Conventional and Accelerated-Solvent Extractions of Green Tea (*Camellia sinensis*) for Metabolomics-Based Chemometrics

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Abstract:

Metabolomics has emerged as an important analytical technique for multiple applications. The value of information obtained from metabolomics analysis depends on the degree to which the entire metabolome is present and the reliability of sample treatment to ensure reproducibility across the study. The purpose of this study was to compare methods of preparing complex botanical extract samples prior to metabolomics profiling. Two extraction methodologies, accelerated solvent extraction and a conventional solvent maceration, were compared using commercial green tea [*Camellia sinensis* (L.) Kuntze (Theaceae)] products as a test case. The accelerated solvent protocol was first evaluated to ascertain critical factors influencing extraction using a D-optimal experimental design study. The accelerated solvent and conventional extraction methods yielded similar metabolite profiles for the green tea samples studied. The accelerated solvent extraction yielded higher total amounts of extracted catechins, was more reproducible, and required less active bench time to prepare the samples. This study demonstrates the effectiveness of accelerated solvent as an efficient methodology for metabolomics studies.

Keywords: Accelerated solvent extraction | *Camellia sinensis* | Design of experiment | Green tea | Metabolomics

Article:

1. Introduction

Metabolomics has developed into an important tool in analyzing large datasets, including those related to disease biomarkers [1], food quality [2], and natural products discovery [3], [4]. Using

metabolomics, the investigator seeks to correlate changes in the qualitative and quantitative chemical profile (incorporating a maximum of observable metabolites) with a corresponding alteration in phenotype as a result of a perturbation to the system [5]. Reproducibility of sample-to-sample preparation and extraction remains one of the most critical steps for rigorous metabolomic studies [6], and the sample extraction procedure is often a bottleneck in metabolomics studies and other analytical investigations. Conventional sample preparation techniques require multiple steps involving solid and liquid transfer [7], and can account for up to one-third of the error of the overall analytical procedure [8]. Thus, robust and reproducible techniques for sample extraction are imperative for reliable metabolomics investigations.

Accelerated-solvent extraction (ASE, also known as pressurized liquid extraction (PLE)) has been touted as a reliable and efficient method of metabolite extraction [9]. Several factors in the accelerated solvent extracton approach increase efficiency. Elevated solvent temperature increases the solubilizing capacity of the solvent, increases diffusion rates for mass transfer into the solvent, and reduces viscosity to ensure deeper penetration of the solvent into the sample matrix. High pressure keeps organic solvents in a liquid phase, even at elevated temperatures, and assists in permeation of the solvent through the sample matrix, maximizing contact with the analyte and facilitating effective extraction [10]. Accelerated solvent extraction enables rapid and effective extractions with dramatically reduced solvent requirements as compared to conventional bench top extraction procedures [9]. The automation of accelerated solvent extractions increases its efficiency and reproducibility, and ASE has become a commonly employed tool for extraction of pesticides from environmental samples [11], food or supplement contaminants [12], and supplement or dietary nutraceuticals [13]. As of yet, however, this technique is not widely employed in natural products chemistry investigations, which tend to rely on more traditional benchtop extraction methodologies.

The goal of this study was to evaluate accelerated solvent extraction for sample preparation in a natural products metabolomics study using green tea [*Camellia sinensis* (L.) Kuntze (Theaceae)] products as a test case. We predicted that ASE would be advantageous for such a study, where reproducible extraction procedures are critical to enable effective comparison among samples that may have very similar metabolite profiles. A design of experiment (DOE) approach was employed to select representative sample conditions using quantification of catechins as the dependent variable. Catechins represent the largest group of polyphenols in green tea leaves and are considered the dominant bioactive phytochemicals in this botanical [14]. This methodology was compared against a conventional benchtop extraction technique, solvent maceration. Our ultimate objective with this study was to explore the utility of accelerated solvent extraction for providing rapid and reproducible extraction of samples prior to metabolomic profiling.

2. Materials and methods

2.1. General materials

All solvents and chemicals used were of spectroscopic grade and obtained from Thermo Fisher Scientific (Waltham, MA, USA). Green tea catechin standards ((+)-catechin, (−)-epicatechin,

(−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG)) were purchased from Chromadex (Irvine, CA, USA).

2.2. General experimental procedures

Ultraperformance liquid chromatography − mass spectrometry (UPLC–MS) data were acquired using a Q Extractive Plus quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific) with an electrospray ionization source coupled to an Acquity UPLC system (Waters, Milford, MA, USA). To collect UPLC–MS data, each sample was reconstituted in MeOH to a concentration of 1 mg/mL, and triplicate 3 μL injections onto the UPLC of each sample were performed. The samples were eluted from the column (Acquity UPLC BEH C_{18} 1.7 μm, 2.1×50 mm, Waters) at a flow rate of 0.3 mL/min using the following binary gradient with solvent A consisting of H_2O (0.1% formic acid added) and solvent B consisting of CH_3CN (0.1% formic acid added): initial isocratic composition of 95:5 (A:B) for 1.0 min, increasing linearly to 0:100 over 20 min, followed by an isocratic hold at 0:100 for 1 min, gradient returned to starting conditions of 95:5 for 2 min, and held isocratically again for 1 min. The mass spectrometer was operated in the positive/negative switching ionization mode over a full scan range of 150 − 2000 Da with the following settings: capillary voltage set at 5 V, capillary temperature set at 300 °C, tube lens offset set at 35 V, spray voltage set at 3.80 kV, sheath gas flow set at 35, and auxiliary gas flow set at 20.

