Application Note

Food & Beverage

The finest LC-EC applications for Food & Beverage analysis

Phenols
Bisphenol A
Catechins
Flavonoids
Phenols
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other polyphenols

Carbohydrates
Monosaccharides
Lactose
Other oligo- and polysaccharides

Vitamins, minerals etc.
A, C, D, E, and K
Iodide
Q10, ubiquinols

Polyphenols and Catechins

- **Gradient HPLC separation with ECD**
- **Analysis of food and beverages**
- **Reproducible and robust**

Summary

A method is presented for the analysis of epicatechin (EC), epigallocatechin (EGC), epigallocatechingallate (EGCG), myricetin, quercetin, kaempferol and cis- and trans- resveratrol.

An ALEXYS Analyzer was used with a binary gradient. Detection limits of 2-5 nmole/L have been obtained.

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 they 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.

This note shows example chromatograms of polyphenols in various food, as obtained with the ALEXYS LC-ECD system running a reversed phase gradient separation.

Method

Separation

Depending on the complexity of the matrix and the components of interest, samples can be analyzed after isocratic or gradient separation. The retention behavior of the catechins is very different in comparison with the polyphenols (see also the retention times under isocratic separation in Table 2), and a gradient separation can be used to detect all in one chromatogram within a reasonable timeframe. Different gradient profiles were tested (Fig 1). Both isocratic as well as gradient separation analyses were evaluated for reproducibility, linearity and detection limit with standards. Gradient separation and conditions given in Table 1 were applied for the sample analyses.

Table 1

LC-ECD conditions

HPLC	ALEXYS Polyphenols Analyzer
Columns	Betabasic™ 18 HPLC Column, 150 x 2.1 mm ID, 3 µm (Thermo Scientific™)
Mobile phase (gradient)	A: 30 mM phosphate buffer pH 2.5 with 5% ACN B: 30 mM phosphate buffer pH 2.5 with 40% ACN Linear gradient profile: 5% (t=0) to 20% (t=10) to 100%B (t=16), 100%B (t=20) + 10 min 5%B for stabilization.
Flow rate	0.2 mL/min
Temperature	35 °C for separation and detection
Backpressure	About 100 bar
$V_{injection}$	5 µL (unless stated otherwise)
Flow cell	SenCell™ with 2 mm GC working electrode and HyREF reference electrode, AST setting 2
Ecell	850 mV
Range	100 nA/V
ADF	0.5 Hz
I-cell	About 55 nA

Detection

A hydrodynamic voltammogram was constructed for all substances under investigation to find the optimal working potential. The best signal to noise ratio was found at 850 mV vs HyREF and this value was applied for all analyses.

