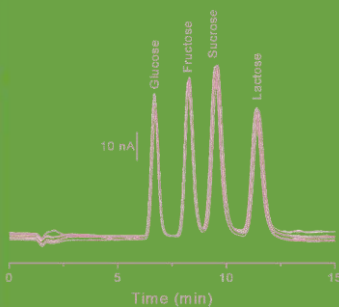# POLYPHENOLS IN WINE AND FOOD

THE FINEST LC-EC APPLICATIONS FOR FOOD & BEVERAGE ANALYSIS EVER PROCESSED

Bisphenol A
Catechins
Flavonoids and phenols
Phenols
Antioxidants

Polyphenols
 Resveratrol
 Epicatechin
 Quercetin
 other polyphenols

Carbohydrates
Iodide
Vitamins A, C, D, E, and K
Q10
ubiquinols

INTRODUCTION

Flavonoids and flavanoids are naturally occurring antioxidant compounds belonging to the phytopolyphenols. Resveratrol, quercetin, and other polyphenolic flavonoids have been reported to be cancer preventive agents and are thought to have therapeutic importance in cardiovascular disease [1, 2]. These polyphenolic substances are not only found in wine, but also green tea, chocolate, cocoa and several other food products.

- Binary gradient HPLC
- Analysis of food and beverages
- Robust & reproducible Analysis

Summary

A method is presented for the analysis of epicatechin (EC), epigallocatechin (EGC), epigallocatechingallate (EGCG), myricetin, quercetin, keampferol and cis and trans resveratrol.
An ALEXYS Analyzer is used with a binary gradient. Detection limits of 2-5 nmole/L have been obtained.

Fig. 1. ALEXYS Polyphenols Analyzer.

Method

Table 1	
LC-EC Conditions	
HPLC	ALEXYS Polyphenols Analyzer
Temp.	35 °C (separation and detection)
Column	ALF 215 150 X 2.1 mm, 3µm
Flow cell	VT-03 with 2 mm GC WE, HyREF
ADF	0.1 Hz

An ALEXYS system with reversed phase gradient HPLC is used in combination with a filtration step (wine and beverages) or double liquid-liquid extraction (chocolate).

Results

Hydrodynamic voltammogram
A hydrodynamic voltammogram has been obtained for all substances under investigation. Best signal to noise ratio was found at 850 mV vs HyREF.

Linearity and reproducibility – isocratic analysis
Depending on the complexity of the matrix, samples can be analysed using gradient or isocratic HPLC. The linearity has been studied in the range 2-100 nM using isocratic HPLC. Correlation coefficient r is better than 0.998 in all cases. From the calibration plot a detection limit of 2-5 nM was found for all 7 polyphenols. Calculation using the signal-to-noise ratio [LOD = 3 n c / s] results in a similar detection limit. Injection volume was 5 µL in these experiments.
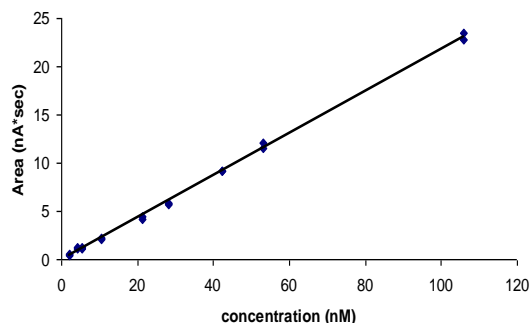

Fig. 2. Calibration plot for epicatechin.

Table 1. Reproducibility of polyphenol analysis at 20 nM

Component	t (min)	%RSD	h (nA)	%RSD
Myricetin	4.04	0.17	0.15	2.2
trans-Resveratrol	5.15	0.15	0.11	4.9
Quercetin	6.29	0.19	0.18	2.1
cis-Resveratrol	7.56	0.20	0.31	1.4
Keampferol	10.63	0.28	0.06	7.0
Epicatechin*	6.88	0.09	0.28	1.02
Epigallocatechin*	3.74	0.12	0.44	1.77

*Both catechins were analysed at 12% ACN all other polyphenols at 29.5% ACN in the mobile phase.

