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Automated method development in HPLC for the quantitative determination of catechins in tea

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Keywords

ChromSwordAuto, UltiMate 3000 RS, Accucore Phenyl-X, aromatic compounds, catechins, tea

Goal

The use of a software-based workflow for automated method development in HPLC proves to be a straightforward approach with a significant reduction in process time. The developed method has been adopted for the quantification of catechins in different teas.

Application benefits

- Accelerated HPLC method development by software assistance
- Thermo Scientific™ Accucore™ Phenyl-X column, the best choice for separation of catechins
- Quantitation of catechins in different tea samples to estimate the uptake of catechins per tea cup

Introduction

For years, there has been a trend toward greater consumption of health-promoting foods and beverages.[1,2] Tea is the most consumed beverage in the world, and black tea and green tea are the most popular types. The catechins in tea may play an important role in health due to their antioxidant potential. It is assumed that white tea contains the highest catechin levels because the leaves are processed under relatively mild conditions. Compared to other teas, the leaves of white tea are only dried and minimally further processed. Green tea is briefly heated or roasted, which may lead to a reduction in catechin content, and the lowest catechin levels are expected in black tea due to its fermentation process, which results in oxidation of catechins.[3]

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The goal of the current work was to develop a simple, fast, and sensitive high-performance liquid chromatography (HPLC) method with ultraviolet detection (UV) for determining catechin levels in different types of tea by applying a software-based automated method development workflow, since method development is always a challenging and time-consuming task for the analyst. In-depth chromatographic knowledge is required for the optimization of gradient elution, especially if complex samples require complicated gradient programs. Through sophisticated algorithms, these software packages are able even to optimize methods with complex multi-step gradients and can significantly help to reduce the required lab time of analysts during the process.

ChromSwordAuto™ 5 software was selected as a tool for automated method development. Four different types of stationary phase and four aqueous mobile phases were screened with either acetonitrile or methanol as the organic solvent. The best condition was then selected for quantification of catechins in tea samples.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Optima™ Acetonitrile LC/MS grade (P/N A955-212)
- Fisher Scientific™ Optima™ Methanol LC/MS grade (P/N A456-212)
- Fisher Scientific™ Formic acid LC/MS grade (P/N A117-50)
- Fisher Scientific™ Acetic acid LC/MS grade (P/N A113-50)
- Fisher Scientific™ Ammonium acetate LC/MS grade (P/N A114-50)
- Fisher Scientific™ Ammonium formate LC/MS grade (P/N A115-50)
- Fisher Scientific™ (-)-Epicatechin gallate (P/N 15473519)
- Fisher Scientific™ (-)-Epigallocatechin (P/N 15414199)
- Fisher Scientific™ (-)-Epigallocatechin gallate (P/N 15178317)
- Fisher Scientific™ (+)-Catechin monohydrate (P/N 15119697)

(-)-Epicatechin, (-)-Gallocatechin, (-)-Gallocatechin Gallate, Caffeine, Gallic acid, and Theobromine were purchased from a reputable vendor.

Equipment

- Syringe filter, regenerated cellulose (RC), Ø 15 mm, 0.2 µm, Fisher Scientific (P/N 10712712)
- Vials (amber, 2 mL), Fisher Scientific (P/N 11545884)
- Snap cap with septum (Silicone/PTFE), Fisher Scientific (P/N 10547445)

Automated method development workflow

The method development workflow included the following tasks:

- Column and mobile phase scouting
- Rapid optimization
- Fine optimization

The ChromSwordAuto Developer module was used for column and mobile phase scouting, rapid optimization, and fine optimization tasks. Column and mobile phase scouting was performed during the rapid optimization task using four different stationary phases, four different aqueous eluents, and two organic eluents (Table 1). Afterwards, fine optimization was carried out with the best combination.

Table 1. Columns, aqueous and organic eluents used for the ChromSwordAuto Developer-rapid optimization task

Columns
Thermo Scientific™ Hypersil GOLD™ aQ (100 x 2.1 mm, 1.9 µm)
Thermo Scientific™ Acclaim™ VANQUISH™ Polar Advantage II (150 × 2.1 mm, 2.2 µm)
Thermo Scientific™ Accucore™ Polar Premium (100 × 2.1 mm, 2.6 µm)
Thermo Scientific™ Accucore™ Phenyl-X (100 × 3 mm, 2.6 µm)
Aqueous Eluent
Water + 0.1% acetic acid, pH 3.3
Water + 0.1% formic acid, pH 2.65
20 mM ammonium acetate, pH 3.8
20 mM ammonium formate, pH 3.8
Organic Eluent
Acetonitrile
Methanol

Preparation of standards

Stock solutions of catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin (GC), and gallocatechin gallate (GCG) were prepared with a concentration of 1 mg/mL in 50% acetonitrile and stored at -20 °C until use.