2.3. Sample selection

Commercial green tea products were selected using readily available product quality reports [15]. Three whole-leaf teas were chosen (T07, T13, and T21), along with the *Camellia sinensis* standard reference material from the National Institute of Standards and Technology (NIST No. 3254) (T26). Tea sample codes are consistent with those used in a previous report [16].

2.4. Extraction methods

The green tea samples were extracted by two methods: conventional benchtop extraction and accelerated solvent extraction. For each extraction methodology, tea samples were extracted in triplicate. For conventional extraction, 200 mg of sample was loaded into a scintillation vial with 20 mL MeOH. Vials were shaken overnight at room temperature, filtered by vacuum filtration, and evaporated under a N_2 stream. Samples were dried to enable determination of the mass of extracted solid per volume, and to provide a more stable format for storage.

Accelerated solvent extractions were carried out with a Dionex ASE 350 (ThermoFisher Scientific). For all extractions, 22 mL cells were charged with 200 mg sample and filled with diatomaceous earth/celite as a neutral matrix. All samples were subjected to three consecutive extractions, which were pooled. Extracted samples were quantitatively transferred and evaporated under N_2.

2.5. D-optimal design and statistical analysis

D-optimal experimental designs maximize the experimental space spanned by a selected number of experiments for a defined model matrix [17]. In the present study, a three-factor, three-level D-optimal design was employed to study the factors influencing the accelerated solvent extraction's ability to obtain the highest quantity of catechins from green tea samples. A D-optimal design was selected to limit the amount of resources (number of runs) required for analysis and investigation of the parameters. Extraction conditions were evaluated using three independent variables: extraction temperature (X_1), cycle time (X_2), and solvent ratio (X_3). Variations in extraction temperature and solvent ratio were straight forward, and based upon previous extraction experience with green tea samples [16]; cycle time represents the contact time between the solvent and sample, and levels of this parameter were altered based upon manufacturer recommendations. The factors were studied at three different levels (coded as −1, 0, and +1), and the D-optimal design resulted in a set of 15 experiments, with three center points for replication (Table 1). The extracted concentration of each catechin (catechin, epicatechin, ECG, EGC, and EGCG) served as the response variables. The sample T07 was used for all D-optimal experiments. Experimental data were fitted to a second-order polynomial model and regression coefficients obtained. The generalized second-order polynomial model used in the response surface analysis is shown in Eq. (1),

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i} \sum_{<i=1}^{3} \beta_{ij} X_i Y_i \quad (1)$$

where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i, and X_j are the independent variables.

Table 1. Experimental matrix for the D-optimal design of experiment. Fifteen total runs were performed with varying levels of the three main parameters (temperature, cycle time, and solvent ratio (Experiments 1–12)). Reponses were coded −1, 0, and +1 representing the low, middle, and high values for each parameter. Experiments 13–15 were identical in their levels to provide replication information.

experiment number	X_1 temperature (°C)	D-optimal code	X_2 cycle time (min)	D-optimal code	X_3 solvent ratio (MeOH: $CHCl_3$)	D-optimal code
1	75	0	7	+1	0:1	−1
2	50	−1	3	−1	1:0	+1
3	100	+1	5	0	1:0	+1
4	75	0	3	−1	0:1	−1
5	100	+1	7	+1	1:0	+1
6	100	+1	3	−1	0:1	−1
7	100	+1	7	+1	0:1	−1
8	50	−1	7	+1	1:0	+1
9	75	0	5	0	1:0	+1
10	100	+1	3	−1	1:0	+1
11	50	−1	5	0	1:0	+1
12	100	+1	3	−1	0:1	−1
13	75	0	5	0	1:1	0
14	75	0	5	0	1:1	0
15	75	0	5	0	1:1	0

2.6. Metabolite quantification

Quantification of the major catechin components of the green tea products used five calibration standards. UPLC–MS analysis was conducted as described in Section 2.2. Standards were prepared in spectrometric-grade MeOH and diluted in a two-fold dilution series ranging from 200 to 0.100 μg/mL before injection. Calibration curves were constructed for both accelerated solvent extractions and conventional extractions by plotting the area of the selected ion chromatogram for each standard versus nominal concentration (Tables S1 and S2). Concentrations of each standard in the extracts were determined by $1/x^2$ weighted least-squares linear regression.