Linearity and reproducibility – gradient analysis
The linearity has been studied in the range 10 -100 nM using a HPLC gradient as shown in Fig. 4C. Correlation coefficient r is better than 0.998 in all cases. A detection limit of 2-5 nM was found for all 7 polyphenols. Calculation using the signal-to-noise ratio [LOD = 3 n c / s] results in a similar detection limit. Injection volume was 5 µL in these experiments.
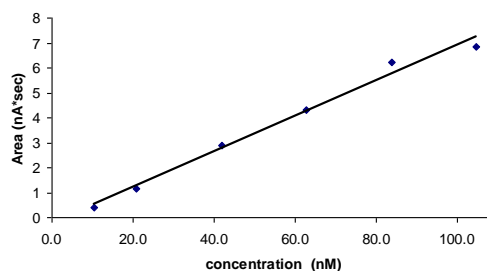

Fig. 3: Calibration curve of Keampferol (10-100 nM).

Table 2: Results of reproducibility study of 40 nM polyphenols

Component	tr (min)	RSD (%)	h (nA)	RSD (%)
Myricetin	17.39	0.01	1.40	1.7
trans-Resveratrol	18.05	0.02	0.36	2.9
Quercetin	18.75	0.01	2.02	0.9
cis-Resveratrol	19.18	0.01	2.40	1.3
Keampferol	19.99	0.04	0.67	2.0

Gradient analysis

Because of the difference in retention behaviour between the catechins and polyphenols a gradient has been applied. Best results were obtained with the gradient in Fig. 4C.
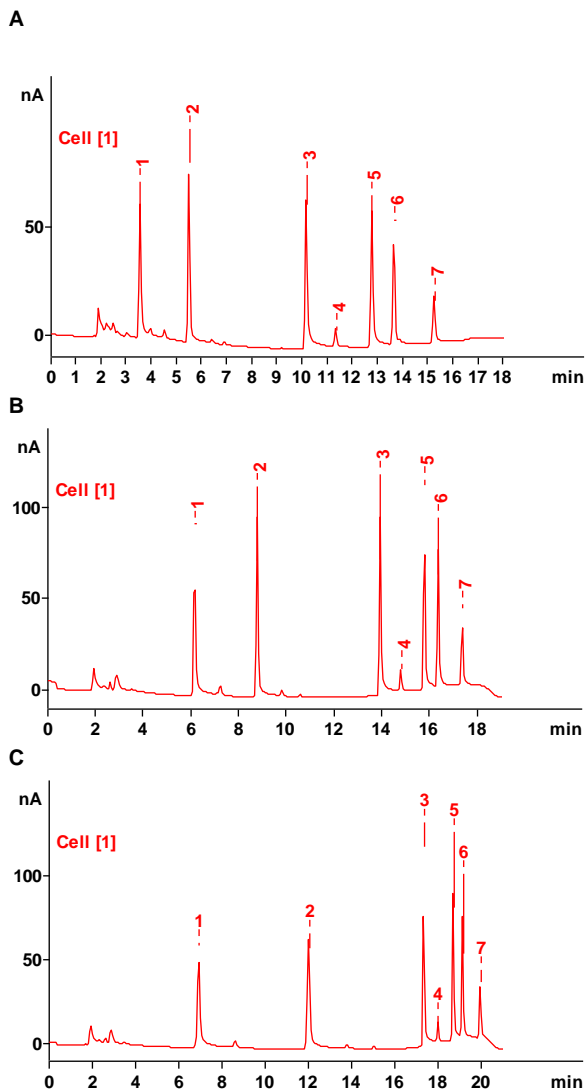
A