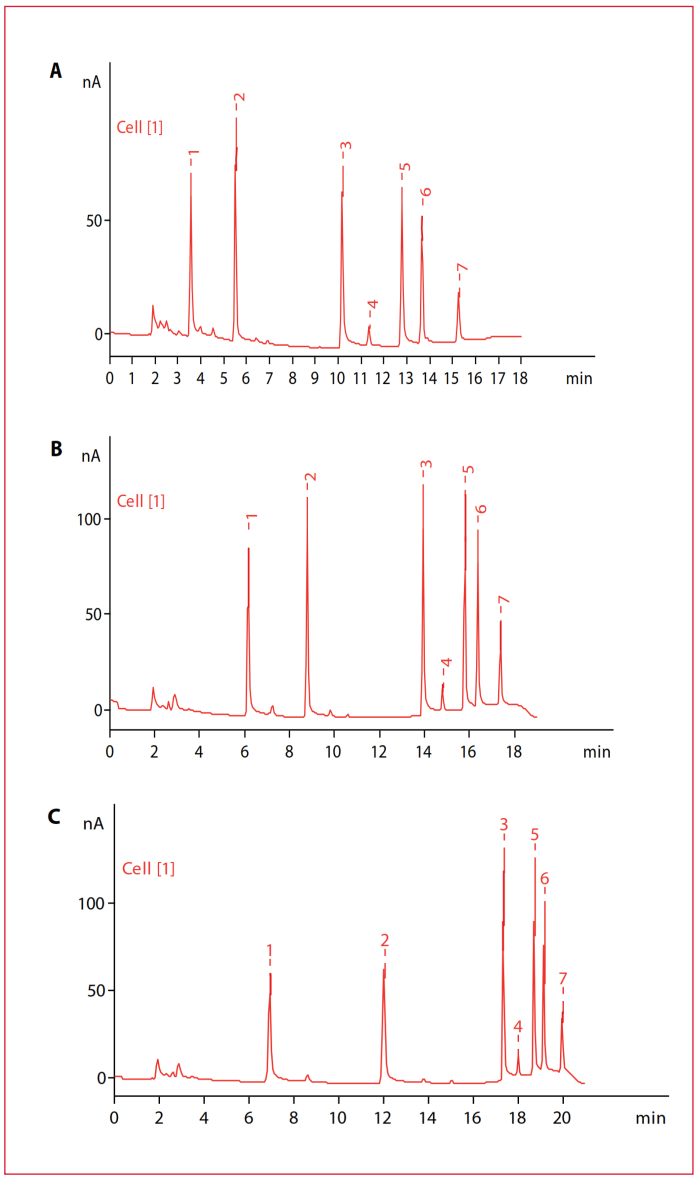

Figure 1: Chromatograms of 2 µM standards separated with 3 different gradient profiles. Peaks: epicatechin (1), epigallocatechin (2), myricetin (3), trans-resveratrol (4), quercetin (5), kaempferol (7) and cis-resveratrol (6).
A: linear gradient from 5% (t=0) to 40% B (t=8)to 100%B (t=14).
B: linear gradient from 5% (t=0) to 20% (t=10) to 100% B (t=16), 100%B (t=17).
C: linear gradient from 5% (t=0) to 20% (t=10) to 100%B (t=16), 100%B (t=20).
All gradients had a 10 min stabilization period at the end of each run, at the initial percentage of 5% B.

Results

Linearity, LOD and reproducibility – isocratic analysis

The linearity was studied in the range 2-100 nM for isocratic separation. Correlation coefficient r was better than 0.998 for all components. Fig. 2 shows the calibration plot for epicatechin as an example.

Based on the calibration plots, a detection limit of 2-5 nM (5 µL injection) was calculated for all 7 polyphenols. Calculation using the signal-to-noise ratio [LOD= 3 n c / s] gave a similar detection limit.

Linearity, LOD and reproducibility – gradient analysis

The linearity was studied in the range 10 -100 nM based on separation as shown in Fig. 1C. Correlation coefficient r was better than 0.998 for all components. Fig. 3 shows the calibration plot for kaempferol as an example.

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.