For the automated method development, mixed and single standard working solutions of 10 µg/mL (mix) and 100 µg/mL (single) were prepared.

Caffeine, gallic acid, and theobromine were prepared as 1 mg/mL stock solutions in 50% acetonitrile. In order to identify additional observed peaks in the tea samples, the three standards were injected with 100 µg/mL.

External calibration was performed in the range of 1 to 100 µg/mL by diluting the stock solutions with the appropriate volume of water. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated by extrapolation from the signal-to-noise (S/N) ratio of the smallest calibration point with 1 µg/mL.

Preparation of samples

White tea, green tea, and black tea were purchased in a local supermarket. One hundred milliliters of hot water was poured over one tea bag and left to stand according to the manufacturer's note on the tea box, as listed in Table 2. Afterwards, the sample was allowed to stand until it reached room temperature and filtered through a 0.2 µm RC membrane.

Table 2. Tea samples analyzed

Tea Type	Tea Amount [g]/ Tea Bag	Brewing Time [min]
White tea (Jasmin)	1.25	3
Green tea	1.75	5
Black tea	1.5	4

For quantitative analysis the samples were diluted 1:5, 1:10, and 1:20, respectively, with water prior to injection. All samples were prepared in triplicate and injected three times each within one day.

Instrumentation

- Thermo Scientific™ UltiMate™ 3000 RS system consisting of:
  - Thermo Scientific™ UltiMate™ 3000 Solvent Rack with 4 Degasser Channels (SRD-3400)(P/N 5035.9245)
  - Thermo Scientific™ UltiMate™ 3000 LPG-3400RS Pump (P/N 5040.0036)
  - Thermo Scientific™ UltiMate™ 3000 WPS-3000TRS Well Plate Sampler (P/N 5840.0020)
  - Thermo Scientific™ UltiMate™ 3000 TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
  - Thermo Scientific™ UltiMate™ 3000 DAD-3000RS Photodiode Array Detector (P/N 5082.0020) with Semi-Micro Flow Cell, SST, 2.5 µL (P/N 6080.0300)

LC conditions of final method used for quantitation

Column:	Accucore Phenyl-X, 100 × 3 mm, 2.6 µm (P/N 27926-103030)
Mobile phase:	A: 20 mM Ammonium acetate, pH 3.8 B: Acetonitrile
Flow rate:	1 mL/min
Gradient:	*Time [min]* / *% B* 0 / 0 3.7 / 2 4.7 / 15 15.4 / 15 16.0 / 90 18.0 / 90 18.1 / 0 22.5 / 0
Column temp.:	30 °C
Autosampler temp.:	10 °C
UV wavelength:	280 nm
UV data collection rate:	5 Hz
UV response time:	1 s
Injection volume:	5 µL
Needle wash:	50% ACN

Data processing and software

ChromSwordAuto 5 software 5.0.437.633 with the modules of ChromSwordAuto Scout for pre-screening experiments and ChromSwordAuto Developer for the automated method development and optimization were used. The ReportViewer module of ChromSwordAuto was used for data analysis and evaluation during the method development process and for reporting.

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software 7.2.8 was used for data acquisition and processing of the quantitative experiments.

Results and discussion

The ChromSwordAuto 5 software was chosen to perform a software-based automated method development for the determination of seven catechins in tea samples. First, the ChromSwordAuto Scout module was used to find and select a suitable sample concentration and detection wavelength for the further method development process. Then, in the ChromSwordAuto Developer module the rapid optimization task was chosen to run all possible column-buffer-solvent combinations. The rapid optimization algorithm was enabled to find the most promising combination of stationary and mobile phase. Afterwards, the sample profiling task for this combination was performed for the fine optimization of separation conditions, while a green tea sample served as a real matrix sample.

The catechins were quantified in three different types of tea (black, white, and green tea). A previous study on catechins in tea samples reports an extraction of the tea leaves,[4] which was used to determine the catechin levels in the leaves.

In this work we focused on the catechin content in one tea cup, if one tea bag is used and the brewing time maintained according to the manufacturers note. Accordingly, the consumption of catechins in one cup of tea was determined.