2.7. Chemometric and statistical analysis

Chemometric analysis was conducted using a slightly modified version of a previously reported method [4]. The untargeted UPLC–MS dataset were analyzed, aligned, and filtered with MZmine 2.20 software (http://mzmine.sourceforge.net/) [18]. Peak detection in MZmine was achieved using the following parameters: noise level (absolute value), 5×10^5counts; minimum peak duration, 0.05 min; tolerance for *m*/*z* variation, 0.05; and tolerance for *m*/*z* intensity variation, 20%. Deisotoping, peak list filtering, and retention time alignment algorithm packages in MZmine were employed to refine peak detection. Finally, the join algorithm integrated all metabolomic profiles into a single data matrix using the following parameters: the balance between *m*/*z* and retention time was set at 10.0 each, *m*/*z*tolerance was set at 0.001, and retention time tolerance size was defined as 0.5 min. The spectral data matrix was exported for analysis, both as a set of peak areas for individual ions detected in triplicate extractions, and as the average peak areas for the triplicate extractions. Chemometric analysis was performed on the datasets (both the individual triplicate data and the average of the triplicates for each sample) using Sirius version 9.0 (Pattern Recognition Systems AS, Bergen, Norway) [19], [20]. Transformation from heteroscedastic to homoscedastic noise was carried out by a fourth root transform of the spectral variables [21]. Statistical comparisons were performed using student's *t*-test (Excel, Microsoft Inc., Redmond, WA), with statistical significance determined at the $p < 0.05$ or $p < 0.01$ level.

Principal component analysis (PCA) was used to provide unsupervised statistical analysis of the green tea samples. Reproduced correlation coefficients (RCC) were calculated from principal component model loadings as described elsewhere to provide a quantitative value for similarity between samples [16]. The correlation coefficient describes the extent to which a given sample (in our case a green tea extract) correlates with any other sample in the dataset after removing noise and other sources of small variation from the data. Coefficient values closer to 1 demonstrate a stronger correlation (i.e. greater similarity) between the two samples.

3. Results and discussion

3.1. Evaluation of accelerated solvent extraction factors

A three-factor, three-level D-optimal experimental design was employed to evaluate the accelerated solvent extraction efficiency of green tea catechins. As noted in *Methods*, three factors were examined: extraction temperature, cycle time, and solvent ratio. This resulted in a

set of 12 experiments, with three center points for replication (Table 1). The reproduced correlation coefficient incorporates the untargeted metabolome profile of each sample into a multivariate statistical model and yields a single quantitative metric of similarity [16]. Three central replicates, representing the variables' parameter mid-points (oven temperature, solvent ratio, and cycle time), were run using identical conditions, and were evaluated as part of the experimental design to provide information on reproducibility (experiments 13–15, Table 1).

A

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1.000	-0.719	0.061	1.000	0.166	1.000	1.000	-0.426	-0.294	-0.287	0.108	1.000	-0.879	-0.988	-0.934
2	-0.719	1.000	0.650	-0.726	0.567	-0.725	-0.725	-0.323	-0.453	-0.459	0.613	-0.723	0.800	0.819	0.920
3	0.061	0.650	1.000	0.050	0.994	0.053	0.051	-0.929	-0.972	-0.974	0.999	0.055	0.530	0.096	0.300
4	1.000	-0.726	0.050	1.000	0.155	1.000	1.000	-0.416	-0.284	-0.277	0.098	1.000	-0.874	-0.989	-0.938
5	0.166	0.567	0.994	0.155	1.000	0.157	0.156	-0.963	-0.991	-0.992	0.998	0.160	0.616	-0.009	0.198
6	1.000	-0.725	0.053	1.000	0.157	1.000	1.000	-0.418	-0.286	-0.279	0.100	1.000	-0.875	-0.989	-0.937
7	1.000	-0.725	0.051	1.000	0.156	1.000	1.000	-0.417	-0.285	-0.278	0.099	1.000	-0.874	-0.989	-0.937
8	-0.426	-0.323	-0.929	-0.416	-0.963	-0.418	-0.417	1.000	0.990	0.989	-0.946	-0.420	0.206	0.279	0.074
9	-0.294	-0.453	-0.972	-0.284	-0.991	-0.286	-0.285	0.990	1.000	1.000	-0.982	-0.289	0.171	0.141	-0.067
10	-0.287	-0.459	-0.974	-0.277	-0.992	-0.279	-0.278	0.989	1.000	1.000	-0.983	-0.282	0.171	0.134	-0.074
11	0.108	0.613	0.999	0.098	0.998	0.100	0.099	-0.946	-0.982	-0.983	1.000	0.102	-0.569	0.049	0.254
12	1.000	-0.723	0.055	1.000	0.160	1.000	1.000	-0.420	-0.289	-0.282	0.102	1.000	-0.876	-0.989	-0.936
13	-0.879	0.800	0.530	-0.874	0.616	-0.875	-0.874	0.206	0.171	0.171	-0.569	-0.876	1.000	0.935	0.965
14	-0.988	0.819	0.096	-0.989	-0.009	-0.989	-0.989	0.279	0.141	0.134	0.049	-0.989	0.935	1.000	0.978
15	-0.934	0.920	0.300	-0.938	0.198	-0.937	-0.937	0.074	-0.067	-0.074	0.254	-0.936	0.965	0.978	1.000

