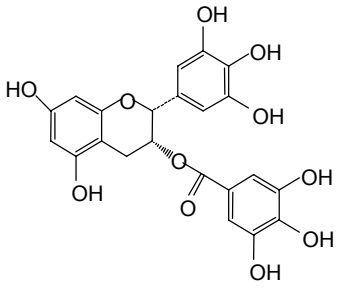
Performance PerSPECtives

Waters® Alliance® HT LC/MS System

Qualitative and Quantitative Analysis of Natural Products

Introduction: Green tea contains significant amounts of the polyphenolic compound (-)-epigallocatechin gallate (EGCG, Figure 1) with reported anti-carcinogenic properties[1] along with many other biological effects. As interest in the human health benefits of EGCG expands, the need for advanced analytical methods for the qualitative and quantitative analysis of these compounds increases. Traditional analytical methods for EGCG and related compounds contained in green tea include TLC, GC, HPLC, and LC/MS. While many of the HPLC methods provide useful information, they are incapable of providing confirmational molecular weight information since identification is based solely upon retention time comparisons. Flow injection Mass Spectrometry does provide molecular weight information, however this non-chromatographic technique cannot differentiate between EGCG and isomers of identical mass. **This Performance PerSPECtive describes how advanced features of Waters Alliance® HT system with on-line mass detection provides a synergistic approach to the rapid analysis of a natural product (EGCG) contained in biological matrices.**

Figure 1: (-)-epigallocatechin gallate (EGCG)
$C_{22}H_{18}O_{11}$ Molecular Weight=458.4



HPLC and Mass Spectrometer Conditions: LC/MS was performed using a Waters Alliance HT HPLC system connected in series to Waters 996 photodiode array (PDA) detector followed by a Waters ZMD Mass Spectrometer fitted with an atmospheric pressure chemical ionization (APcI) probe. Gradient chromatography was performed at 35° C using a Waters XTerra™ MS C18 column (5 µm, 2.1 X 50 mm) at a flow of 1.0 mL/minute using 0.10% formic acid (v:v) in water (solvent A) against CH_3CN (solvent B). Separation and detection conditions are shown in Table 1. All data were collected and processed using MassLynx™ NT software. Samples were prepared for analysis by adding boiling water (100 mL) to green tea leaves (2 g) with gentle stirring, followed by sample filtration and dilution (20X) with water.

To increase sample throughput (decrease cycle time) and increase compound response (enhance peak concentration) a short, narrow-bore column (2.1 x 50 mm) was used at an analytical flow of 1 mL/min. (Note: Cycle time is defined as the sum of run, system equilibration, column equilibration, and vial sampling times.) In addition, the Alliance HT system was run in "Parallel Mode" with rapid equilibration (2 min) between analyses. In Parallel Mode, several processes occur during the chromatographic separation to help speed cycle time (i.e., 1: Injector needle is washed and purged and 2: The next sample is drawn and prepared for injection). To further increase sample throughput, the column is taken out of the flow path following sample elution so that the system tubing can be rapidly flushed with initial condition eluent (5 mL/min) before restoring flow for column re-equilibration (1 mL/min).

Table 1:

HPLC Gradient Conditions

Time (min)	%A	%B
0	95	5
3	95	5
6	75	25
6.1	95	5

Mass Spec APcI Conditions

Source Temp	140 °C
Probe Temp	500 °C
Corona Voltage	3.30 kV
Cone Voltage	30 V
Nebulizing gas	N_2 at 500 L/hr

APcI Mass Spectrometry was performed in the positive ion mode using Single Ion Recording (SIR) at the pseudomolecular weight of EGCG (459.1 m/z). The selected pseudomolecular weight was determined from the MS analysis of an EGCG standard while scanning from 250 to 500 m/z.

How does "Parallel Processing" on the Alliance HT System with short, narrow-bore columns result in higher sample throughput? Typically, HPLC systems running gradient elution methods need to be equilibrated for a total of 3X system volume plus 5X column volume[2]. Using traditional analytical columns (4.6 X 250 mm), column equilibration can add up to 15 minutes (at 1 mL/min) to the total cycle time making the analyses of large numbers of samples difficult. The combination of a short, narrow-bore column (2.1 X 50 mm) at an analytical flow rate combined with the rapid system equilibration, and parallel processing features of the Alliance HT allow both the system and column to be equilibrated in just under 3 minutes. In addition, chromatographic separation or column life are not compromised.

