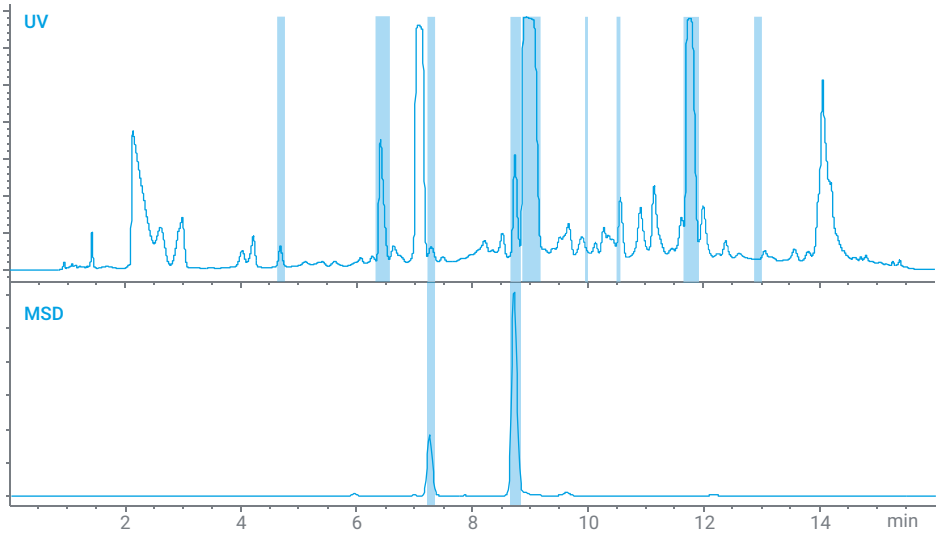
Application Note

Food Testing & Agriculture

Agilent InfinityLab

Purify Complex Samples with High Selectivity

Isolation of green tea catechins using an Agilent 1260 Infinity II Analytical-Scale LC Purification System with mass-based fraction collection



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Abstract

Tea is not just one of the most consumed beverages in the world—its high content of antioxidants also makes it the drink most associated with supposedly health-promoting effects. This application note focuses on the isolation of catechins, the major group of antioxidants found in green tea. For this purpose, an Agilent 1260 Infinity II LC, equipped with an Agilent InfinityLab LC/MSD XT, was extended by a 1260 Infinity II Analytical-Scale Fraction Collector. For the highest specificity, fraction collection was triggered on the mass selective detector signal. Nine fractions were collected, six of which were deemed catechins. Despite the complex composition of green tea, five collected catechins had a purity of 95% or higher. This illustrates the benefit of the highly specific fraction collection by a mass-selective trigger.

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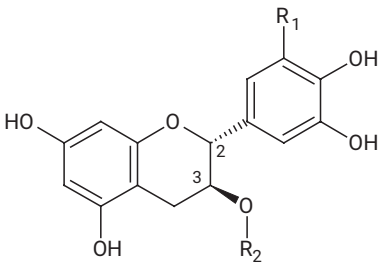
Introduction

As one of the most consumed beverages in the world, tea has long been a focus of research. The wide spectrum of antioxidants found in the leaves of the tea plant *Camellia sinensis* has led to the perception of green tea as a health-beneficial beverage. Of the multitude of supposedly health-promoting compounds found in green tea, flavan-3-ols, or catechins (Figure 1), are most abundantly present, with 25 to 35% by weight in dried leaves.[1]

Because of the complex composition of green tea, quantitative analyses of the constituents are usually carried out with multidimensional chromatography.[2] This application note focuses on the isolation of green tea catechins by preparative HPLC, which is done using a one-dimensional separation with mass selective detection for higher specificity. For this purpose, a 1260 Infinity II LC, equipped with an InfinityLab LC/MSD XT, was extended by a fraction collector. To enable mass-based fraction collection, an active splitter and make-up pump for the flow to the mass selective detector (MSD) were added. These additions are compatible with any analytical Agilent LC/MSD system and do not increase the footprint of the system. Active splitting by a 1290 Infinity II MS Flow Modulator has the benefit of being more flexible and reproducible during operation than a passive split with a split ratio, which is not easy to adjust and changes with the viscosity (i.e., composition) of the mobile phase.