B

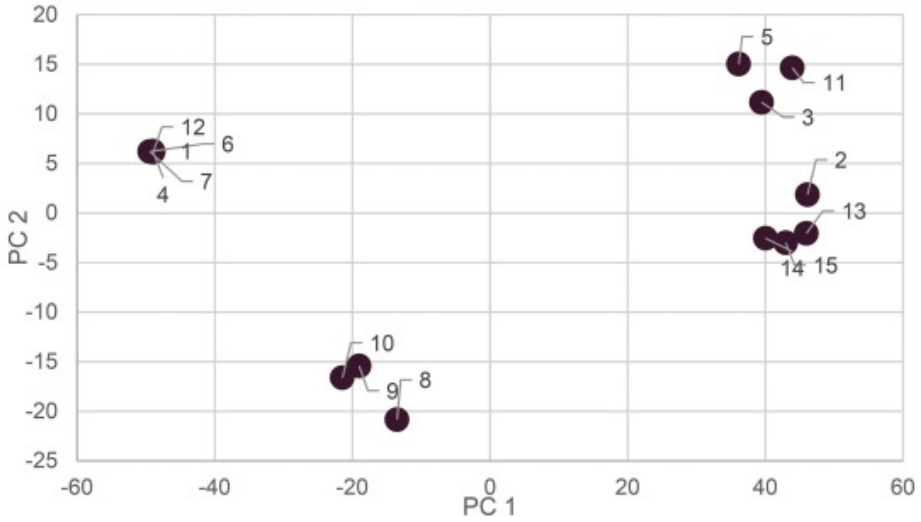


Fig. 1. Similarities between D-optimal experimental design runs. (A) Reproduced correlation coefficient heat map matrix. Correlation was based upon the metabolomic profile for each sample, and calculated from the reproduced correlation coefficient comprised of a three principal component model. Darker shades and correlations closer to 1 represent stronger correlation between samples. (B) PCA scores plot of PC1 vs PC2 (explaining 96.7% of total variance). Numbers represent design experiments (Table 1).

The three central replicates demonstrated a high degree of similarity to one another (Fig. 1A), and were clustered in close proximity to one another in the PCA scores plot (Fig. 1B). Principal component analysis scores plot of PC1 vs PC2 (explaining 96.7% of total variance) for the 15 experiments demonstrated distinct clusterings between experiments with similar reaction conditions. For example, experiments 1, 4, 6, 7, and 12, which used the same 0:100 MeOH:$CHCl_3$ solvent ratio (Table 1), demonstrated tight clustering in the PCA plot (Fig. 1B) as well as high similarity scores in the RCC matrix (Fig. 1A), with a correlation of 1.000 between

the experiments. Other clusters in the PCA scores plot were mirrored by similar reproduced correlation coefficients.

Extraction efficiency (determined by quantification of the five dominant green tea catechins) was correlated against the three factors studied (oven temperature, cycle time, and solvent ratio). A quantitative relationship expressed via a second-order polynomial equation with interaction terms was fitted between the experimental results obtained from the D-optimal experimental design and the input factors. The final equations obtained, in terms of coded factors, are shown in 2–6 below.

$$\text{Catechin} = 49.1 - 3.25X_1 + 0.001X_2 + 18.43X_3 + 1.8X_1X_2 - 2.7X_1X_3 + 0.4X_2X_3 - 3.32X_1^2 + 3.09X_2^2 + 0.01X_3^2 \quad (2)$$

$$\text{Epiatechin} = 66.4 - 3.03X_1 + 0.31X_2 + 21.48X_3 + 10.57X_1X_2 - 0.73X_1X_3 + 2.45X_2X_3 - 1.5X_1^2 - 2.8X_2^2 + 0.01X_3^2 \quad (3)$$

$$\text{EGCG} = 34.0 - 2.43X_1 - 1.11X_2 + 12.26X_3 - 0.02X_1X_2 - 2.4X_1X_3 - 1.1X_2X_3 - 3.1X_1^2 + 0.9X_2^2 + 0.01X_3^2 \quad (4)$$

$$\text{ECG} = 6.77 - 0.45X_1 - 0.19X_2 + 1.91X_3 + 0.19X_1X_2 - 0.41X_1X_3 - 0.15X_2X_3 - 0.60X_1^2 + 0.19X_2^2 + 0.01X_3^2 \quad (5)$$

$$\text{ECG} = 21.85 - 0.31X_1 - 0.12X_2 + 5.70X_3 + 0.39X_1X_3 - 0.78X_1X_3 - 0.91X_2X_3 - 0.52X_1^2 + 1.85X_2^2 + 0.01X_3^2 \quad (6)$$