Software-based automated method development

1) ChromSwordAuto Developer – rapid optimization task

During automated method development four stationary phase materials, four different aqueous eluents, and two organic eluents were tested, as listed in Table 1. A 10 µg/mL standard mixture including the seven catechins served as a sample.

With the rapid optimization algorithm, up to five experiments were performed for each possible combination, resulting in a total of 69 methods. Figure 1 summarizes the best condition of each stationary phase based on the separation results and run time. The results showed that the rapid optimization algorithm could find suitable conditions to separate the analytes on all tested stationary phases, while the combination of the Accucore Phenyl-X column, 20 mM ammonium acetate pH 3.8 buffer, and acetonitrile provided the best separation performance and the shortest run time. The flow rate could be adjusted to 1 mL/min, resulting in a maximum backpressure of 340 bar. Peak 8 in the chromatograms (Figures 1B and 1D) was identified as an impurity of acetate and formate salts, since the aqueous eluents without the buffer show no additional peak on these columns (data not shown). However, the impurity peak is well separated from the target compounds.

The resolution of the critical peak pair (Peak 5 and 6) was 3.8 for the separation on the Accucore Phenyl-X column. Additionally, the method provided the smallest peak width, <0.03 min compared to the other tested conditions. Due to additional polar and steric separation effects, the Accucore Phenyl-X column provides orthogonal selectivity to standard reversed-phase mode. Thus, this unique column phase is the first choice for similar aromatic compounds. In comparison to other well-known phenyl phases, the Accucore Phenyl-X phase exhibits the highest aromatic selectivity, while being fairly well compatible with highly aqueous mobile phases.

The run time of the chosen method was determined to be 9.4 minutes, resulting in a baseline separation of all seven catechins within 4 min (Table 3) using a standard mixture. Peaks were assigned with single standard solutions of 100 µg/mL injected with the same method.

