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Short communication

High-throughput screening of vitamins and natural antioxidants in nutraceuticals from green tea extracts by liquid chromatography coupled to quadrupole orbitrap mass spectrometry

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ABSTRACT

A new analytical method was developed and validated for simultaneous analysis of 52 vitamins and natural antioxidants in nutraceutical products obtained from green tea (*Camellia sinensis*) extracts. The automated extraction procedure was achieved in a simple disposable pipet extraction. Ultra-high performance liquid chromatography and electrospray ionization quadrupole-Orbitrap high-resolution mass spectrometry (UHPLC Q-Orbitrap) was used for the separation and detection of all the analytes. The method was validated by taking into consideration the guidelines specified in European SANCO/12571/2013 Guideline 2013 and Commission Decision 2002/657/EC. The extraction recoveries were in a range of 84.9–112.7%, with coefficient of variation <6.4%. The limits of decision for the analytes were in the range of 0.05–1.91 mg kg^{-1}. The detection capabilities for the analytes were in the range of 0.08–3.18 mg kg^{-1}. The 52 compounds behave dynamic 0.2–200 mg kg^{-1}, with correlation coefficient >0.99. This validated method has been successfully applied on screening of vitamins and natural antioxidants in 136 different commercial nutraceutical products from green tea extracts.

1. Introduction

Currently, nutraceuticals are becoming a part of the daily diet because the incidence of lifestyle diseases worldwide, the life expectancy, and inadequate nutrition due to current habits. Green tea [*Camellia sinensis* (L.) Kuntze] has been a dietary supplement in the United States owing to its health-promoting effect caused from by the bioactive compounds, including caffeine, amino acids, vitamins, and several polyphenols with antioxidant properties [1]. The United States Department of Health and Human Services explains that all types of tea are produced from the *C. sinensis* plants. The extracts of green tea can be presented in tablets or capsules as nutraceuticals [2,3]. Green tea extracts, such as its component vitamins and natural antioxidants, have traditionally been used to prevent and cure nutritional deficiencies, weight loss, lowering cholesterol levels, mental alertness, and protecting skin from sun damage. Although vitamins and natural antioxidants are essential nutrients, nutraceuticals intake of vitamins and natural antioxidants may result in adverse chronic and acute effects, such as diarrhea and hemolytic anemia, which resulted from vitamin C megadosage [4–6]. Standardized testing to assess content uniformity and to describe cross-product variation among the existing formulations is essential for regulatory bodies and industry. Furthermore, the regulatory requirements for precise labeling on nutraceuticals require reliable analytical methods for the quantitative analysis of vitamins and natural antioxidants in foods [7].

However, most of the techniques focus on separating either water- or fat- soluble vitamins and natural antioxidants, and only a few methods are intended to separate hydrophilic and lipophilic vitamins and natural antioxidants simultaneously [8]. In a recent review on methods of natural antioxidants analysis, the need for developing analytical methods to identify and quantify several natural antioxidants and related compounds, such as fat-soluble vitamins in a single chromatographic run was emphasized [9–11]. However, different structures and chemical properties of vitamins and natural antioxidants make the development of a single method for their simultaneous determination more difficult. Nutraceuticals from green tea extracts are dried and concentrated, which creates a greater challenge to the analysis because several interferences from the matrix can be coextracted with the target compounds

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[12]. Concentrations of different vitamins and natural antioxidants vary greatly even in one sample, which requires analytical methods with high sensitivity and selectivity and applicable for large dynamic range [13].

The most frequently reported generic sample-preparation methods are QuEChERS and "dilute and shoot" methods [14,15]. A clear drawback of these strategies is the occurrence of abundant matrix effects, which compromise method selectivity, maintenance frequency, detection limits, and quantitative aspects [16,17]. Nevertheless, the lack of selectivity in generic sample preparation can be compensated by selectivity in instrumental analysis. Several review papers clearly report that high resolution mass spectrometry is a promising approach for routine screening and confirmation of multi-class analytes in complex matrices: to generate detailed structured chromatograms, enhanced product-ion spectra with accurate mass measurement, and reduce the interferences related to the matrices [10,11,18].

The focus of this study was to develop and validate efficient methods for the analysis of vitamins and natural antioxidants in the nutraceuticals from green tea extracts using automated QuEChERS followed by UHPLC/ESI Q-Orbitrap analysis. Response surface methodology was applied to study the behavior of the main generic extraction variables. The 52 vitamins and natural antioxidants were chosen based on EPA guidelines to develop a method. After the method was validated, it was used to analyze commercial nutraceutical products for incurred vitamins and natural antioxidants.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade methanol, chloroform, and acetonitrile were from J.T. Baker (Deventer, Holland). Butylated hydroxyl toluene (BHT), anhydrous magnesium sulfate ($MgSO_4$), acetic acid and sodium acetate were of analytical grade and purchased from Sigma–Aldrich (Steinheim, Germany). Ultrapure Water (resistivity, 18.2 MΩ) was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium). Vitamins and natural antioxidants analytical standards were purchased from Sigma–Aldrich (Steinheim, Germany), Dr. Ehrenstorfer GmbH (Augsburg, Germany), LGC Standards (Teddington, UK), Fluka (Buchs, Switzerland), and Witega (Berlin, Germany). Empty 1 mL disposable pipet extraction (DPX) tips were purchased from Gerstel (Columbia, MD).

