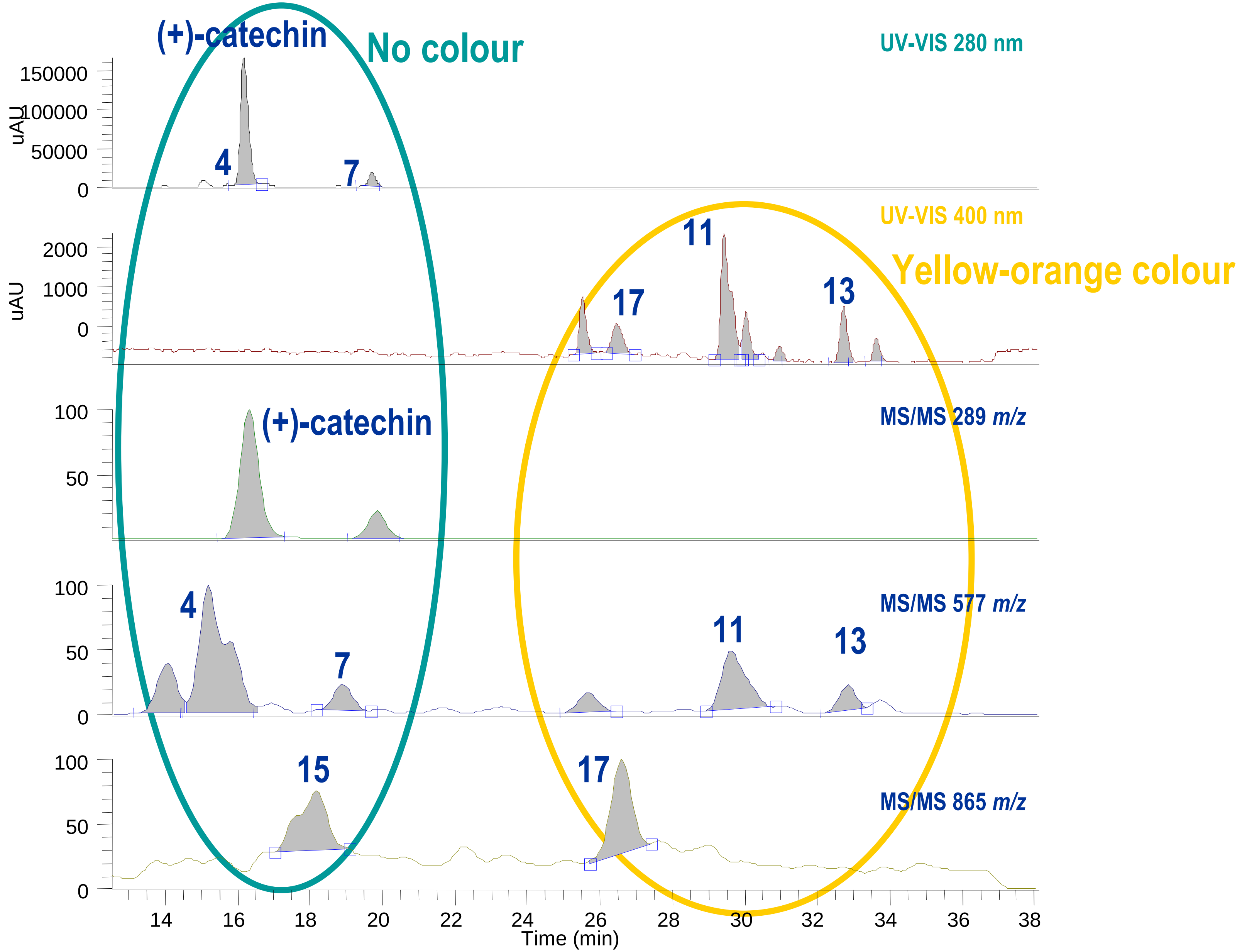
Yellow-brown colour ➚ : impact of temperature and light!