B

C

Fig. 4. Resolving power of the gradient LC system. Analysis of 2 µM standards: epicatechin (1), epigallocatechin (2), myricetin (3), trans-resveratrol (4), quercetin (5), keampferol (7) and cis-resveratrol (6). Top: linear gradient from 5% (t=0) to 40% B (t=8)to 100%B (t=14). Middle: linear 5% (t=0) to 20% (t=10) to 100% B (t=16), 100%B (t=17). Bottom: linear 5% (t=0) to 20% (t=10) to 100%B (t=16), 100%B (t=20). All gradients had a 10 min stabilisation period at the end of each run, at the initial percentage of 5% B.

Detection limits and loadability

In all experiments a 5 µL injection volume has been used. Concentrations of polyphenols in wine and food products is high enough and no need to improve detection limits. However, detection limits can easily be improved by increasing the sample load on column. To illustrate this we studied the detection limit for 5 – 100 µL injection volume using gradient HPLC (details Fig. 4C).

Table 3: Load ability study of 200 nM load on column

	10 µl 20 nM	100 µl 2 nM
EGC	12,99	7,12
EC	10,79	12,66
Myricetin	6,35	6,95
Trans Resveratrol	5,64	8,27
Quercetin	11,70	21,80
Cis Resveratrol	0,36	0,65
Keampferol	2,02	4,15

Table 4: Results of LOD calculation

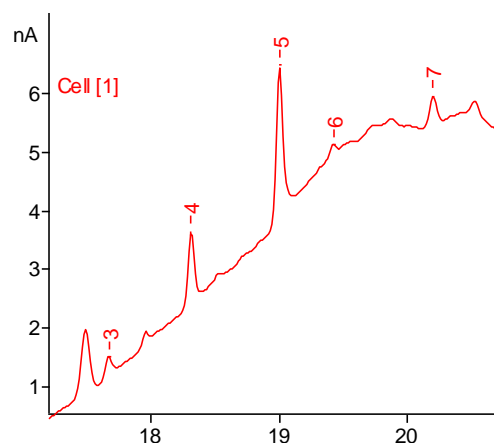|	5 µl	100 µl
EGC	1,8	0,5
EC	1,6	0,3
Myricetin	3,4	0,6
t-Resveratrol	2,1	0,1
Quercetin	2,1	0,2
Keampferol	2,7	0,3

Fig. 5: Analysis of 100 µl, 1 nM myricetin (3), trans-resveratrol (4), quercetin (5), cis-resveratrol (6), and keampferol (7).

The detection limits are considerably improved by increasing the sample load. Due to baseline fluctuations, the improvement of LOD is not linear with injection volume.

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Proven Performance!

Analysis of wine

Several wine samples were analysed after dilution in water and filtration using a Durapore 0.2 µm filter. Injection volume of 5 µL was used in all cases.
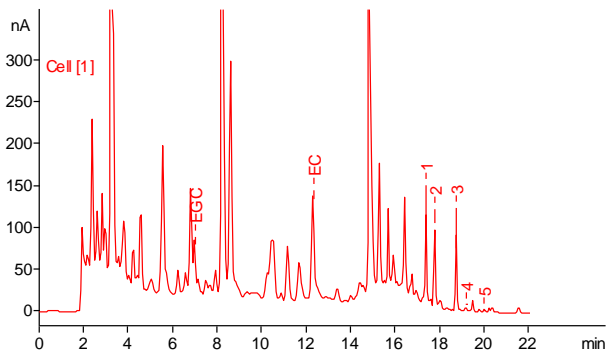