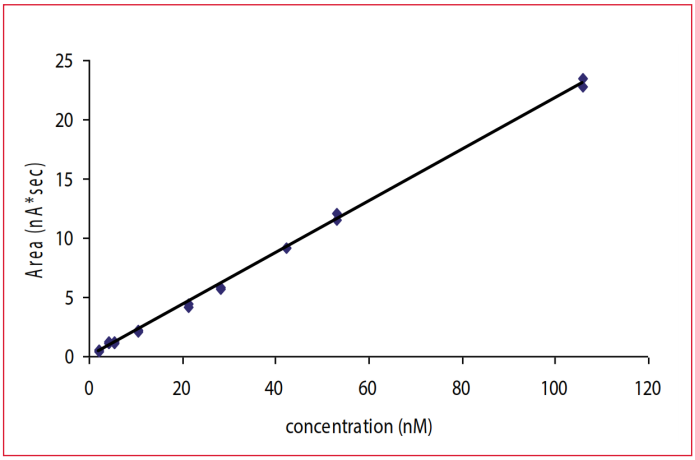

Figure 2: Calibration plot for epicatechin - based on isocratic separation

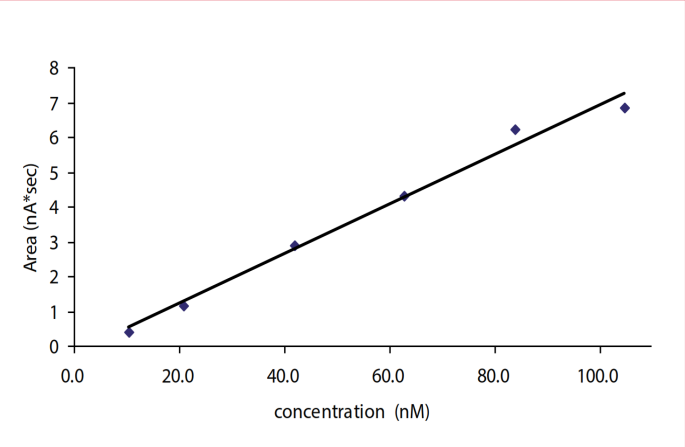

Figure 3: Calibration plot for kaempferol - based on gradient separation

Table 2

Reproducibility of polyphenol analysis (20 nM standards) with isocratic separation

Component	*Retention time*		*Peak height*	
	min	*RSD (%)*	*nA*	*RSD (%)*
Isocratic separation with 29.5 % acetonitrile in the mobile phase				
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
Kaempferol	10.63	0.28	0.06	7.0
Isocratic separation with 12% acetonitrile in the mobile phase				
Epicatechin	6.88	0.09	0.28	1.0
Epigallocatechin	3.74	0.12	0.44	1.8

Table 3

Reproducibility of polyphenol analysis (40 nM standards) with gradient separation

Component	*Retention time*		*Peak height*	
	min	*RSD (%)*	*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
Kaempferol	19.99	0.04	0.67	2.0

Loadability and LOD improvement

The sensitivity of the method is normally sufficient for the analysis of polyphenols in food products. However, here we want to show that the detection limits can easily be improved by increasing the sample load on column. Instead of using an injection volumes of 5 µL we tested up to 100 µL. A chromatogram of a low level standards mix analyzed based on a high injection volume is shown in Fig. 4

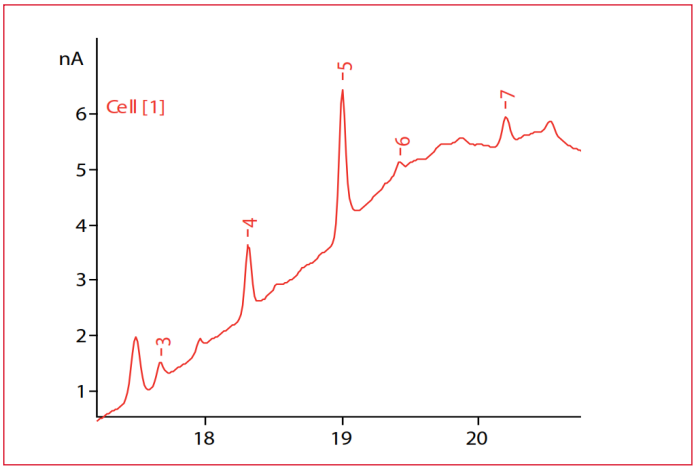

Figure 4: Chromatogram of 1 nM myricetin (3), trans-resveratrol (4), quercetin, (5), cis-resveratrol (6) and kaempferol (7), based on a 100 µL injection. Gradient separation applied as in Fig. 1C..

The detection limits are considerably improved by increasing the sample load (Table 4). However, due to baseline fluctuations, the improvement of LOD is not linear with injection volume.

Table 4

Concentration detetction limits (nM) as calculated for different larger injection volumes

Component	*50 µL*	*100 µL*
EGC	1.8	0.5
EC	1.6	0.3
Myricetin	3.4	0.6
trans-resveratrol	2.1	0.1
Quercetin	2.1	0.2
Kaempferol	2.7	0.3

Sample analysis: tea and tea-related beverage

Several hot and ice tea samples were analyzed. Sample preparation consisted of dilution with mobile phase and filtration over a Durapore 0.2 µm filter before injection. The results of the tea-related analyses shown in Fig 5 - 7 are given in Table 5.