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

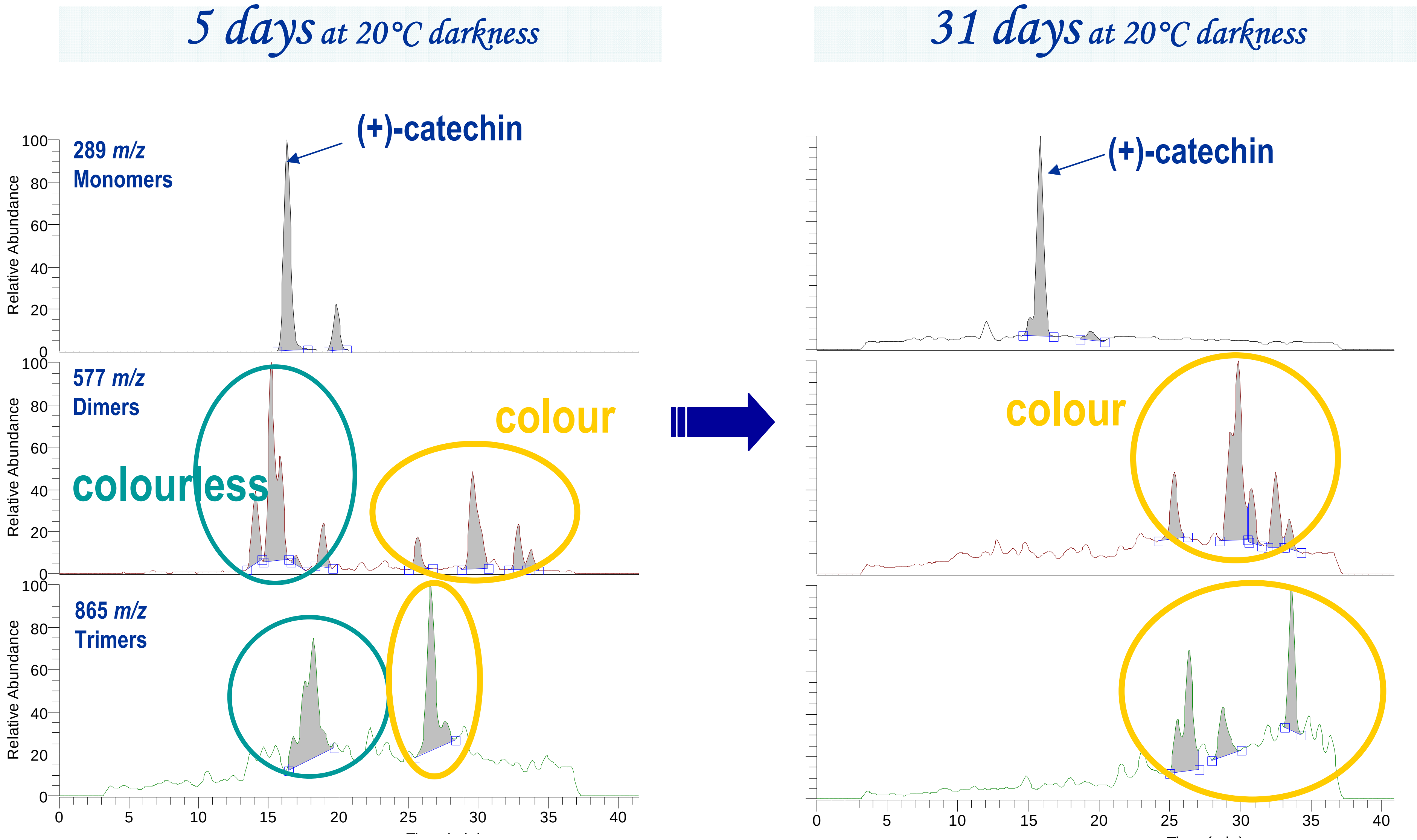
Identification of the coloured compounds