R_1	R_2	C_2	C_3	Compound	M_{mi}
H	H	R	S	(+)-Catechin	290.1
H	H	R	R	(−)-Epicatechin (EC)	290.1
OH	H	R	S	(+)-Gallocatechin (GC)	306.1
OH	H	R	R	(−)-Epigallocatechin (EGC)	306.1
H	Gallate	R	S	(+)-Catechin gallate (CG)	442.1
H	Gallate	S	S	(−)-Epicatechin gallate (ECG)	442.1
OH	Gallate	R	R	(−)-Epigallocatechin gallate (EGCG)	458.1

Figure 1. Chemical structure of catechins found in green tea. M_{mi}, monoisotopic mass.

Experimental

Instrumentation

The system used for the experiments described in this application note consisted of the following modules:

- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1260 Infinity II Isocratic Pump (G7110B) used as make-up pump for the MSD
- Agilent 1260 Infinity II Vialsampler VL (G7129A)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Analytical-Scale Fraction Collector (G1364F)
- Agilent InfinityLab LC/MSD XT (G6135B)

Accurate mass measurements of the collected fractions were conducted using an Agilent 6545 LC/Q-TOF with settings as described in Table 3.

Column

Agilent Pursuit XRs C18, 4.6 × 150 mm, 5 µm (part number A6000150X046)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 or later versions

Solvents

LC-grade acetonitrile and acetone were purchased from VWR (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample

Green tea was purchased at a local grocery store. Extraction was carried out as described in the literature[3] with slight adjustments: Aliquots of 1 g of finely cut tea leaves were extracted by 5 mL of acetone:water (1:1, by volume). Samples were shaken at 750 rpm for two hours at room temperature. After centrifugation for 10 minutes at 14,000 g, supernatants were filtered through an Agilent Captiva premium syringe filter (15 mm, regenerated cellulose membrane, 0.45 µm, part number 5190-5109) and transferred into an injection vial.

Method settings

Table 1. Chromatographic conditions of analytical and preparative runs.

Parameter	Value
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	1.5 mL/min
Gradient	0 min: 5% B 12 min: 25% B 14 min: 98% B 14.1 min: 5% B 16.0 min: stop time
Injection Volume	10 µL
Needle Wash	6 s with 50% acetonitrile in water
Temperature	Ambient
UV Detection	Signal A: 270 nm 10 Hz data rate
MS Detection	Signal 1: positive SIM *m/z* 195.1 *m/z* 291.1 *m/z* 307.1 *m/z* 443.1 *m/z* 459.1 Signal 2: positive scan *m/z* 150 to 600
Split Ratio to MSD	1:100 (Mode M3)
Fraction Collection	Peak-based on MSD signal 1, active from 4.0 to 13.5 min, threshold 25,000 cps

Table 2. MSD spray chamber and fraction collection settings.

Parameter	Value
Make-Up Solvent	0.1% formic acid in methanol/water (70/30)
Make-Up Flow	0.5 mL/min
Ionization Source	Agilent Jet Stream Electrospray
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Drying Gas Flow	12.0 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11.0 L/min
Capillary Voltage	±4,000 V
Nozzle Voltage	±600 V
Target Mass (*m/z*)	291.1; 307.1; 443.1; 459.1
Ion Species	$[M]^+$

Table 3. Agilent 6545 LC/Q-TOF parameters used for fraction reanalysis.