Fig. 6: Chromatogram of 10X diluted Santa Digna cabernet sauvignon (1), 2003, Chile; 1= Myricetin, 2= trans-Resveratrol, 3= Quercetin, 4= cis-Resveratrol and 5= Keampferol
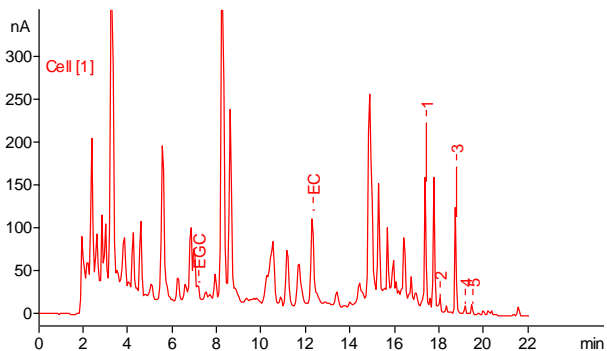

Fig. 7: Chromatogram of 10X diluted wine sample (2): Santa Digna Car-menère, 2003, Chile; 1= Myricetin, 2= trans-Resveratrol, 3= Quercetin, 4= cis-Resveratrol and 5= Keampferol
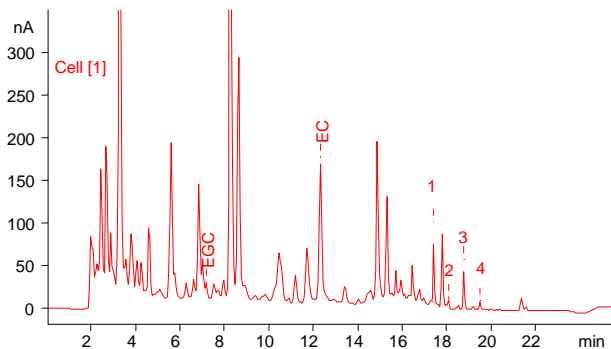

Fig. 8: Chromatogram of 10X diluted wine sample(3): Vin de Pays d'Oc, Vintage unknown, France; 1= Myricetin, 2= trans-Resveratrol, 3= Quercetin, 4= cis-Resveratrol
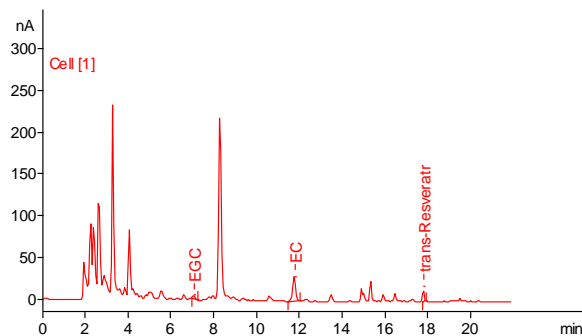

Fig. 9: Chromatogram of 10X diluted wine sample (4): Antech Brut, Blan-quette de Limoux, Vintage unknown, France

Table 5: Results of analysis of catechins and polyphenols in wine (all concentrations in mg/L)

sample	EGC	EC	Myr	Res	Quer	Kea
1	0.5	10	4.9	0.7	5.8	0.4
2	0.7	11	7.4	1.2	8.1	0.7
3	0.6	16	3.5	0.7	2.8	0.2
4	<0.1	0.2	-	<0.1	trace	-

Analysis of beverages

For analysis of tea, the procedure is performed as normally tea is made, extraction with hot water. A tea bag (ca. 2gr) is left for 5 minutes in 200 mL hot water. The tea cooled and diluted with mobile phase and filtered using a Durapore 0.2 µm filter and 5 µL was injected.
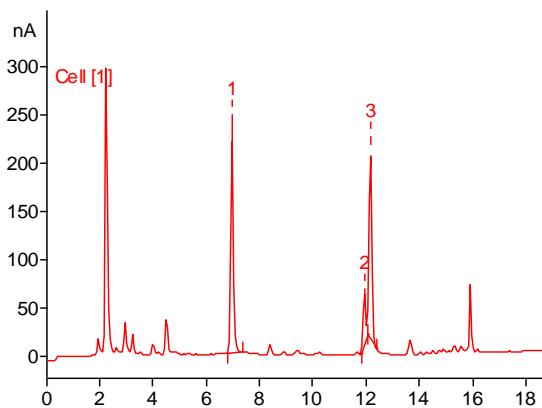