Table 5

Concentration of catechins in tea beverages (mg/L)

sample	*EGC*	*EC*	*EGCG*	*Quercetin*
Ice tea	100	15	91	trace
Green tea	130	24	117	trace
Black tea	9	3	8	-

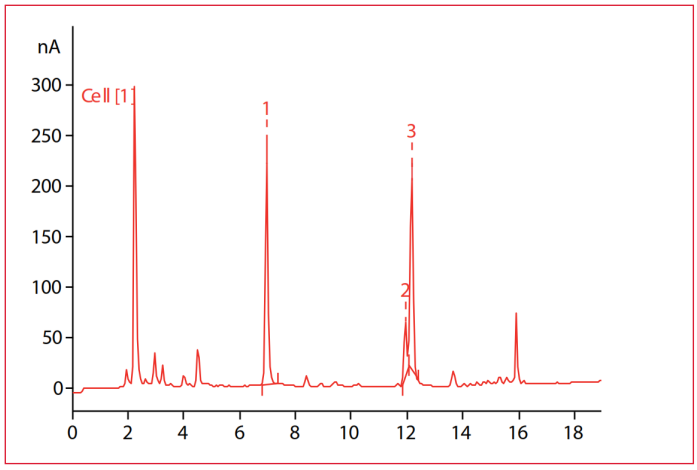

Figure 5: Chromatogram of 50x diluted and filtered ice tea (Lipton green ice tea, Unilever). Peaks: 1= EGC, 2= EC, 3=EGCG.

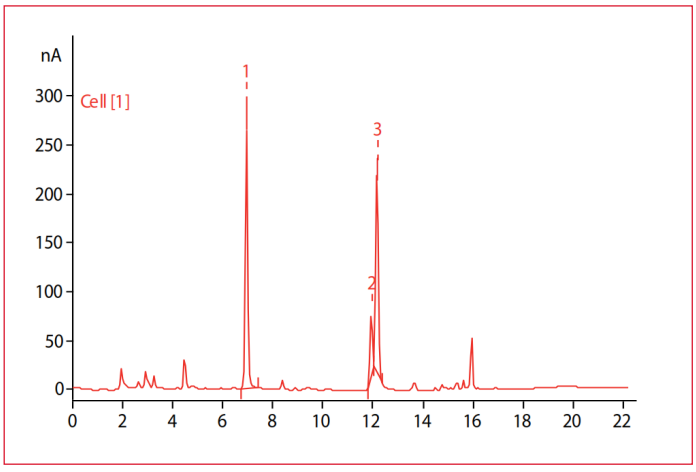

Figure 6: Chromatogram of 50x diluted and filtered hot green tea (bag of ca. 2 g Pickwick green tea seeped for 5 min in 0.2 L hot water). Peaks: 1= EGC, 2= EC and 3= EGCG.

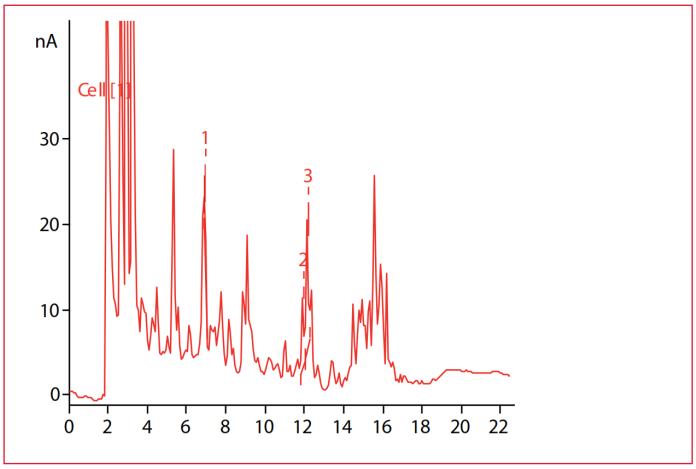

Figure 7: Chromatogram of 10x diluted and filtered hot black tea (bag of ca. 2 g Zonnatura black tea seeped for 5 min in 0.2 L hot water). Peaks: 1= EGC, 2= EC and 3= EGCG.

