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Direct screening of malonylginsenosides from nine Ginseng extracts by an untargeted profiling strategy incorporating in-source collision-induced dissociation, mass tag, and neutral loss scan on a hybrid linear ion-trap/Orbitrap mass spectrometer coupled to ultra-high performance liquid chromatography

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ABSTRACT

Specific analytical approaches that enable untargeted profiling of modified metabolites are in great need. An untargeted profiling strategy, by integrating in-source collision-induced dissociation (ISCID)-MS^1, mass tag-MS^2, and neutral loss scan-MS^3, is established on a linear ion-trap/Orbitrap mass spectrometer coupled to ultra-high performance liquid chromatography. This strategy is applied to screen malonylginsenosides from three reputable *Panax* species (*P. ginseng*, *P. quinquefolius*, and *P. notoginseng*). In light of the preferred neutral elimination of CO_2 and entire malonyl substituent ($C_3H_2O_3$) in the negative electrospray ionization mode, a pseudo-neutral loss scan (PNL) method was established by applying ISCID energy 40 V in MS^1, mass tag 43.9898 Da oriented CID-MS^2 at normalized collision energy (NCE) 30%, and neutral loss 43.9898 Da-triggered high-energy *C*-trap dissociation-MS^3 at NCE 70%. The PNL approach achieved a high coverage of targeted malonylginsenosides but introduced less false positives. It displayed comparable performance to a precursor ions list-driven targeted approach we have reported in the profiling and characterization of malonylginsenosides, but could avoid complex data processing. Totally 178 malonylginsenosides were characterized from the roots, leaves, and flower buds of *P. ginseng*, *P. quinquefolius*, and *P. notoginseng*, and most of them possess potentially new structures. The compositions of malonylginsenosides identified from these three *Panax* species are similar, and only malonylginsenoside Rb2 and some minor may have potential chemotaxonomic significance. In conclusion, we provide a potent analytical strategy for the direct and efficient screening of modified metabolites, which may have broad applications in the fields of metabolomics, drug metabolism, and natural product research.

1. Introduction

Comprehensive profiling of metabolites (metabolomics) or peptides (proteomics) can be performed in an untargeted or targeted mode [1,2]. An untargeted profiling strategy generally takes advantage of high-resolution mass spectrometry (HRMS) full-scan spectra to capture the features of a whole metabolome (or proteome). In contrast, targeted profiling is mostly based on selective ion monitoring (SIM) or selective reaction monitoring (SRM), displaying improved sensitivity and broader dynamic linearity range. Systematic profiling and elucidation of a class of metabolites or chemical components that are involved in a pathway, such as the modified metabolites, have become vital in a variety of research fields, such as to unveil the biosynthesis pathways, to discover bioactive natural products, and to identify phase-II drug metabolites. Targeted detection of interested metabolites in an untargeted mode is emerging as a new trend in analytical chemistry.

Aiming to achieve specific profiling and characterization of the modified metabolites or other components that can undergo neutral eliminations, two strategies can be implemented by means of

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neutral loss (NL)-oriented methods. In the case of the first strategy, various data-dependent acquisition (DDA), data-independent acquisition (DIA), all-ions fragmentation (AIF), and MS^E approaches can be utilized to record the signals (precursors and product ions) of all metabolites, and *in silico* NL filtering tools are then applied to screen targeted components from the acquired MS^n or even full-scan HRMS spectra [3–7]. NL filtering of the full-scan data by combining in-source collision-induced dissociation (ISCID) has been demonstrated as a potent survey scan in profiling various modification-specific metabolites (*e.g.* acetyl, glucosyl, glucuronidated metabolites, and ribose conjugates) [8]. Regarding the second strategy, constant neutral loss (CNL; on a triple quadrupole mass spectrometer or a hybrid triple quadrupole/linear ion-trap mass spectrometer) or pseudo-neutral loss (PNL)-triggered MS^n (on a high-resolution linear ion-trap/Orbitrap mass spectrometer; LTQ-Orbitrap) can be utilized to directly profile and characterize the modified metabolites/peptides of interest [9–15]. Comparatively, the PNL approach established on LTQ-Orbitrap is more powerful than the CNL methods in untargeted profiling of the modified metabolites, because of its ability of high-resolution MS^n measurement. According to the available literature, the PNL method can be established (on LTQ-Orbitrap) by setting a multistep scan circle that consists of ISCID-MS^1 and mass tag-triggered MS^2 [11], or multiple scan events involving full-scan MS^1, DDA-MS^2, and NL-MS^3 [13,15]. Among these available MS scan methods, ISCID enables a rapid scan rate and the acquisition of rich information of both precursors and the product ions. The ISCID data can be processed by either *in silico* algorithms [8] or be used to trigger subsequent MS^n fragmentation to achieve the targeted metabolites characterization [16]. By enabling the "mass tag" function, the LTQ-Orbitrap mass spectrometer determines the charge state of ionized species in MS^1 and converts the *m/z* values into masses. If a mass pair consistent with the defined mass tag(s) exists in the full-scan spectrum (with the ion intensity higher than the threshold), the instrument turns off the ISCID energy and triggers multi-stage activation MS/MS of the selected ion pairs [11]. NL scan measures the mass difference in MS^n data. Those product ions in accordance with predefined mass(es) are selected for further fragmentation [13,15]. Between these two different scan combinations, the former approach (ISCID-MS^1/mass tag-MS^2) can bring in false positives due to the rather complex signal species present in the full-scan spectra, while the latter setting (full-scan MS^1/DDA-MS^2/NL-MS^3) may be restrained in coverage mainly due to DDA recording in MS^2.