The value of R^2, a measurement for fitness of the regression models, ranged from 0.86 to 0.99 (Table 2), indicating the experimental data were in a good agreement with predicted values (Fig. S1). In observing the regression model equations oven temperature (X_1) had a negative effect on catechin extraction, though it was not significant for epicatechin and EGC (Table 2). Cycle time (X_2) did not yield any significant effects, and the regression coeffieicnt was variable across all five measured catechins. In contrast, solvent ratio (X_3) had a positive influence on the catechin concentrations, and was statistically significant ($p < 0.05$) across all five catechins. The absolute value for the coefficient of X_3 is larger than that of either X_1 or X_2. This indicates that the linear term influence of X_3 is more significant than those of X_1 and X_2. Some interactions were also observed between factors (Table 2), though they were not universally significant across all catechins measured. The D-optimal design was not sufficient to optimize the extraction factors; however, the responses provided insight into the key parameters that affected extraction. Based upon the D-optimal design and the authors' previous experience with green tea extraction [16], the final factors selected for the green tea screening were: oven temperature 75 °C; cycle time 5 min, and a 100% MeOH solvent composition.

3.2. Comparison of accelerated solvent and conventional extraction methods

Accelerated solvent extractions of green tea samples, using the final extraction conditions (75 °C, 100% MeOH, and 5 min cycle time), yielded significantly greater mass for all four tea samples tested compared to conventional extraction (Table 3). In general, the accelerated solvent extraction method yielded approximately 27–70% more material than conventional benchtop extraction (Table 3). This is significant to natural products analysis, as having enough material for isolation after metabolomics analysis is often a critical limiting factor in analysis. Obtaining

additional starting material from accelerated solvent extraction to facilitate subsequent isolation efforts would be a benefit.

Table 2. Analysis of variance (ANOVA) for the second order polynomial models .

Model	**(+)-catechin**			**(−)-epicatechin**			**EGCG**		
	RC	**SE**	***p*-value**	**RC**	**SE**	***p*-value**	**RC**	**SE**	***p*-value**
	49.1	2.1	<0.0001	66.4	1.0	0.0006	34.0	2.1	<0.0001
X_1	−3.25	0.15	0.0306	−3.03	0.56	0.6099	−2.43	0.18	0.0852
X_2	0.001	0.15	0.9992	0.31	0.56	0.9575	−1.11	0.18	0.3822
X_3	18.43	0.15	<0.0001	21.48	0.56	0.0088	12.26	0.18	<0.0001
X_{12}	1.8	1.1	0.1536	10.57	0.55	0.1027	−0.02	1.1	0.9860
X_{13}	−2.7	1.1	0.0546	−0.73	0.55	0.9010	−2.4	1.1	0.0880
X_{23}	0.4	1.1	0.7391	2.45	0.55	0.6784	−1.1	1.1	0.3850
X_1^2	−3.32	0.47	0.0004	−1.5	2.3	0.5412	−3.1	4.8	0.0007
X_2^2	3.09	0.47	0.5125	−2.8	2.3	0.2494	0.9	4.8	0.0987
X_3^2	0.01	0.47	1.0000	0.01	2.3	1.000	0.001	4.8	1.0000
R^2	0.989			0.885			0.974		
CV%	0.59			2.3			0.37		

Model	**ECG**			**EGC**		
	RC	**SE**	**p-value**	**RC**	**SE**	**p-value**
	6.77	0.28	<0.0001	21.85	0.85	0.0013
X_1	−0.45	0.15	0.0269	−0.31	0.21	0.9862
X_2	−0.19	0.15	0.2638	0.12	0.21	0.9586
X_3	1.91	0.15	<0.0001	5.7	0.21	0.0366
X_{12}	0.19	0.15	0.2478	0.39	0.2	0.1036
X_{13}	−0.41	0.15	0.0382	0.78	0.2	0.7278
X_{23}	−0.15	0.15	0.3648	0.91	0.2	0.6826
X_1^2	−0.6	0.62	<0.0001	0.52	0.86	0.7819
X_2^2	0.19	0.62	0.0906	−1.85	0.86	0.0727
X_3^2	0.01	0.62	1	0.01	0.86	1
R^2	0.987			0.982		
CV%	0.48			0.62		

RC Regression coefficient, *SE* Standard error, *CV%* Coefficient of variation, *EGCG* (−)-epigallocatechin gallate, *ECG* (−)-epicatechin gallate, *EGC* (−)-epigallocatechin.

Table 3. Mass yields of the accelerated solvent and conventional extractions. Asterisks indicate significant differences between accelerated solvent and conventional extraction methods. Tea codes are taken from a previous reference [16].

sample number	**accelerated solvent extraction mass (mg)**	**conventional extraction mass (mg)**
T07	58.4 ± 1.2*	34.2 ± 1.3
T13	54.48 ± 0.52*	31.88 ± 0.15
T21	57.8 ± 1.3*	45.6 ± 1.0
T26	81.15 ± 0.32*	57.2 ± 1.2

* $p < 0.01$.