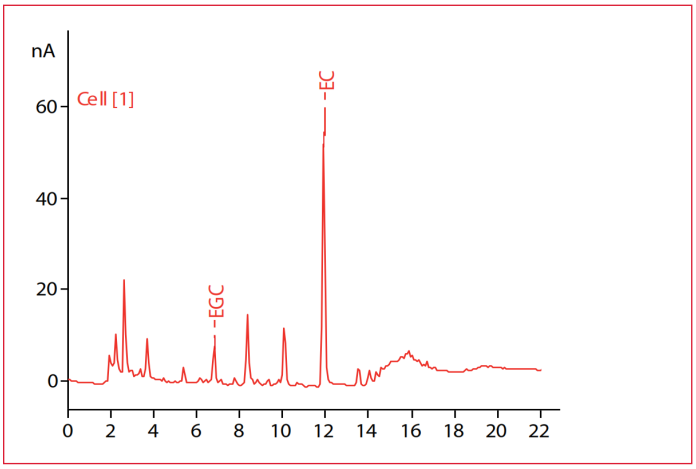

Figure 8: Chromatogram of milk chocolate (Trèsor, the Convenience company), after extraction, dilution and filtration. Peaks: 1= EGC, 2= EC, 3=EGCG.

Sample analysis: chocolate

Several chocolate samples were analyzed. Sample preparation consisted of extraction and removal of lipids before injection. An amount of 5 g chocolate was crushed and weighed. To the crushed material, a volume of 15 mL hexane was added and vigorously shaken. The lipid extraction was repeated 4 times. After the last hexane extraction, 0.5 g from the solid residue was dissolved in 5 mL extraction buffer (acetone : water : acetic acid = 70 : 29.5 : 0.5) and shaken for 30 s. The sample was diluted 50x with mobile phase and filtered over a Durapore membrane filter (0.2 µm) before injection. The recovery of the sample preparation procedure was investigated and a recovery of 85- 115%.was found for each standard compound.

The results of the chocolate analyses shown in Fig 8 - 10 are given in Table 6.

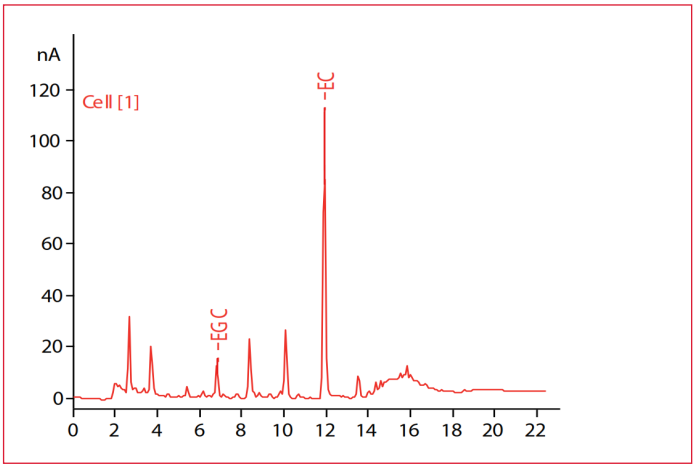

Figure 9: Chromatogram of dark chocolate (Albert Heijn), after extraction, dilution and filtration. Peaks: 1= EGC, 2= EC, 3=EGCG.