Parameter	Value
Ionization Source	Dual Agilent Jet Stream Electrospray
Nebulizer Pressure	45 psig
Gas Temperature	250 °C
Drying Gas	11.0 L/min
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12.0 L/min
Capillary Voltage	±4,500 V
Fragmentor	180 V
Skimmer	45 V
Octopole 1 RF Voltage	750 V
Acquisition Range	*m/z* 120 to 1,000
MS Acquisition Rate	1 spectrum/sec
Reference Masses	*m/z* 121.050873 *m/z* 922.009798

Results and discussion

A green tea sample was extracted and separated by a shallow gradient on a 1260 Infinity II LC. Figure 2 shows a chromatogram of the UV detector, which demonstrates the complex composition of the sample. The vastness of different compounds requires multidimensional liquid chromatography if single compounds are to be quantified.[2] This publication, however, focuses on the purification of the main catechins, which constitute only a minor part of all green tea ingredients. For this purpose, fraction collection was triggered by the mass selective detector (MSD) signal, which yielded a higher selectivity than the UV detector signal. The instrument setup diverted the main flow to the UV detector and the fraction collector. Aliquots of the

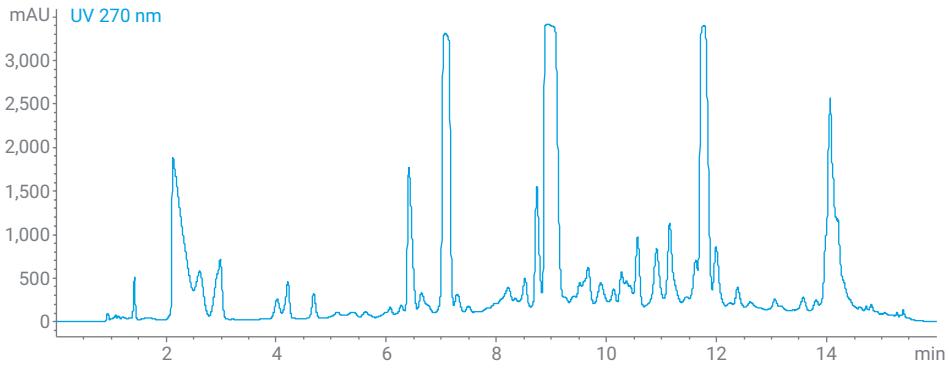


Figure 2. UV chromatogram (270 nm) of the green tea extract.

main flow were periodically transferred to the MSD by an active splitter controlled by the LC method. Adjustments of the split ratio were therefore easily possible, and an optimum split was found at 1:100. The make-up flow to the MSD was set to 0.5 mL/min and provided a constant composition along the LC gradient to ensure optimum ionization.

Figure 3 depicts an overlay of the UV chromatogram with the different single ion monitoring (SIM) chromatograms of the MSD. The single traces were used to detect caffeine and the main catechins found in green tea[1] (see Table 4). Each catechin trace can in theory produce two signals: one for each epimer, (+)-catechin and (−)-epicatechin, and accordingly for their respective hydroxylated or gallic acid-conjugated derivatives (see Figure 1). The traces of *m/z* 307.1 and 459.1, however, contained more than two signals, of which three have been collected.