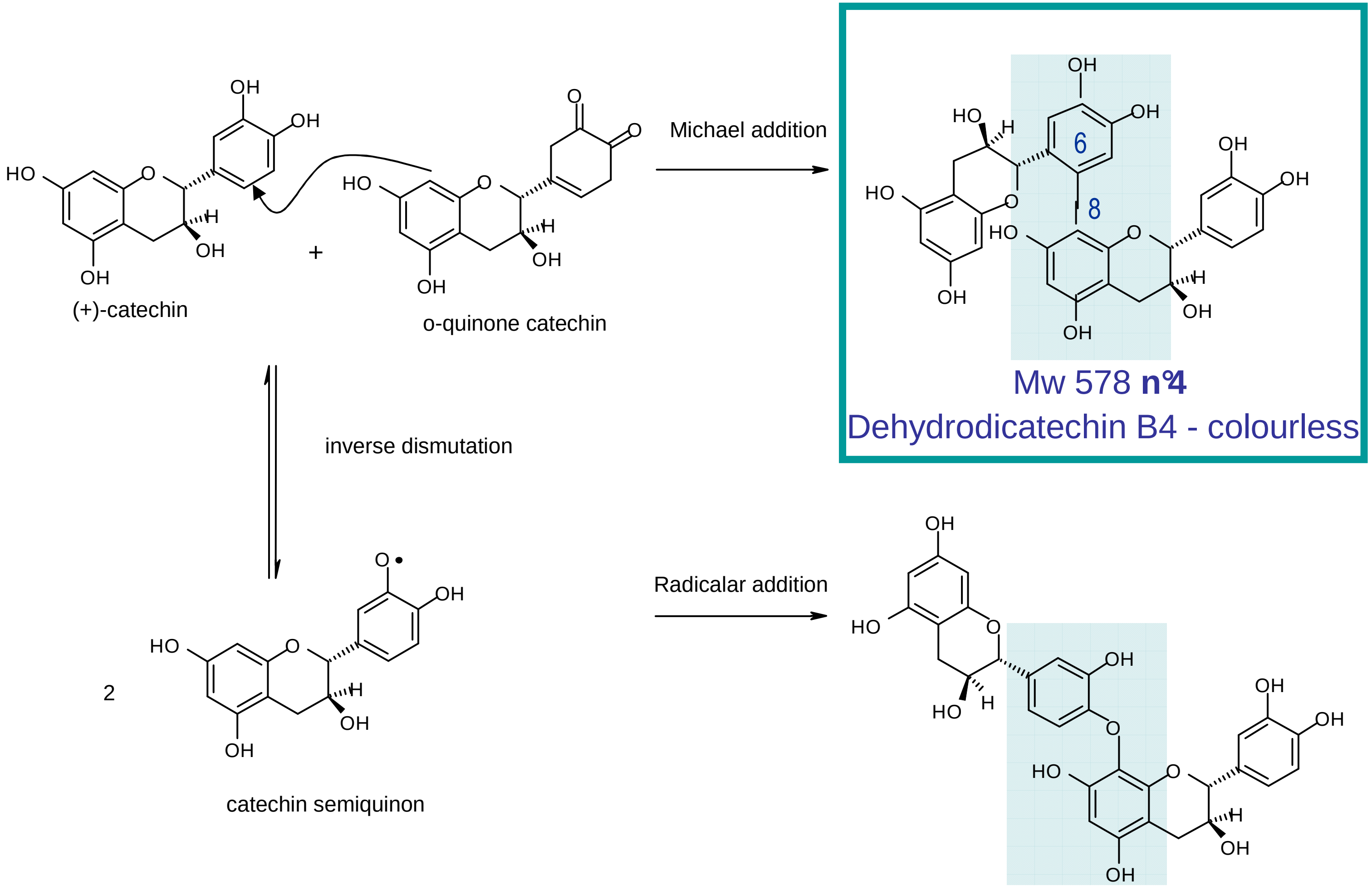
Colourless and yellow-brown new structures