Individual catechin metabolites (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), and (+)-gallocatechin (GC) were quantified from the metabolomic profiles for the samples under both extraction conditions (Fig. 2, Tables S1 and S2). Accelerated solvent extraction (Fig. 2, light bars) yielded significantly higher quantities of (−)-epicatechin gallate and (−)-epicatechin across all four tea samples. Across all six metabolites, the accelerated solvent extraction had generally higher

extraction levels, even if some samples did not reach statistical significance, indicating that accelerated solvent extraction produced higher yields of catechins compared to conventional extraction. The standard deviation among replicate samples (as indicated by the magnitude of the error bars in Fig. 2) was similar between the two extraction approaches, although the two were performed by researchers with vastly different levels of experience in natural product extraction; the benchtop workflow was executed by researchers with >30 years combined experience in natural product chemistry, while the accelerated solvent extraction was performed by a researcher with <1 month of training. The observation that similar repeatability can be achieved with an accelerated solvent extraction that is comparable to trained benchtop researchers highlighted a positive benefit of the automated technique in controlling for interpersonal variability and removing subtle technical aspects of the experimental technique that could introduce variability and reduce reproducibility [22].

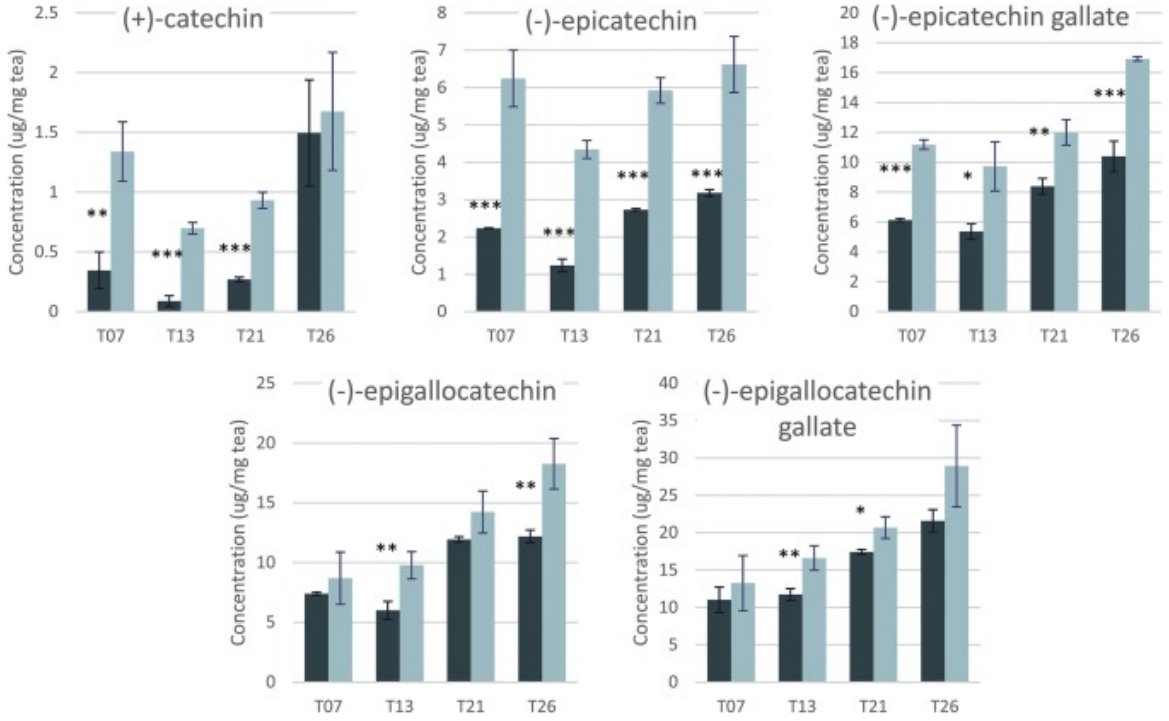


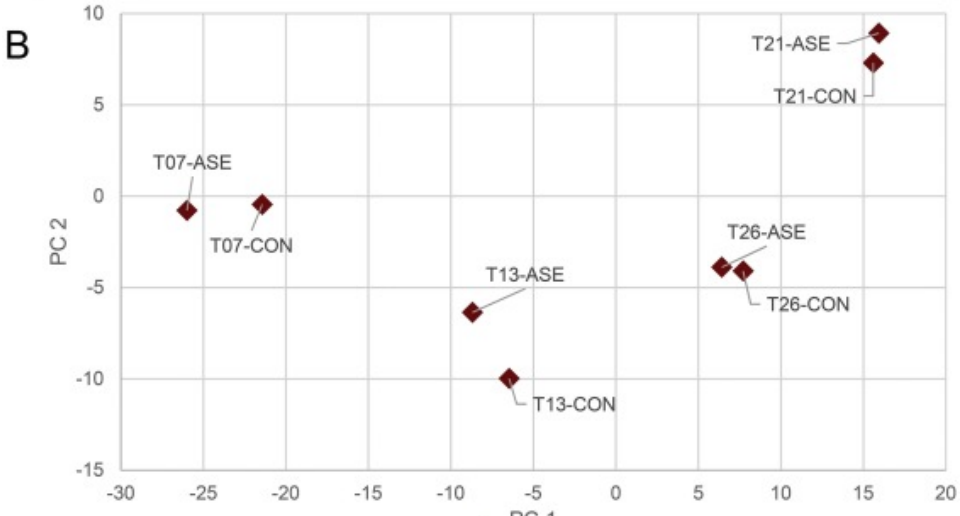
Fig. 2. Comparison of quantified metabolites from accelerated solvent extraction (light blue) and conventional extraction (dark blue) methods for four green tea samples (T07, T13, T21, and T26). Asterisks denote significant difference between metabolite quantified between two extraction methods, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See Table S3 for quantitation data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Accelerated solvent extraction and conventional extraction methodologies produced extracts with very similar metabolite profiles (Fig. 3). Principal component analysis highlighted the close clustering of the two extraction approaches (Fig. 3A). PC1 and PC2 explained 38.8% and 15.6% of the variation, respectively. The observed scores plot suggested highly similar profiles, which was supported by the quantified reproduced correlation coefficients for the individual metabolomic profiles (Fig. 3B). Correlation between the two extraction techniques ranged from 0.82–0.95 for each of the four tea products evaluated, indicating a high degree of similarity