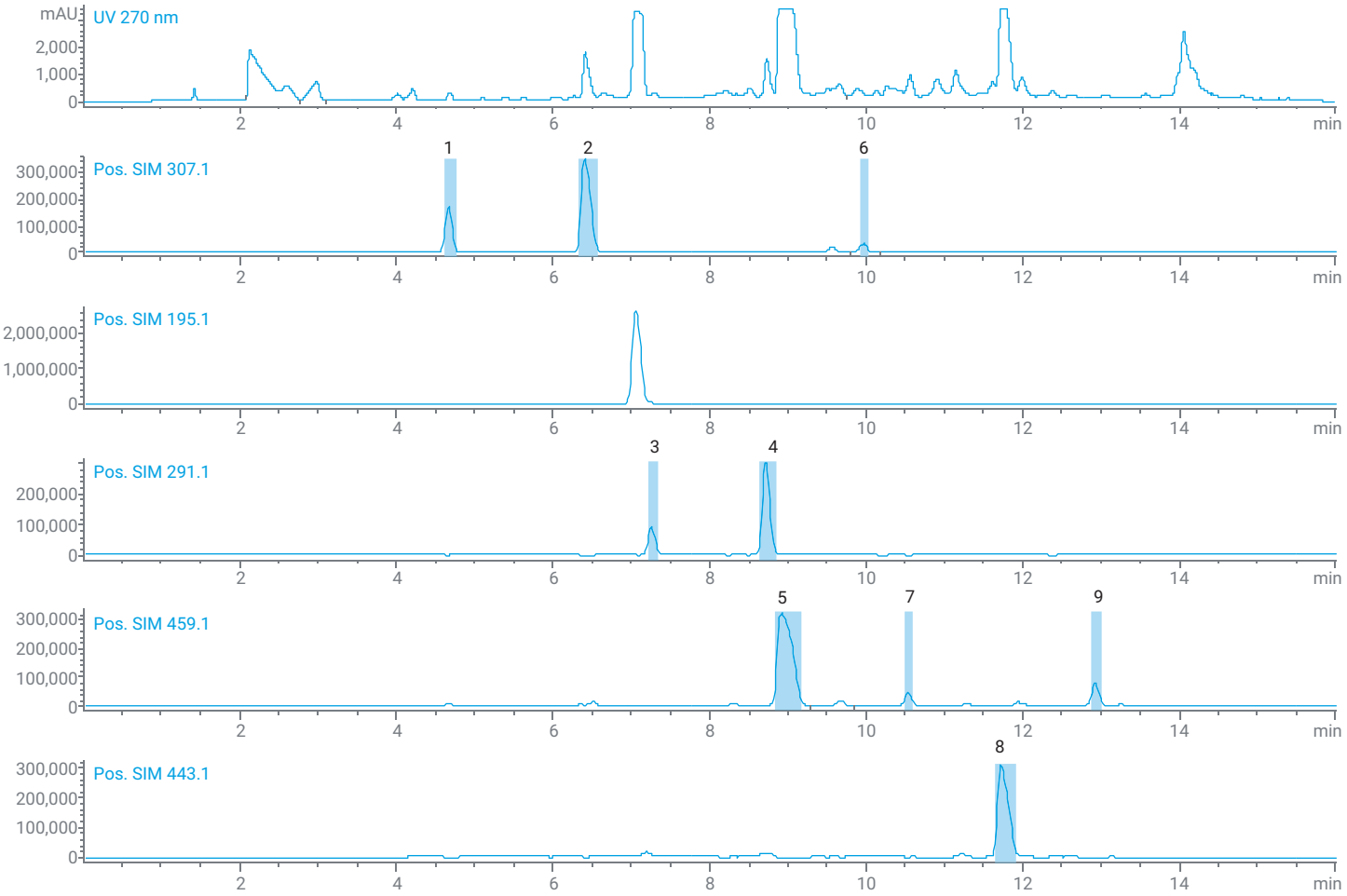


Figure 3. Overlay of UV and SIM chromatograms of the green tea analysis. Dedicated SIM signals for the catechins were used to trigger highly specific fraction collection. Blue bars and numbers refer to fraction collection events and fraction numbers, respectively.

Table 4. SIM ions and their corresponding compounds in positive ionization mode used for monitoring only (caffeine) or fraction collection.

m/z	Epimers	Compounds	Fractions
195.1	1	Caffeine	0
291.1	2	(+)-Catechin, (−)-epicatechin	2
307.1	2	(+)-Gallocatechin (GC), (−)-epigallocatechin (EGC)	3
443.1	2	(+)-Catechin gallate (CG), (−)-epicatechin gallate (ECG)	1
459.1	2	(−)-Epigallocatechin gallate (EGCG), (+)-gallocatechin gallate (GCG)	3

One of the three signals at *m/z* 307.1 is significantly less prominent than the other two, leading to the assumption that, besides (+)-gallocatechin (GC) and (−)-epigallocatechin (EGC), a structurally different impurity with similar mass has been collected in fraction 6.