Fig. 10: Chromatogram of 50X diluted Lipton green ice tea, Unilever, 1= EGC, 2= EC, 3=EGCG
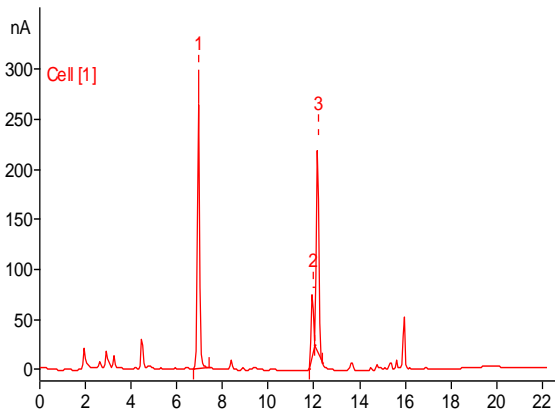
Fig. 11: Chromatogram of 50X diluted green tea, Pickwick; 1= EGC, 2= EC and 3= EGCG
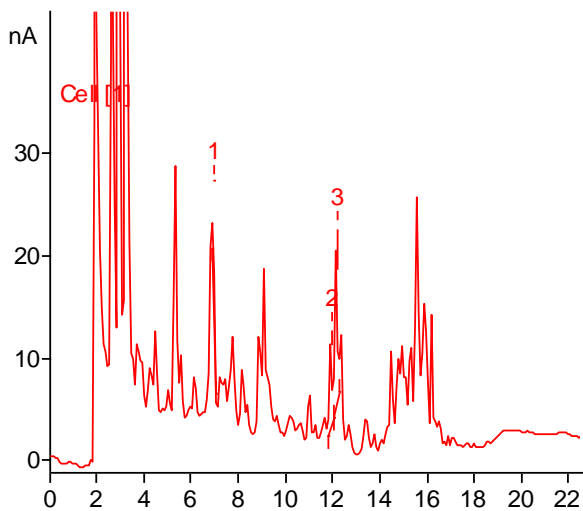

Fig. 13: Chromatogram of Milk chocolate (Trèsor, the Convenience company), extracted and diluted 50X ; 1= EGC, 2= EC

Fig. 12: Chromatogram of 10X black tea, Zonnatura; 1= EGC, 2= EC and 3= EGCG

Fig. 14: Chromatogram of Dark chocolate (Albert Heijn), extracted and diluted 50X ; 1= EGC, 2= EC

Analysis of chocolate

Chocolate contains lipids which were extracted before analysis. An amount of 5 g chocolate is crushed and weighed. To the crushed material, a volume of 15 mL hexane is added and vigorously shaken to extract lipids from the sample. This lipid extraction is performed 4 times to ensure all lipids are removed. After the last hexane extraction, 0.5 g was taken from the solid residue and dissolved in 5 mL extraction buffer (acetone : water : acetic acid = 70 : 29.5 : 0.5). The mixture was shaken for 30 s and diluted in mobile phase and filtered using a Durapore membrane filter (0.2 μm).