Degradation of (+) catechin

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





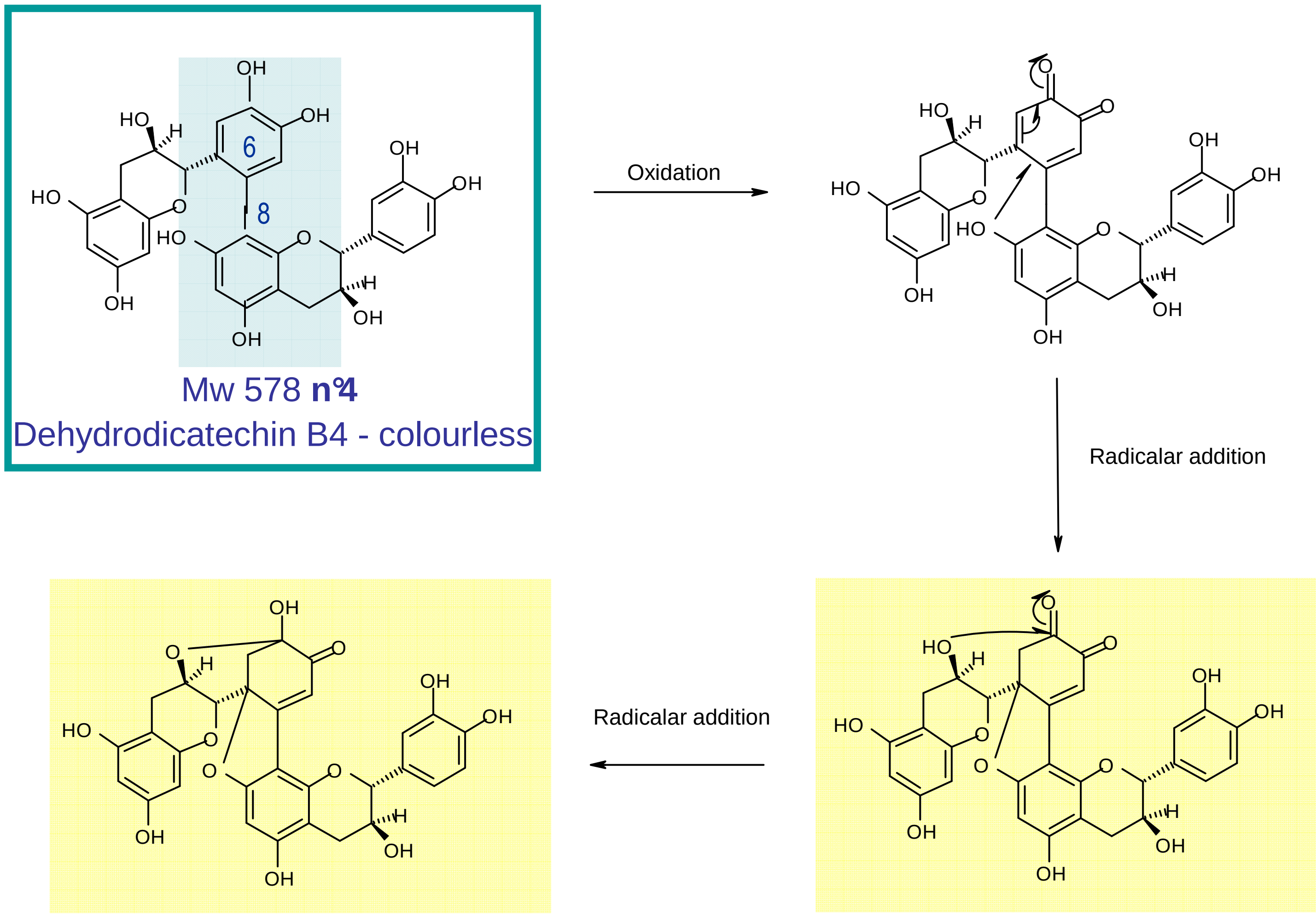
Mw 578 n°4

Dehydrodicatechin B4 - colourless