The *m/z* 459.1 trace only contains one dominant signal, which was collected in fraction 5 and is assumed to be (−)-epigallocatechin gallate (EGCG), a catechin derivative found abundantly in green tea.[2] A few minor signals are visible in the trace, two of which were above the threshold and have been collected (fractions 7 and 9). Reanalysis of these fractions, however, did not show any of the typical in-source fragmentation ions at *m/z* 291.1 and 307.1 that were found in fraction 5 (see Figure 4). It is therefore likely that fractions 7 and 9 are structurally different components with masses similar to the green tea catechins.

The two fractions collected on the *m/z* 291.1 signal are most likely (+)-catechin and (−)-epicatechin. Although no absolute identification was pursued in this experiment, previous quantitative work on green tea with identification by reference standards[2] led to the assumption that the early-eluting smaller signal originated from (+)-catechin. The close elution of caffeine with peak 3, as well as

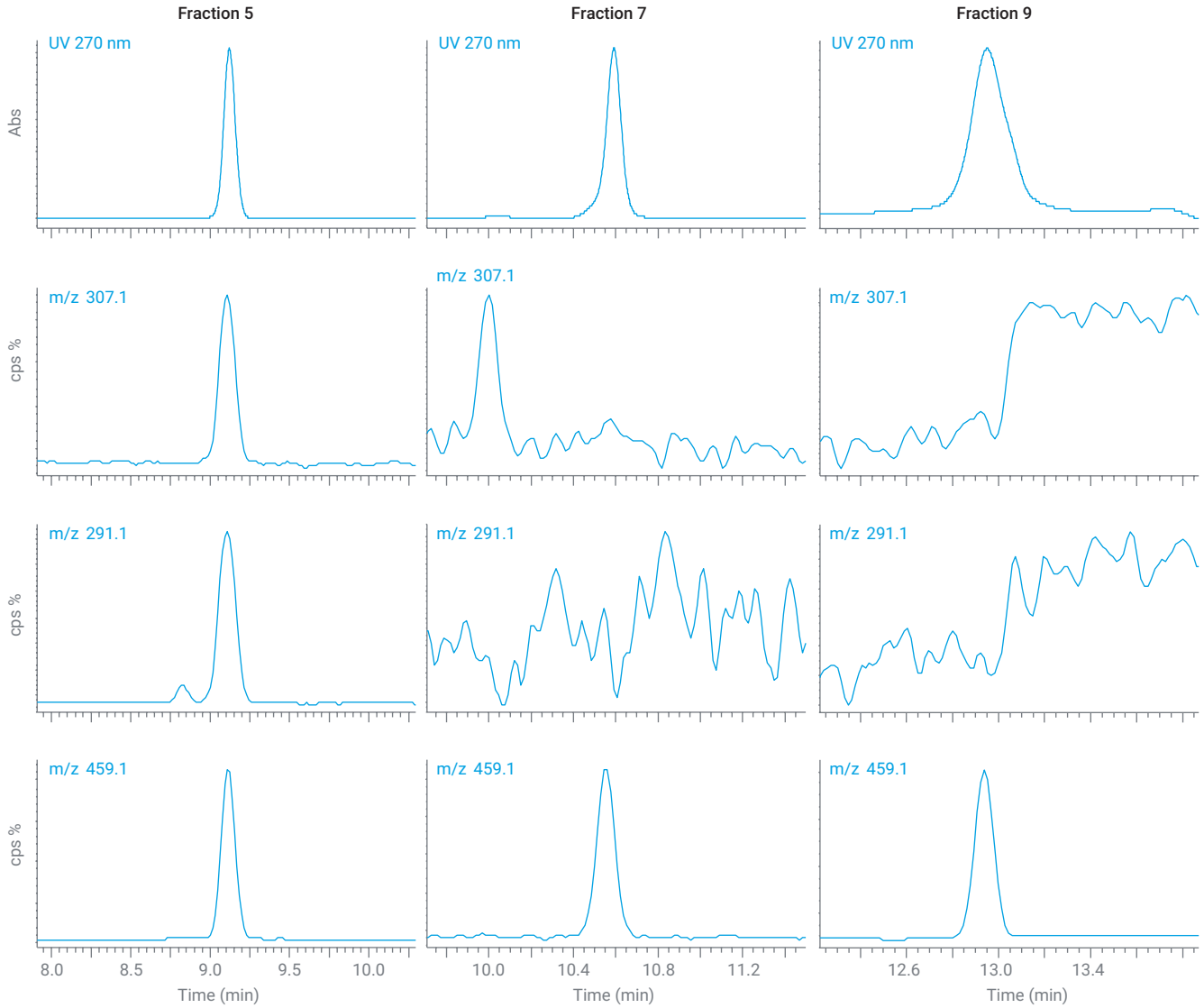


Figure 4. Comparison of UV and MSD traces of fractions 5, 7, and 9, all triggered by *m/z* 459.1. Only fraction 5 shows signals at *m/z* 307.1 and 291.1, which are fragments of *m/z* 459.1.

peak 4 with peak 5, demonstrates the importance of an MSD as trigger source to achieve highest selectivity for fraction collection. Whether the MSD signal enabled a clean cut during fractionation of these peak pairs was determined by fraction reanalysis.