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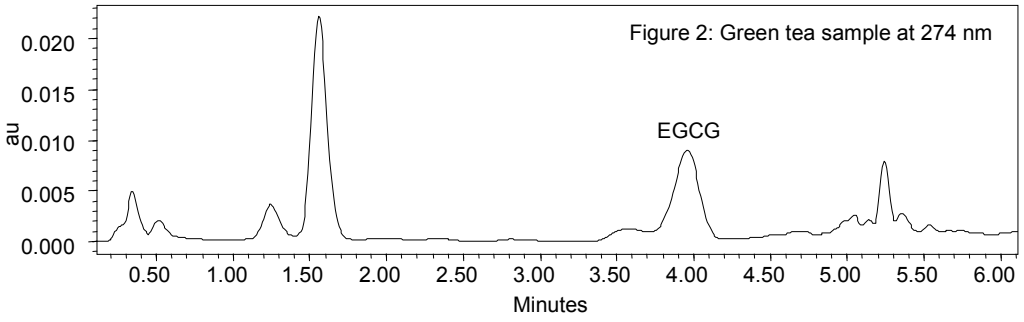
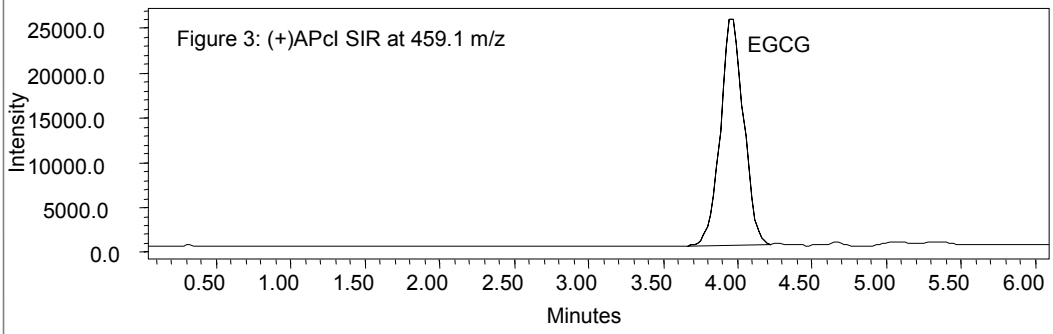
Why use Mass Spectrometry? Mass spectrometry yields a high degree of compound specificity in the analyses of complex samples. Figure 2 shows the analysis of a green tea sample using only HPLC with UV detection at 274 nm. While Integration of the EGCG peak is possible, interference from leading and tailing peaks compromises the ability to obtain accurate quantitation. By comparison, integration of the single EGCG peak using SIR at 459.1 yields superior results (Figure 3). In addition, mass spectrometry provides a confirmational molecular weight information compared to traditional HPLC methods that rely upon retention time comparisons between sample and standard runs for compound identification.





The Synergistic Value of LC/MS Methods: LC/MS techniques provide both retention time and molecular weight information for the analysis of EGCG in green tea. Although direct infusion (flow injection) of the sample into the mass spectrometer would provide accurate quantitation, erroneous values could result due to the presence of isomers in the sample that have the same molecular weight as EGCG. For example, the compound gallocatechin gallate ($C_{22}H_{18}O_{11}$, molecular weight=458.4) is also present in green tea. Presence of this compound could interfere with the accurate quantitation of EGCG if a chromatographic separation did not proceed quantitation by mass detection. However, gallocatechin gallate is adequately resolved from EGCG using the described reversed-phase technique (4.70 min for gallocatechin gallate vs. 3.85 min for EGCG) making the accurate quantitation of the HPLC resolved EGCG possible.

Calibration and Quantitation: A calibration curve was made for EGCG from 5.0 to 100.0 μg/mL. The resulting data yielded an excellent calibration curve using a quadratic equation (r^2 value greater than 0.9998). To ensure low levels of EGCG carryover between injections, the sample manager of the Alliance HT system was washed with a mixture of acetonitrile and water (1:1) (6 seconds injector port wash and a 15-second exterior needle wash). Following the wash, the injector was flushed with 600 μL of purge solvent (0.10 % formic acid). Because this process takes place in parallel during the sample analysis, incorporation of the needle wash step does not increase cycle time. A blank injection performed immediately after the injection of the 100 μg/mL standard showed no detectable carryover from the EGCG standard (data not shown).

Summary:

- Waters Alliance HT system with on-line mass detection provides a synergistic approach to the rapid analysis of natural products (such as EGCG) contained in biological matrices.
- Chromatography prior to SIR mass detection allows isomers to be adequately separated from target compounds resulting in superior EGCG quantitation compared to use of non-LC/MS techniques (e.g., Flow Injection analysis).
- Use of short, narrow-bore columns at analytical flow rates combined with the advanced capabilities of the Waters Alliance HT System (i.e., Parallel Mode of operation) increases sample throughput and peak response by decreasing cycle time between analyses.

1: Fujiki, H.; Yoshizawa, S.; Horiuchi, T.; Suganuma, M.; Yatsunami, J.; Nishswaki, S.; Okabe S.; Nishswaki-Matsushima, R.; Okuda, T.; Sugimura T. Anticarcinogenic Effects of (-)-Epigallocatechin Gallate. *Prev. Med.* 1992, *21*, 503-509.
2: Li, J.B.; Morawski, J. Strategies for Faster Gradient Chromatography. *LC•GC* 1998, *16(5)*, 468-476.