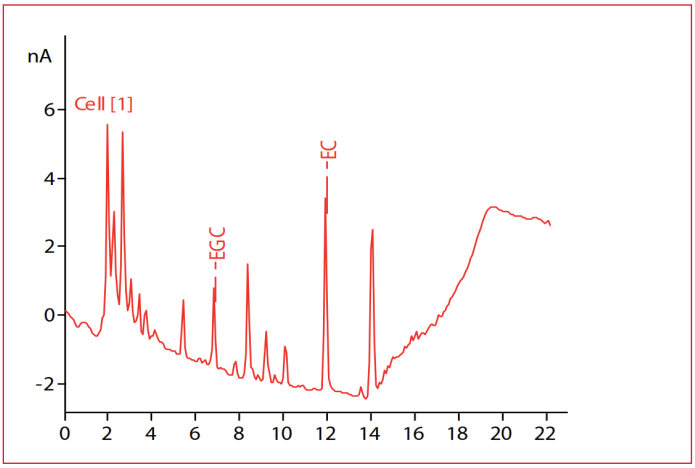

Figure 10: Chromatogram of Nutella (Nestle), after extraction, dilution and filtration. Peaks: 1= EGC, 2= EC, 3=EGCG.

Table 6

Concentration of catechins in chocolate (mg/100 g)

sample	*EGC*	*EC*	*EGCG*	*Quercetin*
Dark chocolate	8	45	-	trace
Milk chocolate	5	12	-	trace
Nutella	0.3	0.4	-	-

Sample analysis: wine

Several wine samples were analyzed. Sample preparation consisted of dilution with water and filtration over a Durapore 0.2 µm filter before injection.

The results of the analyses shown in Fig 11 - 14 are given in Table 7.

Table 7

Concentration of catechins and polyphenols in wine (mg/L)

Wine sample #	*EGC*	*EC*	*Myr*	*Res*	*Quer*	*Kae*
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	-

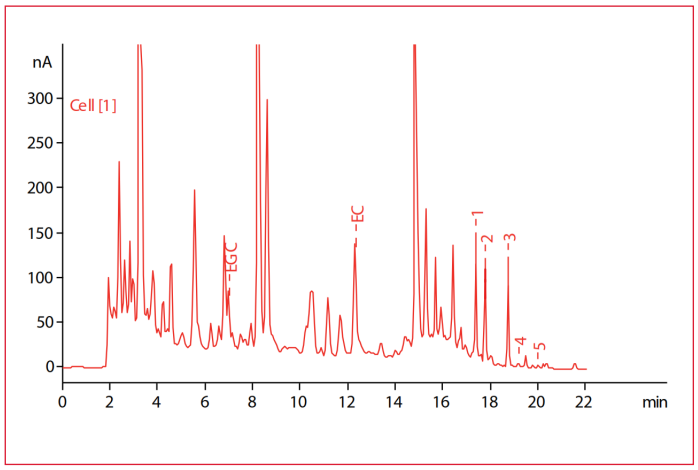

Figure 11: Chromatogram of 10x diluted and filtered wine (Santa Digna cabernet sauvignon 2003, Chile - sample '1'). Peaks: 1= myricetin, 2= trans-resveratrol, 3= quercetin,4= cis-resveratrol and 5= kaempferol.

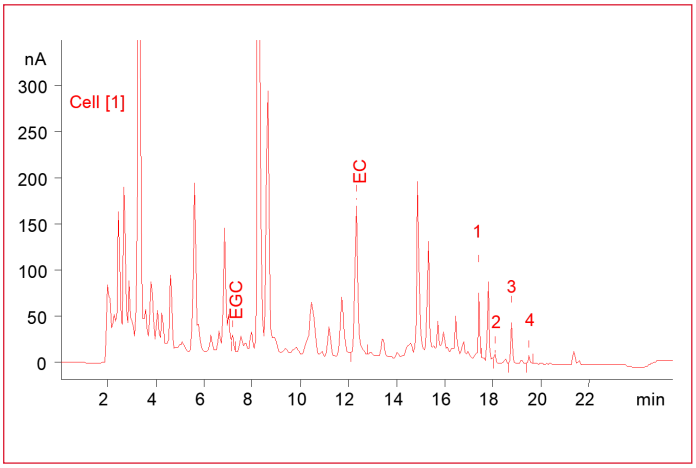

Figure 13: Chromatogram of 10x diluted and filtered wine (Vin de Pays d'Oc, vintage unknown, France - sample '3'). Peaks: 1= myricetin, 2= trans-resveratrol, 3= quercetin, 4= cis-resveratrol and 5= kaempferol.