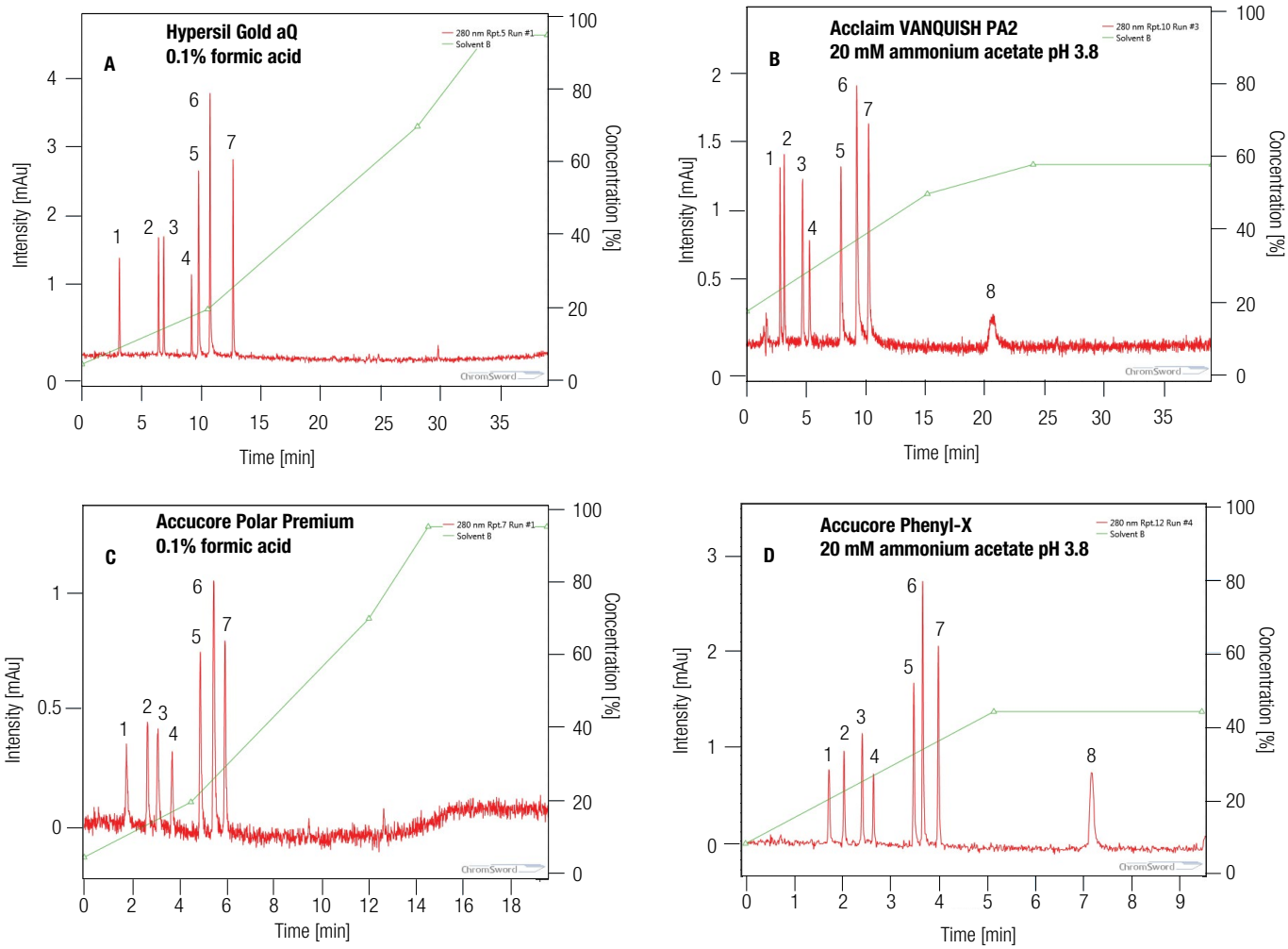

Figure 1. Summary of best methods obtained on each column, with respect to the ideal eluent composition (buffer/acetonitrile) after running rapid optimization in ChromSwordAuto Developer (the green line represents the gradient profile)

Table 3. Peak assignment of the seven catechins of Figure 1D

Peak #	Retention Time [min]	Compound	
1	1.7	GC	Gallocatechin
2	2.0	EGC	Epigallocatechin
3	2.3	C	Catechin
4	2.6	EC	Epicatechin
5	3.4	EGCG	Epigallocatechin gallate
6	3.6	GCG	Gallocatechin gallate
7	3.9	ECG	Epicatechin gallate

2) ChromSwordAuto Developer – fine optimization and sample profiling task

For the fine optimization, the ChromSwordAuto Developer module in sample profiling mode (gradient and isocratic) was chosen. To include possible matrix interferences, all three types of tea were injected and green tea selected for further optimization because it showed the highest number of matrix peaks.

GC, EGC, EC, EGCG, and ECG could already be qualitatively identified in the sample. The missing or very low intense catechins C and GCG were spiked into the sample prior injection with a concentration of 100 µg/mL. Figure 2 shows chromatograms obtained before and after the fine optimization of the gradient profile for the selected combination of the column and mobile phase.

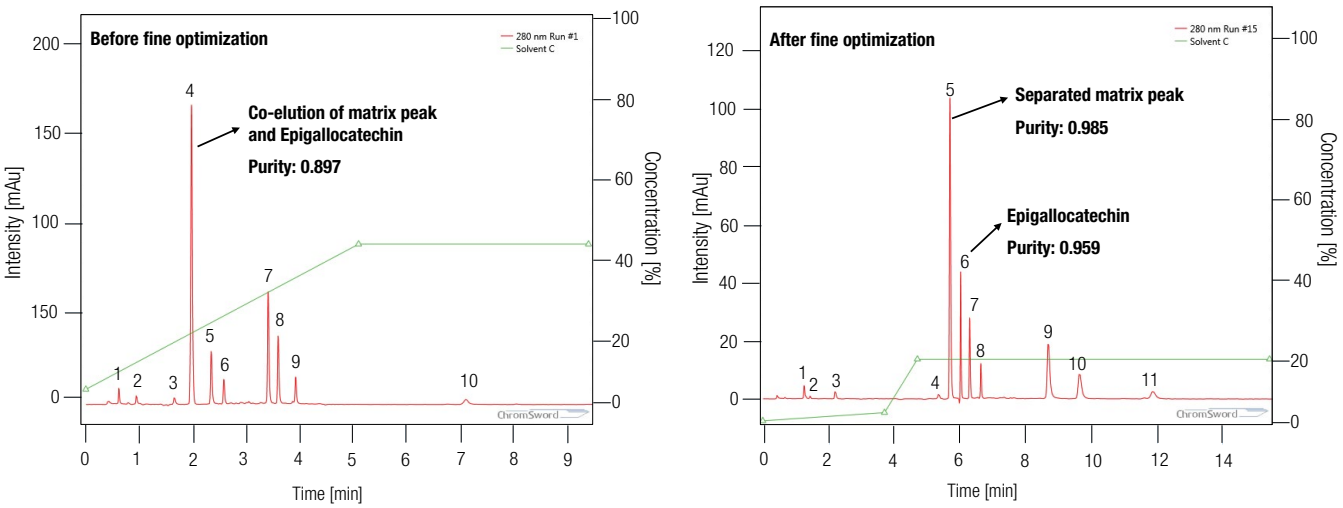

Figure 2. Comparison of separation results before and after fine optimization (the green line represents the gradient profile)