between the two methods in extracting a representative metabolomic profile. This was further illustrated by the similarity of the mas spectral profiles (Fig. 3C).

A

	T07_CON	T07_ASE	T13_CON	T13_ASE	T21_CON	T21_ASE	T26_CON	T26_ASE
T07_CON	1.000	0.871	-0.527	-0.462	0.898	0.065	-0.703	-0.922
T07_ASE	0.871	1.000	-0.629	0.818	-0.530	0.475	-0.566	0.235
T13_CON	-0.527	-0.629	1.000	0.821	-0.256	-0.789	0.958	0.235
T13_ASE	-0.462	0.818	0.821	1.000	-0.799	-0.053	-0.075	0.341
T21_CON	0.898	-0.530	-0.256	-0.799	1.000	0.950	-0.394	-0.815
T21_ASE	0.065	0.475	-0.789	-0.053	0.950	1.000	-0.583	0.316
T26_CON	-0.703	-0.566	0.958	-0.075	-0.394	-0.583	1.000	0.949
T26_ASE	-0.922	0.235	0.235	0.341	-0.815	0.316	0.949	1.000



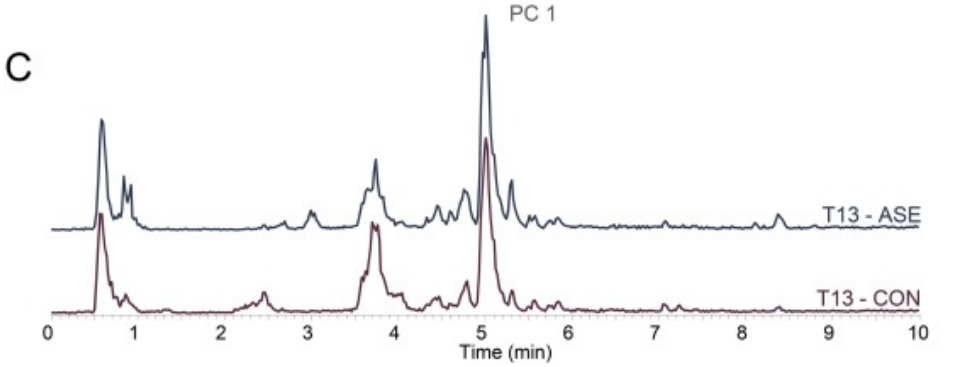


Fig. 3. Similarities between accelerated solvent extraction (ASE) versus conventional benchtop extraction (CON) for each tea product tested. Each data point represents the average of triplicate extractions. (A) PCA scores plot of the green tea extracts. (B) Reproduced correlation coefficient heat map matrix. Reduced correlation coefficients were based upon the metabolomic profile for each sample, and calculated from the three-component PCA model. Darker shades and correlation coefficients closer to 1.00 represent stronger correlation between samples. (C) Comparison of representative liquid chromatography-mass spectral profiles from accelerated solvent extraction (T13-ASE) and conventional benchtop extraction (T13-CON) methods.

4. Conclusion

The use of the accelerated solvent extraction, using 100% MeOH at 75 °C as a solvent system and a 5-min cycle time, is a suitable technique for the extraction of green tea products for metabolomic analysis. Compared to conventional extraction methodologies, accelerated solvent extraction samples possessed a similar metabolomic profile as analyzed by PCA, RCC, or visual comparison of chromatographs (Fig. 3). While the profiles were similar, the overall yield in the accelerated solvent extraction method was higher (Table 3), resulting in higher quantites of catechins per mass of tea sample used compared to conventional benchtop extraction (but not catechin quantity per mass extract). Both techniques were characterized by high extraction efficiency, reproducibility and recovery. In addition, accelerated solvent extraction was easy to perform, reducing the active time required for extraction and the number of steps for extraction (Table 4). Overall, our data suggest that ASE is a particularly suitable method for high-throughput screenings and metabolomic analyses of botanical supplements or natural products.

Table 4. Extraction variables and efficiencies for accelerated solvent extraction versus conventional extraction technique.

