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An integrated strategy for establishment of curcuminoid profile in turmeric using two LC–MS/MS platforms

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A B S T R A C T

Turmeric and curcuminoids are used as natural food coloring and functional food additives in various parts of the world. In this study, ninety-six curcuminoids were fully characterized using a targeted curcuminoid profile, which established by integrated use of two complementary LC–MS/MS platforms (liquid chromatography–quadrupole time of flight mass spectrometry (LC–QTOF-MS/MS) and liquid chromatography–quadrupole linear ion trap mass spectrometry (LC–QTRAP-MS/MS)). The curcuminoid profile was represented in the form of a multiple reaction monitoring (MRM) mode based on LC–QTRAP-MS/MS analysis. It facilitated the qualitative and relative quantitative analysis of curcuminoids in a single injection. Meanwhile, the profile was successfully applied to the quality evaluation of raw materials of turmeric from different regions in China and Myanmar. The structural identification procedures of curcuminoids and the integrated strategy provide a suitable method to analyze targeted plant metabolites which occur in a high number but sharing either structural similarities or similar functional groups.
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1. Introduction

Metabolites are the end products of cellular regulatory processes that exist in humans and other living systems [1]. Among these various types of molecules, functional secondary metabolites of plant are known to be unique sources for pharmaceuticals, food additives, flavors and other industrial materials [2]. Turmeric (*Curcuma longa* L.) is a tropical herb in the Zingiberaceae family, which is native to Southern Asia. The curcuminoids are the major functional secondary metabolites in turmeric and curcumin has been identified as the active principle of turmeric [3]. Chemically, it is a bis-α, β-unsaturated β-diketone that exhibits keto-enol tautomerism [4]. Turmeric and its pigment curcuminoids are used as natural food coloring in various parts of the world [5]. As functional food additives, curcuminoids demonstrated a remarkable variety of beneficial pharmacological activities [6] and are widely used as carminative, laxative, anthelmintic and a cure for liver ailment in Traditional Chinese Medicine [7,8]. In addition to our previous reports [9–11], analytical methods are unavailable regarding the systematic discovery of curcuminoids in turmeric. Therefore, an integrated strategy using two complementary LC–MS/MS platforms was developed in the present study. Since other minor curcuminoids, such as dihydro and tetrahydro-curcuminoids have attracted increasing research attention due to their significant bioactivites shown in several in *vitro* systems [12]. The dramatic improvement of the method for qualitative analysis of these minor ingredients is instrumental for functional food and pharmaceutical applications. In our previous study, the metabolic changes of serum lysophosphatidylcholines were found in the preventive effects of turmeric on high-fat diet-induced hyperlipidaemic mice using a

Abbreviations: LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; QTOF, quadrupole time of flight; QTRAP, hybrid triple-quadrupole linear ion trap; RDBE, ring double bond equivalents; MRM, multiple reaction monitoring; IDA, information dependent acquisition; EPI, enhanced product ion scan; CID, collision-induced dissociation.

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targeted metabolomic approach by integrated use of two complementary LC–MS platforms [13]. In the present study, this targeted metabolomic approach was better modified and proved to be highly efficient for characterizing curcuminoids in turmeric.

Separation, purification and identification of plant secondary metabolites have been proved to be the most reliable method to analyze these components [14]. However, the range and number of discrete molecular structures produced by plant is huge and complicated. Most secondary metabolites are not easily to be isolated due to their trace content, although they can be detected by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Currently, LC–MS/MS is one of the most promising analytical technique for analysis of plant secondary metabolites due to its sensitivity, selectivity and efficiency [15–18]. It is ideally suited for metabolomic analysis, which can provide detailed insights into the changes of plant secondary metabolites.

Two different strategies of untargeted and targeted analysis are commonly used for plant secondary metabolites profiling. The untargeted analysis can be performed with high resolution mass spectrometers [e.g., LC–QTOF-MS/MS] based on full MS scan and data-dependent MS/MS scan analysis, which provide accurate mass measurement for both precursor ions and product ions as well as product ion spectra for structural identification [19]. For targeted analysis, certain subgroups of metabolites can be analyzed using selective MS scan methods [e.g., LC–QTRAP-MS/MS with a multiple reaction monitoring (MRM) mode] [20]. The two strategies represent the breadth-first (qualitative) and depth-first (quantitative) screening approach, respectively. The time-of-flight mass spectrometers are able to measure target ions with high mass resolution, which can be used to acquire the elementary formula of the analyte [21]. However, the matrix effects in complex samples, can often deteriorate the selectivity of low-abundant targeted analytes in QTOF-MS/MS analyses. LC–QTRAP-MRM method is recently undergoing a renaissance within the metabolomics community for its superior sensitivity, selectivity and wide linear dynamic range [22,23]. This mode also has the potential to screen qualitatively for structural similarities of analytes [24]. Therefore, the profile should be represented in the form of LC–QTRAP-MRM mode through a targeted strategy.