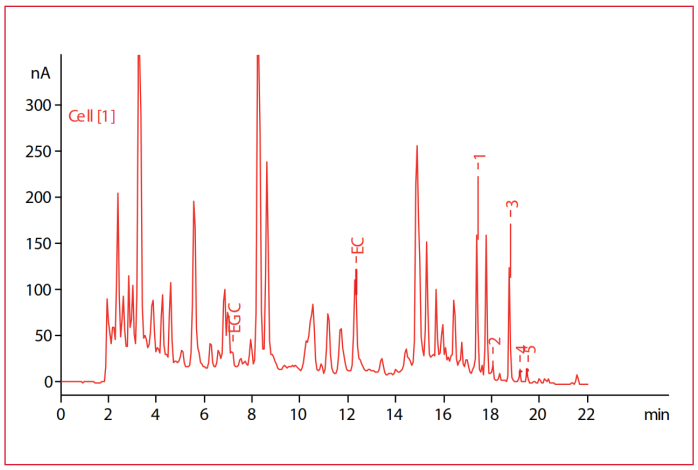

Figure 12: Chromatogram of 10x diluted and filtered wine (Santa Digna Carmenère, 2003, Chile - sample '2'). Peaks: 1= myricetin, 2= trans-resveratrol, 3= quercetin, 4= cis-resveratrol and 5= kaempferol.

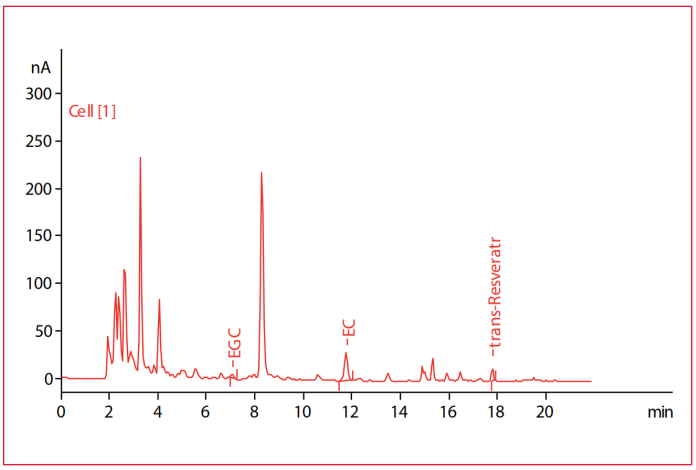

Figure 14: Chromatogram of 10x diluted and filtered wine (Antech Brut, Blanquette de Limoux, vintage unknown, France - sample '4'). Peaks: 1= myricetin, 2= trans-resveratrol, 3= quercetin, 4= cis-resveratrol and 5= kaempferol.

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

Conclusion

The ALEXYS Polyphenols Analyzer with gradient separtion is most suitable for analysing multiple polyphenols in complex food and beverage samples. The method was shown to be sensitive and reproducible.

Recommended LC-ECD hardware

The advised analytical configuration for this application is the ALEXYS Analyzer with gradient pumps, ECD and SenCell with glassy carbon electrode and HyREF reference.

Figure 15: Recommended instrument configuration for the applications: the ALEXYS Analyzer.
The system consists of P6.1L gradient pumps with integrated degasser, an AS6.1L autosampler, and the DECADE Elite electrochemical detector.

Ordering information

Detector only	
176.0035A	DECADE Elite SCC electrochemical detector
116.4320	SenCell 2 mm GC HyREF
Recommended ALEXYS analyzer	
180.0094P	ALEXYS Polyphenols Analyzer
Software	
195.0035#	Clarity CDS single instr. incl LC, AS module

#) optional: Antec ECD drivers for use with Chromeleon CDS , OpenLAB CDS or OpenLAB Chemstation CDS are available.

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