Extraction process variable	**Accelerated solvent extraction**	**Conventional extraction**
Extraction time per sample (min)	20 min	overnight
active bench time per sample (weighing/loading, filtering) (min)	5 min	25 min
Solvent usage per sample (mL)	35 mL	20 mL

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2017.07.027.

References

[1] M. Urpi-Sarda, E. Almanza-Aguilera, S. Tulipani, F.J. Tinahones, J. Salas-Salvadó, C. Andres-Lacueva, Metabolomics for biomarkers of type 2diabetes mellitus: advances and nutritional intervention trends, Curr. Cardiol. Risk Rep. 9 (2015) 12.

[2] T.K. McGhie, D.D. Rowan, Metabolomics for measuring phytochemicals and assessing human and animal responses to phytochemicals, in food science, Mol. Nutr. Food Res. 56 (2012) 147–158.

[3] D. Krug, R. Müller, Secondary metabolomics: the impact of mass spectrometry-based approaches on the discovery and characterization of microbial natural products, Nat. Prod. Rep. 31 (2014) 768–783.

[4] J.J. Kellogg, D.A. Todd, J.M. Egan, H.A. Raja, N.H. Oberlies, O.M. Kvalheiam, N.B. Cech, Biochemometrics for natural product research: comparison of data analysis approaches and application to identification of bioactive compounds, J. Nat. Prod. 79 (2016) 376–386.

[5] S.G. Oliver, M.K. Winson, D.B. Kell, F. Baganz, Systematic functional analysis of the yeast genome, Trends Biotechnol. 16 (1998) 373–378.

[6] M.Y. Mushtaq, Y.H. Choi, R. Verpoorte, E.G. Wilson, Extraction for metabolomics: access to the metabolome, Phytochem. Anal. 25 (2014)291–306.

[7] R.E. Major, Trends in sample preparation, LC•GC Europe 16 (2003) 2–8.

[8] R.E. Major, Sample prep perspectives, LC•GC Europe 4 (1991) 10–14.

[9] L. Wang, C.L. Weller, Recent advances in extraction of nutraceuticals from plants, Trends Food Sci. Tech. 17 (2006) 300–312.

[10] B.E. Richter, B.A. Jones, J.L. Ezzell, N.L. Porter, Accelerated solvent extraction: a technique for sample preparation, Anal. Chem. 68 (1996) 1033–1039.

[11] X. Huang, X. Zhao, X. Lu, H. Tian, A. Xu, Y. Liu, Z. Jian, Simultaneous determination of 50 residual pesticides in Flos Chrysanthemi using accelerated solvent extraction and gas chromatography, J. Chromatogr. B 967(2014) 1–7.

[12] P. Vazquez-Roig, Y. Picó, Pressurized liquid extraction of organic contaminants in environmental and food samples, TrAC Trends Anal. Chem.71 (2015) 55–64.

[13] C. Bergeron, S. Gafner, E. Clausen, D.J. Carrier, Comparison of the chemical composition of extracts from Scutellaria lateriflora using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70%ethanol extraction, J. Agr. Food Chem. 53 (2005) 3076–3080.

[14] D. Wang, J. Lu, A. Miao, Z. Xie, D. Yang, HPLC-DAD-ESI–MS/MS analysis of polyphenols and purine alkaloids in leaves of 22 tea cultivars in China, J. Food Compos. Anal. 21 (2008) 361–369.

[15] Consumer Reports, Tonic in a teapot, Cons. Rep. 68 (2003) 40–43.

[16] J.J. Kellogg, T.N. Graf, M.F. Paine, J.S. McCune, O.M. Kvalheiam, N.H. Oberlies, N.B. Cech, Comparison of metabolomics approaches for evaluating the variability of complex botanical preparations: green tea (Camellia sinensis) as a case study, J. Nat. Prod. 80 (2017) 1457–1466.

[17] L. Eriksson, E. Johansson, C. Wikström, Mixture design − design generation PLS analysis, and model usage, Chemometr. Intell. Lab. 43 (1998) 1–24.

[18] T. Pluskal, S. Castillo, A. Villar-Briones, M.;ˇ Oreˇsic, MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data, BMC Bioinf. 11 (2010) 395.

[19] O.M. Kvalheim, H.Y. Chan, I.F.F. Benzie, Y.T. Szeto, A.H. Tzang, D.K. Mok, F. Chau, Chromatographic profiling and multivariate analysis for screening and quantifying the contributions from individual components to the bioactive signature in natural products, Chemom. Intell. Lab. Syst. 107 (2011) 98–105.

[20] O.M. Kvalheim, T.V. Karstang, Interpretation of latent-variable regression models, Chemom. Intell. Lab. Syst. 7 (1989) 39–51.

[21] O.M. Kvalheim, F. Brakstad, Y. Liang, Preprocessing of analytical profiles in the presence of homoscedastic or heteroscedastic noise, Anal. Chem. 66 (1994)43–51.

[22] J. Loscalzo, Experimental irreproducibility: causes, (Mis)interpretations, and consequences, Circulation 125 (2012) 1211–1214.