Mw 578 n°7

Dehydrodicatechin B - colourless

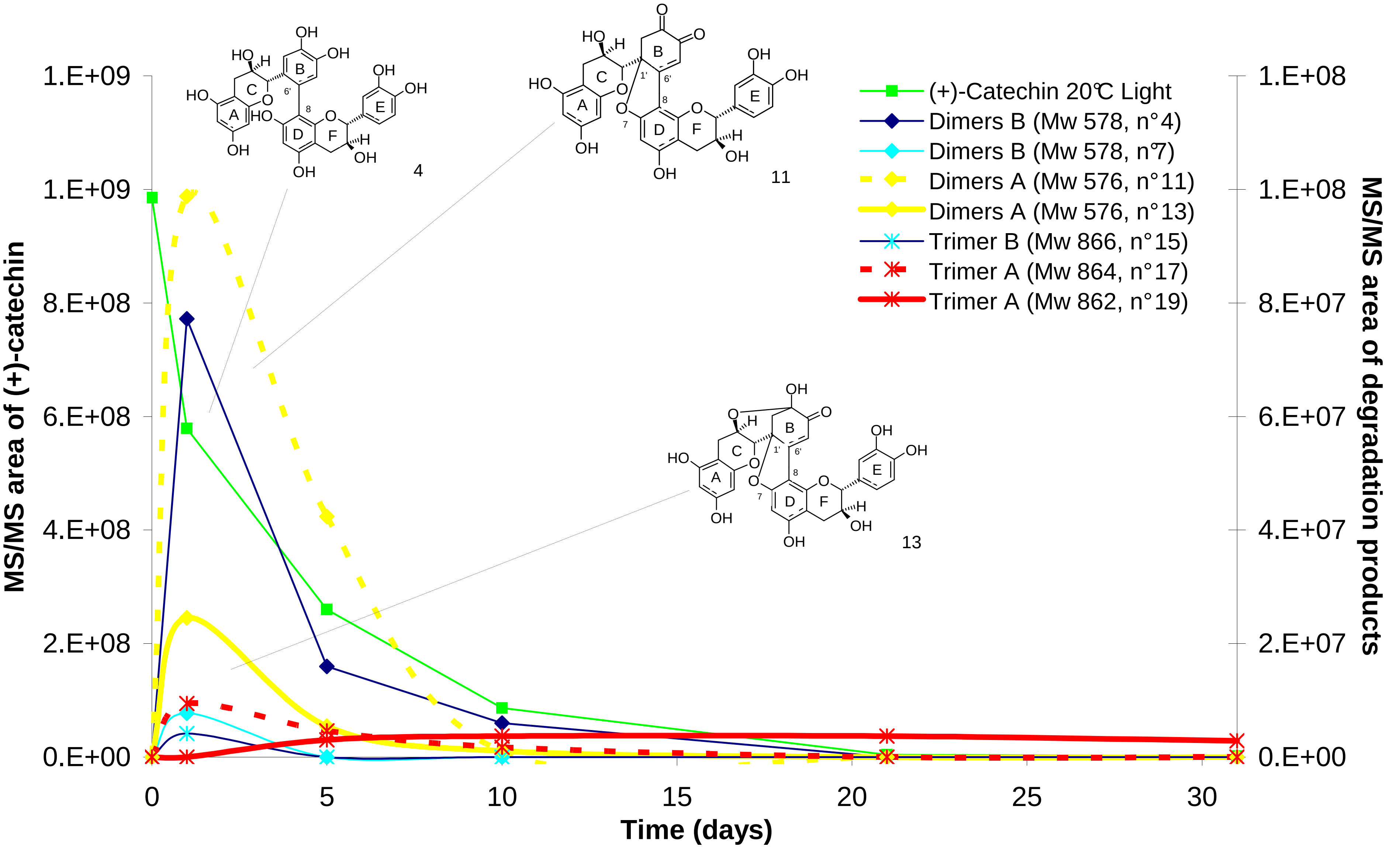
Guyot et al., 1996, *Phytochemistry, Vol. 42, No. 5, pp. 1279-1288*