The recovery of the procedure was studied using the same procedure for a standard solution of all polyphenols. A recovery of 85-115%.was found for each standard compound.
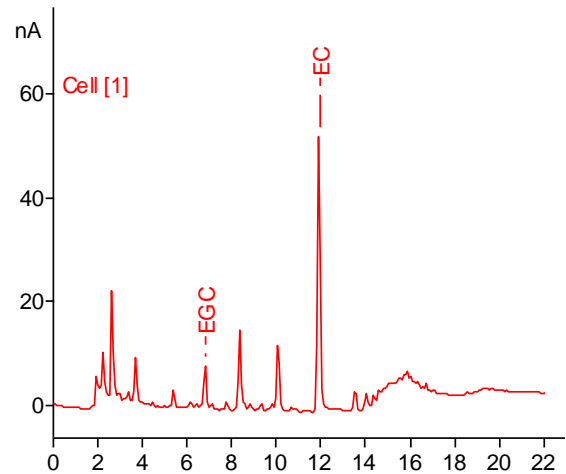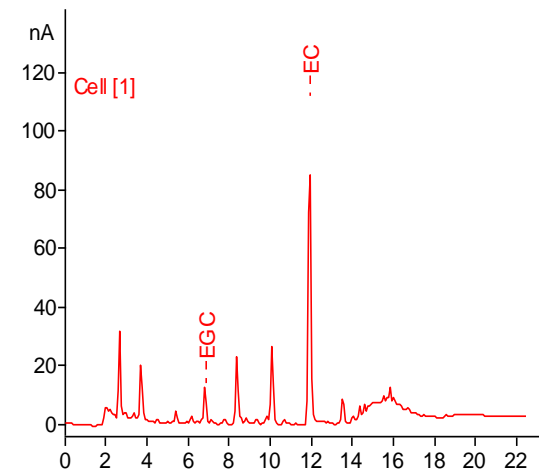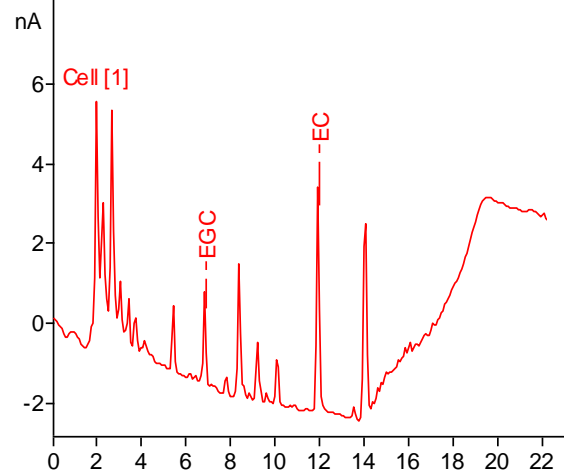

Fig. 15: Chromatogram of Nutella (Nestle), extracted and diluted 50X ; 1= EGC, 2= EC

Table 6: Results of analysis of beverages and chocolate

	EGC	EC	EGCG	Quercetin
Lipton tea	100	15	91	trace
apple juice	-	1	-	-
green tea	130	24	117	trace
black tea	9	3	8	-
Chocolate dark*	8	45	-	trace
Chocolate milk*	5	12	-	trace
Nutella*	0.3	0.4	-	-

All concentrations in mg/L, except *, in mg/100g

PART NUMBERS

180.0094A	ALEXYS Polyphenols Analyzer
250.1120	ALF-215 column, 150x2.1mm, 3um C18

Conclusion

An ALEXYS Polyphenols Analyzer has been used for the analysis of polyphenols in wine and food products. Gradient HPLC is most suitable for analysing multiple polyphenols in complex matrix.

At 5µL injection volume a detection limit of 2- 5 nM has been obtained, which can be improved to 0.1 – 0.5 nM by increasing the injection volume to 100 µL. At 20 nM an RSD of 2% for peak heights and areas was found.

References

1. I Kolouchova-Hanzlikova, K Melzoch, V Filip, J Smidrkal; *Rapid method for Resveratrol determination by HPLC with electrochemical and UV detections in wine; Food Chemistry*; 87 (2004) 151-158
2. M Careri, C Corradini, L Elviri, I Nicoletti, I Zagnoni; *Direct HPLC analysis of quercetin and trans-Resveratrol in red wine, grape, and winemaking byproducts*; J Agric. Food Chem. 51 (2003) 5226-5231
3. JF Hammerstone, SA, Lazarus, HH Schmitz; *Procyanidin content and variation in some commonly consumed foods*; J. Nitr. 130 (2000) 2086S-2092S