To enhance both coverage and specificity in profiling the targeted modified metabolites, we develop a new untargeted profiling strategy that incorporates ISCID full-scan MS^1, mass tag-driven MS^2, and NL-triggered MS^3 (Fig. 1). The first scan is set to record the richest HRMS data of both precursors and the ISCID resultant product ions. MS^2 scan by enabling mass tag could dissociate both the mass pairs (calculated from the ISCID data) to validate the true occurrence of predefined neutral loss. Those ions meeting the NL criteria (showing predefined mass difference between MS^1 and MS^2) are further fragmented by MS^3 to produce product ions for the final structural elucidation. By these settings, ISCID-MS^1 could ensure a high coverage of targeted components, while mass tag-MS^2 and NL-MS^3 are beneficial to reducing false positives and simplifying subsequent data interpretation.

Natural products derived from the *Panax* genus, particularly *P. ginseng*, *P. quinquefolius*, and *P. notoginseng*, *etc.*, are highly popular from a global scope serving as herbal medicines, healthcare products, and food supplements [17,18]. Abundant and specific dammarane triterpenoid saponins (known as the ginsenosides) have shown beneficial effects to humans on central nervous system and cardio/cerebrovascular system associated with the tonifying properties of Ginseng [19–21]. Some ginsenoside compounds and their transformed products have been reported with the anti-cancer effects [22,23]. The structure of a ginsenoside typically consists of a dammarane triterpenoid sapogenin (few exceptions with the oleanonic acid or octillol sapogenin) and no more than six sugar(s) (*e.g.*, glucose, glucuronic acid, rhamnose, xylose, and arabinose) [24–26]. Ginsenosides also suffer from acyl modification, such as the acetyl ($+C_2H_2O$), malonyl ($+C_3H_2O_3$), butenoyl ($+C_4H_4O$), and octenyl ($+C_8H_{12}O$), *etc.* Malonylginsenosides, which are rich and structurally diverse in the flower buds of various *Panax* species, have been demonstrated as potential antidiabetic molecules [27–29]. In our precious work, we isolated fifteen new malonylginsenoside compounds from the flower buds of *P. ginseng* [30] and reported an improved untargeted metabolomics approach to comprehensively characterizing new malonylginsenoside molecules [31]. Malonylginsenosides may be a perfect model to develop an improved PNL strategy that can enable the direct profiling and characterization of modified metabolites.

Table 1
Information of the samples of *P. ginseng*, *P. quinquefolius*, and *P. notoginseng* analyzed in this study.

No.	Label	Samples	Producing regions	Collection time
1	RPG	root of *P. ginseng*	Jilin, China	2016.07
2	LPG	leaf of *P. ginseng*	Jilin, China	2016.04
3	FPG	flower bud of *P. ginseng*	Jilin, China	2016.08
4	RPQ	root of *P. quinquefolius*	Wisconsin, USA	2016.11
5	LPQ	leaf of *P. quinquefolius*	Jilin, China	2016.09
6	FPQ	flower bud of *P. quinquefolius*	Jilin, China	2015.04
7	RPN	root of *P. notoginseng*	Guangnan, China	2016.03
8	LPN	leaf of *P. notoginseng*	Shiping, China	2016.08
9	FPN	flower bud of *P. notoginseng*	Wenshan, China	2016.08

The aim of the current work is to develop an LTQ-Orbitrap MS-based PNL approach enabling the direct, efficient characterization of modified metabolites in an untargeted mode. As a proof of concept, its performance is validated by comprehensive screening of malonylginsenoside structures from nine Ginseng extracts (the roots, leaves, and flower buds of *P. ginseng*, *P. quinquefolius*, and *P. notoginseng*). According to the preferred neutral eliminations of the malonyl substituent in the negative electrospray ionization (ESI) mode, key parameters in establishment of the PNL approach are optimized to obtain the best condition suitable for malonylginsenoside screening. Due to the integration of the advantages of mass tag-driven MS^n and NL-triggered MS^n acquisitions, hopefully, the established strategy has the potential to being a highly specific, universal vehicle that facilitates the untargeted profiling and characterization of modified metabolites in a variety of fields such as metabolomics, drug metabolism, and natural product research.

2. Experimental

2.1. Chemicals and materials

A total of fifteen malonylginsenoside compounds isolated from the flower buds of *P. ginseng* [30], including m-floral-Re1 (**1**), m-floral-Re2 (**2**), m-Rb1 (**3**), m-Rb2 (**4**), m-Rc (**5**), m-Rd (**6**), m-floral-Rb2 (**7**), m-floral-Rc1 (**8**), m-floral-Rc2 (**9**), m-floral-Rc3 (**10**), m-floral-Rd1 (**11**), m-floral-Rd2 (**12**), m-floral-Rd4 (**13**), m-floral-Rd5 (**14**), and m-floral-Rd6 (**15**), were used as the reference compounds (Fig. 2). Acetonitrile (Merck, Darmstadt, Germany) and ammonium acetate (Sigma-Aldrich, MO, USA) were both HPLC grade. Ultra-pure water (18.2 MΩ cm at 25 °C) was prepared by a Millipore Alpha-Q Water purification system (Millipore, Bedford, USA). Information regarding the raw materials of three different parts (root, leaf, and flower bud) of three *Panax* species (*P. ginseng*, *P. quinquefolius*, and *P. notoginseng*) is provided in Table 1.

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