2.2. Solution preparation

Stock solutions of individual standards were prepared, depending on the specific solubility properties, by dissolving 250–1000 mg of compound in 25 mL of toluene, chloroform, methanol, or acetonitrile for calibration and fortification standards, and stored at −20 °C in the dark. A multi-compound working standard solution (100 mg L^{-1} concentration of each compound) was prepared by dilution of the stock solutions with methanol and stored under refrigeration at 4 °C. A $^2H_6$-all-trans-retinol solution (10 mg L^{-1}) was prepared as internal standard (IS) in the same way as the stock standard solutions.

2.3. Instrumentation

The UHPLC/ESI Q-Orbitrap system consisted of an Accela 1250 UHPLC system coupled with a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). All automated sample preparation steps for the vitamins and natural antioxidants determination were performed using a dual-head multipurpose sampler (MPS XL) equipped with a CF-100 dual position centrifuge, ^{m}VORX vortex, and DPX Option (Gerstel, Columbia, MD). The system was controlled by Exactive Tune 1.1 and Xcalibur 2.3 software (Thermo Fisher Scientific, San Jose).

The analytical column used was a 100 mm × 2.1 mm, 1.9 μm, Hypersil Gold aQ C-18 connected to a 10 mm × 2.1 mm, 1.9 μm, Accucore C-18 aQ guard column (Thermo Fisher Scientific, San Jose). UHPLC mobile phase A was 0.1% formic acid and 4 mM ammonium formate in water, and mobile phase B was 0.1% formic acid and 4 mM ammonium formate in methanol. The following gradient was used: 0 min, 100% A; 1 min, 100% A; 7 min, 0% A; 12 min, 0% A; 13 min, 100% A, until the end of the run at 15 min, operating at a flow rate of 0.3 mL min^{-1}. The optimized electrospray ionization source temperature was set at 350 °C, the capillary temperature at 320 °C, the electrospray voltage at 3.5 kV and 3.0 kV for positive and negative modes, respectively. Sheath and auxiliary gas were 18 and 3 L min^{-1}.

2.4. Sample Preparation

A total of 136 different green tea (*C. sinensis*) nutraceutical products were obtained from different retail commercial outlets. Green tea extract tablets or capsules were homogenized with an IKA Grinder Tube Mill (Staufenberg, Germany) for 60 s and stored at 4 °C prior to analysis.

For the extraction, 1.0 g of each sample was weighed into a 10 mL tube. Gerstel MPS XL configured for automated sample preparation. 5 mL of an acetonitrile/water solution (84/16, *v*/*v*) with 0.01% BHT and 1% acetic acid was added as an extraction solvent and the tube was tightly capped and vigorously mixed for 1 min using the ^{m}VORX vortex at its maximum speed (3200 rpm). 1.00 g of anhydrous magnesium sulfate and 0.30 g of sodium acetate were added to the DPX tip (5 mL), to induce phase separation. 2 mL of sample was then aspirated into a DPX tip three times from the bottom followed by an equilibration time of 30 s. The DPX tip acts as a filter removing the salt particulate matter from the solution. Then the solution was immediately vortexed for 1 min, and then centrifuged for 5 min at 2264 × *g*. An aliquot of the final upper layer (200 μL) was transferred into a Mini-UniPrep vial, to which 10 μL of 10 mg L^{-1} IS, 290 μL of methanol, 500 μL of 8 mM ammonium formate buffer were added. 5 μL of the diluted extract was injected into the UHPLC Q-Orbitrap system for analysis.

3. Results and discussion

3.1. Optimization of the extraction procedure

This study focused on performing automated extraction of reduced sample volumes coupled with UHPLC Q-Orbitrap to provide high throughput analysis "one sample at a time". In preliminary investigation, pH, extraction solvent volume and water content in the mixture solvent were the variables with the most significant effects. Therefore, the three variables were further optimized using a central composite design (CCD, Table S1, Electronic Supplementary Material). The optimal values of response *Y* (individual recovery of interest compounds) were obtained by solving the regression equation and by analyzing the response surface contour plots. The significance of parameter and the goodness of fit of the regression model estimates were determined through appropriate statistical methods. Design Expert trial version 9.0 was used (Stat-Ease Inc., Minneapolis, MN). Fig. 1 is a representative model (for pyridoxine, representing the behavior for all compounds) presenting a suitable correlation $R^2 = 0.9912$ (the regression was statistically significant at the 99.12% confidence level), significant Model *F*-value and no lack of fit *F*-value. Therefore, the generated response surfaces suggested that the best extraction