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An enhanced strategy integrating offline two-dimensional separation and step-wise precursor ion list-based raster-mass defect filter: Characterization of indole alkaloids in five botanical origins of Uncariae Ramulus Cum Unicis as an exemplary application

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ABSTRACT

Comprehensive chemical profiling is of great significance for understanding the therapeutic material basis and quality control of herbal medicines, which is challenging due to its inherent chemical diversity and complexity, as well as wide concentration range. In this study, we introduced an enhanced strategy integrating offline two-dimensional (2D) separation and the step-wise precursor ion list-based raster-mass defect filter (step-wise PIL-based raster-MDF) scan by tandem LTQ-Orbitrap mass spectrometer. A comprehensive analysis of indole alkaloids in five botanical origins of Uncariae Ramulus Cum Unicis (Gou-Teng) was used as an exemplary application. A positively charged reversed phase (PR) × conventional RP LC system in different pH conditions was constructed with the orthogonality of 74%. A theoretical step-wise PIL among 310–950 Da with the step-size of 2 Da was developed to selectively trigger fragmentations and extend the coverage of potential indole alkaloids. Simultaneously, by defining parent mass width (PMW) of the step-wise PIL to ±55 mDa, a raster-MDF screening was achieved in the acquisition process. Additionally, subtype classification and structural elucidation were facilitated by a four-step interpretation strategy. As a result, a total of 1227 indole alkaloids were efficiently exposed and characterized from five botanical origins of Gou-Teng, which showed high chemical diversity. A systematic comparison among five species was first performed and only 66 indole alkaloids were common. For method validation, three new alkaloid *N*-oxides were isolated and unambiguously identified by NMR. The present study provides a novel data-dependent acquisition method with improved target coverage and high selectivity. The integrated strategy is practical to efficiently expose and comprehensively characterize complex components in herbal medicines.

1. Introduction

Herbal medicines are gaining more attentions nowadays, since their important roles in preventing and curing diseases, especially chronic, systematic diseases and population aging [1–3]. However, there is still a long way to the worldwide prevalent use of herbal medicines, mainly due to ambiguity in the therapeutic material basis and difficulty in quality control [3]. Since then, it has been well acknowledged that it is a top priority to comprehensively profile chemical compositions of herbal medicines. And the inherent chemical diversity and complexity, as well as wide concentration range render rapid separation and structural elucidation challenging [4].

With the advent of state-of-the-art instruments, liquid chromatography coupled to mass spectrometry (LC–MS) increasingly stands out in analyzing complex chemical system due to its powerful separation capacity, remarkable sensitivity and high throughput [5–7]. Great efforts have been made to improve the productivity of chemical characterization in aspects of chromatographic separation, MS data acquisition and mining. With regard

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to chromatographic separation, two-dimensional (2D) LC combining orthogonal separation mechanisms attracts special attentions thanks to its increased peak capacity, higher resolution and selectivity. It has been proven powerful in analyzing complex components of herbal medicines, especially trace ones [8]. MS/MS or MS^n fragmentation information, offered by tandem MS (such as Q-TOF, LTQ-Orbitrap, Q-trap, IT-TOF, *etc.*), is essential for detailed structure elucidation of chemical components. Data-independent acquisition (DIA), MS^E and MS/MS^{ALL} with SWATH, although providing high-throughput fragmentation information, need further deconvolution and have difficulty in assignment of fragments [9]. Intelligent data-dependent acquisition (DDA) methods based on selection criteria including ion intensity, precursor ion list (PIL) [10], exclusion list (EL) [11], neutral loss (NL) or product ion (PI) [12], multiple reaction monitoring (MRM) [13] and mass defect filter (MDF) [14] recently have been explored to improve sensitivity and selectivity in acquiring MS/MS or MS^n data. However, it is well acknowledged that DDA scans inevitably miss information of unexpected structures and usually need multiple injections [15]. Alternatively, various targeted post-acquisition data mining techniques, such as MDF [15], neutral loss filter (NLF), product ion filter (PIF) and isotope-pattern filter (IPF) [16], have emerged for simplifying characterization process [17]. Whereas, unavailable MS/MS or MS^n spectra of minors hinder further characterization of the screened components. Thus, it is necessary to develop integrated approaches combining advantages of DDA and targeted data-acquisition mining. PIL and MDF dependent acquisitions are capable of analyzing trace components in complex matrices [18]. And the latter is specially achieved on the Triple-TOF 5600+ system [14,18]. Simultaneously, as a data mining technique, MDF generally is realized based on softwares, such as AB SCIEX Peakview [19], Waters Metabolynx XS [20], Thermo Scientific Metworks [21], and Microsoft excel [22]. Different algorithms containing classical rectangular MDF and modified MDF have been successively designed to find an acceptable compromise between decreasing false positives and expanding target coverage [10,22]. Additionally, post-run data analysis with MDF followed by PIL-triggered fragmentations was also developed for characterization of target components [10,23]. However, a tedious pre-analysis containing an exploratory run, software-aided peak extraction and MDF screening is necessary and the generated PILs are sample-specific.