The peak purity was used to assess the quality of the separation based on UV spectral data. Table 4 summarizes the peak purities before and after fine optimization. It was found that the purity value for EGC was determined to be 0.897 before the fine optimization step, while all other compounds showed at least a peak purity of 0.941 or better. This leads to the assumption that EGC co-eluted with another analyte with the method obtained by the rapid optimization task. After applying sample profiling, a major matrix component was successfully separated from EGC and the peak purity of EGC increased to 0.959. The run time extended to 15.4 min, while all compounds were separated within 12 min (Table 4). Gallic acid, theobromine, and caffeine were injected to the final method as they are known to be common analytes in tea samples. In the chromatogram after the fine optimization, Peak 2 could be assigned to gallic acid, Peak 3 to theobromine, and Peak 5 to caffeine. Caffeine was identified to be the main matrix peak in the sample, which completely co-eluted with EGC before the fine optimization step.

In summary, the new method was developed within 5 days (calculation based on 24 hours/ day for instrument time and 8 hours/per day for analyst time). A summary of the required instrument time and analyst time during the whole process is shown in Table 5.

Table 4. Retention time and peak purity before and after fine optimization (sample profiling) of assigned catechin analytes

Compound	Before Fine Optimization			After Fine Optimization		
	Peak #	Retention Time [min]	Peak Purity	Peak #	Retention Time [min]	Peak Purity
GC	3	1.7	0.992	4	5.4	0.944
EGC	4	2.0	0.897	6	6.0	0.959
C	5	2.3	0.983	7	6.3	0.986
EC	6	2.6	0.994	8	6.6	0.995
EGCG	7	3.4	0.941	9	8.7	0.989
GCG	8	3.6	0.971	10	9.6	0.997
ECG	9	3.9	0.994	11	11.9	0.968

Table 5. Summary of the time required for automated method development of catechins by using ChromSwordAuto 5 software

Experiment	Instrument Time [h]	Analyst Time [h]
Developer-rapid optimization	42	16
Developer-fine optimization	8.5	8

The instrument time consists of the run time of experiments, equilibration, and solvent purging times.

The analyst time can be divided into three categories:

- Before the process: preparation of mobile phase and samples, setting-up the instrument
- During the process: checking data for adjustments, restarting experiments if necessary, preparation of additional mobile phase if needed to finish all experiments
- After the process: data evaluation to select the best method for the separation of catechins in tea samples

We assume that for manual method development the time required to find the optimal method for all screened stationary and mobile phase would have been much longer. In addition, the success of a manual method development depends crucially on the experience of the analyst, while software-based automated method development uses sophisticated algorithms to optimize the separation, without user interaction.

Quantitation of catechins in tea samples

External calibration was performed in the range of 1 µg/mL to 100 µg/mL using reference standards dissolved in water. Linearity was found to be excellent with R^2 = 0.9984–0.9995. LOD and LOQ were estimated by using the smallest calibration point as a reference and extrapolating the concentration for LOD to S/N 3 and LOQ to S/N 10. Low limits could be achieved, with the exception of gallocatechin, which shows 10-times higher values (Table 6). However, higher levels of catechins are expected in the samples, well above the indicated LOD and LOQ values.

Looking at the chromatograms (Figure 3), all seven catechins were detected in all three samples, but in quite different amounts. In order to obtain the content of catechins in one cup of tea, a cup volume of 200 mL was assumed and the brewing time stated by the manufacturer on the tea bag was respected. The results obtained are presented in Figure 4.