Figure 5 shows chromatograms of the fraction reanalysis of peaks 3, 4, and 5. Based on the peak area at 270 nm, fraction 3 was 83% pure. The major impurity of this fraction was caffeine, as could be expected by the EICs shown in Figure 3. To achieve higher purity for this fraction, a second purification run or a change of separation conditions of the first purification run would be necessary.

Fractions 4 and 5 had a resolution of 0.70 in the UV signal, and showed slight overlapping peaks in the EIC traces (not shown). Reanalysis of these fractions revealed that the mass-based trigger enabled good separation of these compounds: fraction 4 was 95% pure, fraction 5 even >99%. This demonstrates the benefits of higher selectivity when fractions are triggered on a mass signal.

Fractions 1 and 2, collected by the MSD trace of GC/EGC, were both cleanly cut from the surrounding peaks, exhibiting purities of >99% each (not shown). Fraction 8 was collected with a purity of 97%. This fraction was collected on the mass trace for CG/ECG; no further experiments to determine the identity have been undertaken, though.

Fractions 6, 7, and 9, as mentioned above, were unlikely to be green tea catechins. To support this hypothesis, these fractions were subjected to an accurate mass analysis by an Agilent 6545 LC/Q-TOF. Experimental masses of the molecular ions in the fractions deviated 150 ppm or more from the accurate mass of the green tea catechins (data not shown). GC/EGC and GCG/EGCG isomers were therefore ruled out as the underlying compounds in these fractions.