The genus *Uncaria* contains about 34 species. The majority are widely used as traditional medicines worldwide for the treatment of fevers, headaches, gastrointestinal illness, hypertension, epilepsy, wounds and ulcers, *etc.* [24,25]. Indole alkaloids are abundant and bioactive ingredients, and there have been about 120 indole alkaloids phytochemically isolated from the genus *Uncaria* [24]. Chinese Pharmacopoeia (2015 edition) legally collects the stems and hooks of five species, *U. rhynchophylla* (UR), *U. hirsuta* (UH), *U. macrophylla* (UM), *U. sinensis* (USI) and *U. sessilifructus* (USE), as Uncariae Ramulus Cum Unicis (Gou-Teng) used for hypertension, epilepsy, convulsion, fevers, headaches and dizziness, *etc.* [26]. In few available reports on chemical analysis of indole alkaloids in five botanical origins of Gou-Teng, amounts of minor components were usually ignored [27,28]. For the ultimate purpose of systematic chemical profiling and classification of five botanical origins of Gou-Teng, in the previous works, the authors conducted the isolation of indole alkaloids from UR, UH and USI [10,21,29], and also developed two analytical methods based on UPLC/LTQ-Orbitrap MS for rapid discovery of new indole alkaloids [10,21].

Herein, an enhanced strategy based on offline comprehensive 2D LC coupled with high-resolution LTQ-Orbitrap MS is explored and applied to systematically analyze indole alkaloids in five botanical origins of Gou-Teng (Fig. 1A). An orthogonal 2D LC system is constructed to efficiently expose and separate indole alkaloids. And, a step-wise PIL-based raster-MDF scan method is developed by defining a theoretical step-wise PIL and optimal parent mass width (PMW) to selectively trigger fragmentations (Fig. 1B, taking precursors of m/z 350–400 for illustration). DE-step-wise PIL-CID/MS^2-HCD/MS^3 (with dynamic exclusion enabled, step-wise PIL triggered collision-induced dissociation-MS^2 and high-energy *C*-trap dissociation-MS^3) data is acquired on UPLC/LTQ-Orbitrap mass spectrometer. The rapid characterization is carried out according to an established four-step interpretation strategy. Ultimately, targeted isolation of potentially new compounds is conducted for method validation and new natural products discovery. The novelty of this approach in MS^n data acquisition involves: 1) comprehensive coverage of potential target components based on a theoretical step-wise PIL; 2) raster-MDF screening implemented in the process of data acquisition.

2. Experimental

2.1. Chemicals and reagents

Forty-four indole alkaloids (**C1**-**C44**) isolated and unambiguously identified by authors were used as the reference standards (shown in Fig. S1). The detailed information of these alkaloids was given in Table S1. Methanol and acetonitrile purchased from Merck KGaA (Merck, Darmstadt, Germany) were of HPLC-grade. ACS-grade ammonium hydroxide (28–30 wt.% solution in H_2O, J&K, Beijing, China) was used for sample preparation. HPLC-grade formic acid (Burdick & Jackson, Honeywell International, Inc., USA; **FA**) and ammonium hydroxide solution (~10% in H_2O, Sigma-Aldrich, St. Louis, MO, USA; **AHS**) were separately used as chromatographic additives of the ^1D and ^2D chromatographic separation. Ultra-pure water was prepared in house by a Millipore Alpha-Q water purification system (Millipore, Bedford, USA).

Twenty batches of the stems and hooks of UR, UH, UM, USI and USE, four batches of each species, were collected and authenticated based on the botany traits as recorded in China Flora (http://frps.eflora.cn/frps?id) and the corresponding descriptions were offered in Table S2. The voucher specimens were deposited at the author's laboratory in Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China.

2.2. Sample preparation

A base-assisted extraction was applied. In detail, an aliquot of 1.0 g fine powder of each batch material was accurately weighed and infiltrated with 3 mL ammonium hydroxide for 30 min in a 100-mL conical flask with cover. The materials were then immersed and extracted in 50 mL methanol for 30 min with ultrasound assistance (35 kHz, 350 W). After being centrifuged at 4000 rpm for 10 min, the supernatant of four-batch materials from one species, 45 mL of each batch, were mixed and evaporated to dryness under the reduced pressure. The residues of each species were re-dissolved and diluted to a constant volume in a 10-mL volumetric flask with 75% aqueous methanol. After another centrifugation at 14000 rpm for 10 min, the test solutions of UR, UH, UM, USI and USE were obtained and stored at 4 °C before analysis. A Quality Control (**QC**) sample used for the establishment of 2D LC system, orthogonality evaluation, ^2D retention time alignment and optimization of PMW was further obtained by pooling equal volume of the test solution from each species and diluting by 10 folds with 75% aqueous methanol.

2.3. Offline comprehensive 2D LC separation by positively charged RP × conventional PR

The offline comprehensive 2D LC separations of indole alkaloids in five species (UR, UH, UM, USI and USE) were performed

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