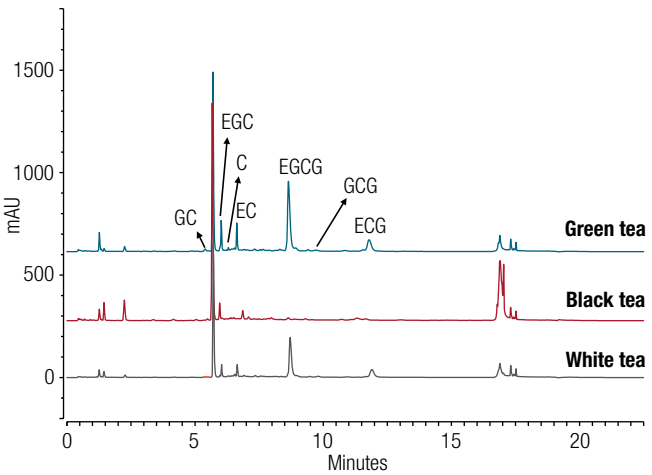

Figure 3. Overlaid chromatograms of three tea samples with assigned catechin compounds (blue: green tea, red: black tea; gray: white tea)

Table 6. Calibration results and LOD, LOQ values

Compound	Calibration Range [µg/mL]	R^2	LOD [µg/mL]	LOQ [µg/mL]
GC	1–100	0.9993	2.5	8.0
EGC	1–100	0.9995	0.6	2.0
C	1–100	0.9994	0.1	0.2
EC	1–100	0.9992	0.1	0.3
EGCG	1–100	0.9994	0.2	0.7
GCG	1–100	0.9984	0.2	0.6
ECG	1–100	0.9992	0.2	0.7

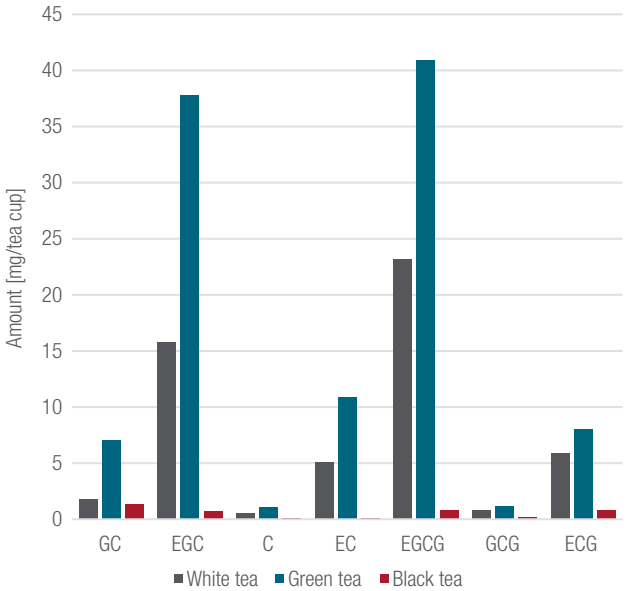

Figure 4. Quantitative results of all seven catechins in each tea sample

The total amount of all seven catechins was determined with 107 mg/tea cup for green tea and 53 mg/tea cup for white tea. Black tea showed the lowest amount with 4 mg/tea cup.

In green and white tea, EGCG and EGC showed the highest concentrations, while GC was the dominant species in black tea. The lowest amounts observed were C and GCG, except for black tea, which showed the lowest amount for EC.

In analyzing the three tea samples, it was expected to find the highest catechin content in the white tea and the lowest in black tea sample. The leaves for white tea are minimally processed, while in the production of black tea the leaves are full fermented, which causes oxidation of the catechins and therefore decreases the catechin content. The content in green tea is expected between these two types, as the leaves are roasted briefly, which may affect the catechin levels.[3]

Contrary to the expectations, the highest total amount of catechins was observed for green tea. It should be noted, that one green tea bag had the highest tea content and that the brewing time was longer than for the others (Table 2). This finding is in agreement with another study on catechins in white, green, and black tea, where the leaves were extracted and the catechin levels compared.[4]

The method offers excellent separation of the analytes and matrix substances on the Accucore Phenyl-X column and is well-suited for routine quantification of catechins in different tea types.

Conclusions

- ChromSwordAuto 5 software provides fully automated method development and optimization on an UltiMate 3000 HPLC system.
- Software-based automated method development was implemented easily and quickly for the determination of seven catechins.
- Sophisticated algorithms allow straightforward method development even for non-experienced analysts and are quite time-saving compared to manual development.
- With its additional orthogonal selectivity, the Accucore Phenyl-X column is the first choice for the separation of similar aromatic compounds such as catechins and can withstand highly aqueous mobile phase conditions required for the current application.
- The highest total catechin content was found for green tea with 107 mg/tea cup, followed by white tea at 53 mg/tea cup, and black tea with 4 mg/tea cup.

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