In the present study, an integrated strategy for establishment of curcuminoid profile by integrating two complementary LC–MS/MS platforms was systematically explored in order to identify more subsets of curcuminoids in turmeric. The established curcuminoid profile based on a LC–QTRAP-MRM mode can enable the full characterization of all identified curcuminoids in a single injection. Then the relative quantitative analysis was successfully applied to the quality evaluation of the raw materials of turmeric from different regions based on the established curcuminoid profile.

2. Materials and methods

2.1. Chemicals, materials and reagents

Dried Rhizome of Turmeric from Sichuan, Yunan, Guangdong, Fujian and Guanxi in China and Myanmar were purchased from huge-hunter agriculture and products Co., LTD (Gaoyao city, Guangdong, China). The turmeric were identified and authenticated by Professor Keli Chen at the Hubei University of Chinese Medicine. The voucher specimens were deposited at the herbarium of Huazhong University of Science and Technology.

HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was produced by a Milli-Q water system (Millipore, Bedford, MA, USA). Formic acid ($\geq$98%) of analytical grade was purchased from Sinopharm Chemical Reagent Co., LTD (Shanghai, China). The reference standards of ten curcuminoids were in-house isolated and identified by comparing the accurate mass measurement of their protonated molecular ions, characteristic MS/MS fragmentation patterns, ^{13}C NMR and ^{1}H NMR data with their corresponding compounds reported in the literatures (Table A.1, Figs. A.1 and A.2). The purity grade (>98%) of the standards were tested using HPLC with a UV detector.

2.2. Preparation of turmeric extract and standard solutions

Turmeric rhizomes were ground into a fine powder. One gram of turmeric powder was added with 10 mL of 80% aqueous methanol and vigorously vortexed for 2 min followed by sonication for 5 min. The mixture was centrifuged and the supernatant was carefully removed as an extract. The extraction process was repeated twice with the solvents of 10 mL 50% aqueous methanol and 10 mL 100% methanol, respectively. Plant extract was performed according to the previous report with a little modification [25]. Three separate extracts were combined and directly analyzed by LC–MS/MS. All the procedures were operated away from light in order to avoid the photodegradation of the curcuminoids.

Ten curcuminoid standards were dissolved in methanol to make individual stock solutions at the concentrations of 100 μg mL^{-1}. Then, each stock solution of the ten standards was mixed and diluted with methanol to prepare a final mixed standard solution at a concentration of 100 ng mL^{-1} for direct infusion and LC–MS/MS analysis.

2.3. LC–QTOF-MS/MS and LC–QTRAP-MS/MS analysis

The UHPLC system consisted of a LC-30AD solvent delivery system, a SIL-30AC autosampler, a CTO-30A column oven, a DGU-30A3 degasser, and a CBM-30A controller from Shimadzu (Kyoto, Japan). A Welch Ultimate UHPLC XB-C18 column (100 mm × 2.1 mm, 1.8 μm) was used at a flow rate of 0.3 mL min^{-1}. The injection volume was 4.0 μL. Mobile phase A was water-formic acid (1000: 1, v/v) and mobile phase B was acetonitrile. The following binary gradient with linear interpolation was used: 0.01 min, 20% B; 10 min, 35% B; 30 min, 55% B; 40 min, 95% B; 43 min, 95% B; 43.1 min, 20% B; 46 min, 20% B. The column oven and autosampler temperatures were maintained at 40 °C and 4 °C, respectively.

The LC–QTOF-MS/MS analysis was carried out using a Triple TOF^{TM} 5600 system with a Duo Spray source in the positive electrospray ion mode (AB SCIEX, Foster City, CA, USA). The QTOF-MS parameters were optimized as follows: ion source temperature, 600 °C; ion spray voltage, 5000 V; curtain gas, 25 psi; nebulizer gas (GS 1), 50 psi; heater gas (GS 2), 50 psi; declustering potential (DP), 80 V. The collision energy (CE) was set at 30 or 50 eV and the collision energy spread (CES) was 10 eV for TOF MS/MS. The mass ranges were set at m/z 100–1200 for TOF MS scan, 50–1200 for TOF MS/MS experiments. The most intensive of 8 ions from each full MS scan were selected as precursor ions for MS/MS fragmentation in the information dependent acquisition (IDA) experiments. Dynamic background subtraction was used to match the IDA criteria.

The LC–QTRAP-MS/MS analysis was performed on an AB Sciex QTRAP 4000 system in the positive electrospray ion mode (AB SCIEX, Foster City, CA, USA). For QTRAP-MS, the parameters were optimized as follows: ion source temperature, 600 °C; ion spray voltage, 4500 V; curtain gas, 30 psi; nebulizer gas (GS 1), 50 psi; heater gas (GS 2), 50 psi; declustering potential (DP), 80 V; a collision cell exit potential, 10 V; a dwell time, 8 ms. The CE and CES were set at 35 eV and 10 eV, respectively. MRM-enhanced product ion scan (EPI) were conducted to monitor predicted MRM transitions for each potential curcuminoid. Then the qualitative analysis of curcuminoids was performed and each specific MRM transition and

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