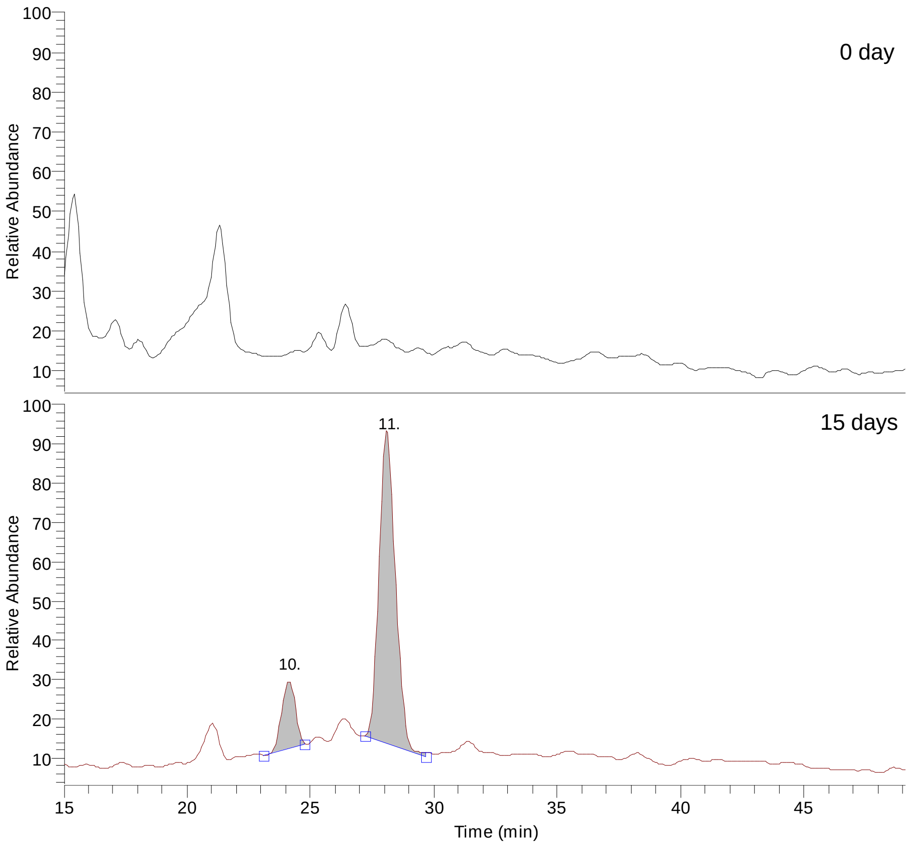
Mw 576 **n°13**
Dehydrodicatechin A- yellow-brown

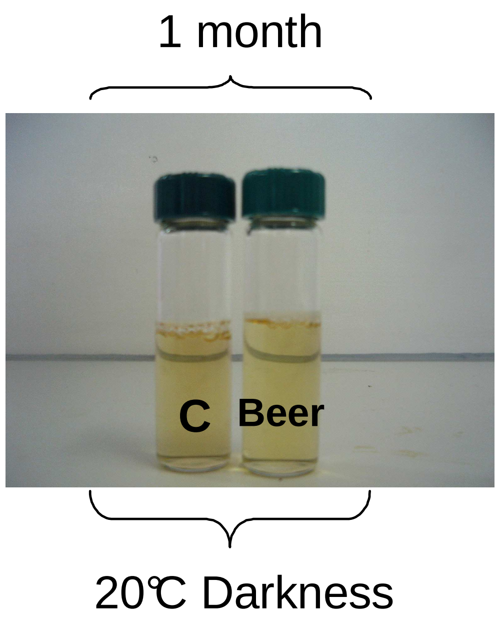
Mw 576 **n°11**
Dehydrodicatechin A-yellow/brown

Dimers and trimers still transformed to other orange coloured compounds



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 dehydrodicatechin B4.

Conclusions on fresh beer

Small flavanoids (Sephadex LH20 extract)

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

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

Hop /Malt

➔ P1: (+)-catechin > (-)-epicatechin > (-)-gallocatechin and (-)-epigallocatechin

P2: B3 (C-4α-8-C) > B1[N] (E-4α-8-C) > B4[N] (C-4α-8-E) & B3 (GC-4α-8-C)

P3: C2[N] (C-4α-8-C-4a-8-C) & GC-4α-8-C-4α-8-C[N] & GC-4α-8-GC-4α-8-C[N]

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

Conclusions on the colour of aged beer

- ➔ Strongly increased with O_2 and light !! PET bottles !!
- ➔ Issued from catechin degradation

Catechin ➔ Dehydrodi(tri)catechins B ➔ Dehydrodi(tri)catechins A

Colourless — Yellow-Brown

⇩

Still to be identified

Brown-Orange compounds