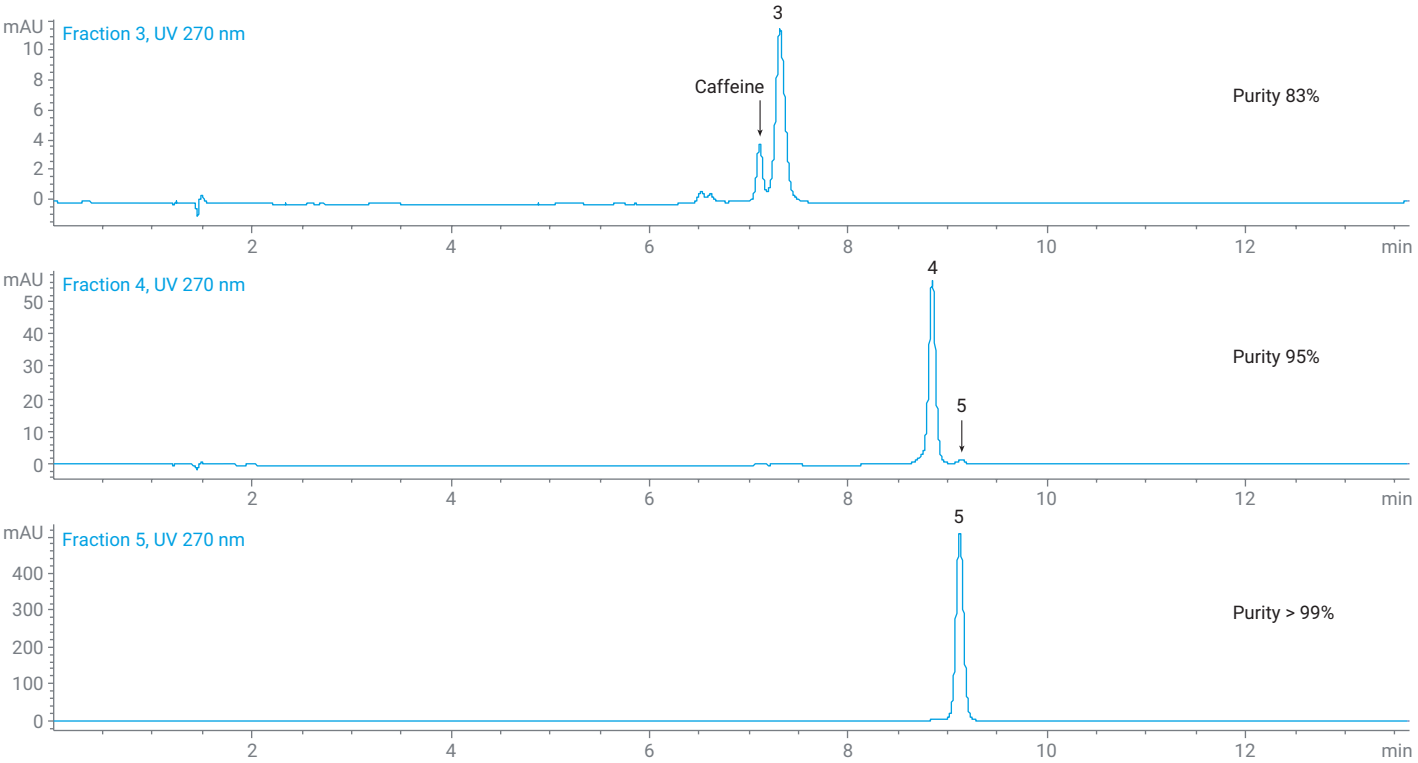


Figure 5. Chromatogram overlay (UV 270 nm) of reanalysis runs of fractions 3, 4, and 5. The strong caffeine signal has contaminated fraction 3; fractions 4 and 5, however, are cleanly cut by the mass-selective trigger.

Conclusion

Green tea was extracted and separated using an Agilent 1260 Infinity II LC with mass selective detector. Adding a 1260 Infinity II Analytical-Scale Fraction Collector and a 1290 Infinity II MS Flow Modulator enabled mass-based fraction collection of green tea flavon-3-ols. The specific mass-based trigger permitted collection of six catechin derivatives, five of which were collected with high purity (>95%). One compound coeluted partly with caffeine but could still be cut out with 83% purity. Three other compounds collected with lower intensity turned out not to be catechin derivatives. Being able to adjust the split ratio to the MSD by a simple method setting was beneficial for an optimum signal that did not change with the composition or viscosity of the mobile phase and contributed to successful fraction collection. This demonstrates that constituents of even complex natural products can be isolated by the specific trigger of an MSD.

References

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3. Turkmen, N. *et al.* Effect of Extraction Conditions on Measured Total Polyphenol Contents and Antioxidant and Antibacterial Activities of Black Tea. *Molecules* **2007**, *12(3)*, 484–496.

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