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Analytical Methods

# Multi-targeted screening of botanicals in food supplements by liquid chromatography with tandem mass spectrometry

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ABSTRACT

Safety, quality and composition assessments of food supplements based on botanical ingredients are of major concern, as they have usually not been through a rigorous testing process as required for the approval of therapeutic phytopreparations. Therefore, an efficient multi-targeted method was developed to screen selected botanicals of interest in herbal food supplements. Liquid chromatography coupled with a hybrid triple quadrupole linear ion trap was used for this purpose. Botanicals were characterised by means of appropriate biomarkers, which were unambiguously identified by mass spectrometry using an information dependent acquisition experiment which combined a multiple reaction monitoring survey with dependent enhanced product ion scans. During this procedure, product ion scans of targeted analytes were generated at three collision energies and compared with an in-house library of MS/MS spectra acquired from reference standards of all biomarkers. This generic method enables detection, identification and quantification of 98 biomarkers intended to characterise 79 selected plants.

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## 1. Introduction

Botanicals and botanical preparations intended for human consumption are widely marketed with various health claims, and consumption of Herbal Food Supplements (HFS) is increasing all over the world. This commercial success is largely due to a growing interest in products supporting a healthy lifestyle and for naturally derived ingredients. These preparations are easily available to consumers through several distribution channels, e.g., over the counter in pharmacies, supermarkets, herbalist shops or via the Internet.

According to most current legislation, dietary supplements are concentrated sources of nutrients intended to supplement the normal diet with a nutritional or physiological effect and are considered as foodstuffs. Thus, they have generally not been through a rigorous drug testing process as for registered therapeutic phytochemicals. However, incorporation of medicinal herbs or other active botanicals is frequently encountered in food supplements, which are often sold with inappropriate health claims to promote natural health benefits. Therefore, in this context, there is a need to conduct control analyses to confirm the presence of declared botanicals and exclude the presence of undeclared, unauthorised or toxic botanicals in HFS. Screening of toxic botanicals may be of utmost importance in guaranteeing food safety when there might be problems associated with possible contamination or confusion among different species (misidentification). Indeed, the inadvertent presence of toxic plants or toxins in herbal preparations can induce dramatic consequences (Byard, 2010; Cheze, Gaillard, & Pépin, 2000; Ize-Ludlow et al., 2004). Recently, the European Food Safety Authority (EFSA) built up a considerable compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern (European Food Safety Authority, 2009). However, this compendium, composed of different European lists of botanicals, has no legal status and cannot be used as a regulatory basis to flag botanicals of concern that should go through a careful quality control process or may not be incorporated in food supplements. In line with the European legislation, food supplements sold in Switzerland are not allowed to bear any therapeutic activity and are merely composed of vitamins, minerals, essential fatty acids and/or amino acids. Nevertheless, formulations with therapeutically active botanicals are expanding on the Swiss market and derived phytopreparations are often under the guise of food supplements to circumvent an official registration process to be recognised as drugs. Thus, to properly delimit botanicals which can be integrated in food derived products (e.g., for aromatic purposes) from those with therapeutic effects that cannot be incorporated in HFS, a compendium has been build up by the Swiss authorities (Federal Office of

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Public Health, 2010). This guidance lists over 300 edible plants, among which 71 contain therapeutically active ingredients and require a marketing authorization, or must be handled on a regulatory basis as phytopreparations. Furthermore, this list points out the types of formulations (e.g., herbal tea, spices, capsules, tablets) of the remaining plants that can be used.

Presently, plants can be identified using several different methods. Microscopy or biochemical approaches can be used but they are not well adapted for screening plant mixtures. Chemical methods exist for detection and characterisation of plants, but they are usually specific for a particular species or dedicated to a class of compounds (Chen, Wu, Tan, Zhu, & Chai, 2011). To fill this current gap, a generic method was developed for multi-targeted screening of biomarkers (BMs) with the aim of characterising plant species in HFS. The analytical approach employed liquid chromatography coupled to tandem mass spectrometry with an information dependent acquisition step which generates fingerprint MS/MS spectra based on previous investigations published for the detection of drugs in human fluids (Mueller, Weinmann, Dresen, Schreiber, & Gergov, 2005) or to screen pharmaceuticals in dietary supplements (Lee & Lee, 2011).

## 2. Materials and method

### 2.1. Botanicals, chemicals and reagents

All plants were purchased from Dixa (St. Gallen, Switzerland) except *Digitalis purpurea* L. and *Podophyllum* sp., were provided from the collection of the Laboratory of Pharmacognosy and Phytochemistry at the School of Pharmaceutical Sciences in Geneva (Switzerland).

BMs and internal standards (ISTD) were purchased either from Ambinter (Paris, France), Chromadex (Irvine, CA, USA), Cil (Andover, MA, USA), Dr. Ehrenstorfer (Augsburg, Germany), Phytolab (Vestenbergsgreuth, Germany), Roth (Karlsruhe, Germany) or Sigma (Buchs, Switzerland).

Ammonium formate was purchased from Sigma and methanol (technical grade, LC grade and hypergrade for LC–MS) from Merck (Darmstadt, Germany).

Water was purified with a Milli-Q system from Millipore (Molsheim, France).

### 2.2. Herbal food supplements

Food supplements were bought in supermarkets and herbalist shops in Switzerland. Different types of formulations were purchased; a majority were powders, pills, tablets or capsules, and some others were in liquid form (drinkable ampoule or oral solution to dilute). Health claims such as weight loss, anti-ageing, immune system booster, vascular protection or skin care were frequently mentioned on the packaging.

### 2.3. Sample preparation

A homogeneous sample (200 mg) was extracted by sonication for 10 min in 10 ml of water–methanol (1:1, v/v). The resulting suspension was centrifuged for 10 min at 4000 rpm, at 25 °C. Clomipramine and fipronil were used as chromatographic standards in the positive and negative ionisation modes, respectively. Two millilitres of the supernatant and 50 μl of ISTD at 20 mg/l (in methanol–water 1:1, v/v) were diluted to 5 ml with methanol-ammonium formate buffer (5 mM, pH 4) (1:1, v/v). The solution was filtered through a 0.2 μm PTFE filter and used for further LC–MS analyses.

### 2.4. HPLC–ESI-MS/MS

High Performance Liquid Chromatography (HPLC) analysis was performed with an Ultimate 3000 instrument from Dionex (Olten, Switzerland). For the separation, a 50 × 2.0 mm, 2.5 μm RP Synergi Polar analytical column was used (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of water (phase A) and methanol (phase B) for the negative ionisation mode, and methanol and water with 5 mM ammonium formate buffer at pH 4 for the positive ionisation mode. In both modes, the column temperature was maintained at 30 °C and the flow rate was set at 250 μl/min. The sample injection volume was 5 μl. The solvent gradient started with 10% of phase B for 0.5 min and then was linearly increased to 97% over 12 min, kept constant for 7.5 min and then decreased over 0.1 min back to 10% phase B. Column re-equilibration time was set at 5 min.

Detection and quantification were performed with a 3200 Q-trap hybrid mass spectrometer from ABSciex (Applied Biosystems, Darmstadt, Germany), equipped with an ESI source.

MS parameters in the positive ionisation mode were set as follows: curtain gas at 25 psi, collision gas level at medium, ion spray voltage at 4200 V, source temperature at 500 °C, ion source gas 1 at 50 psi and ion source gas 2 at 40 psi. MS parameters were the same in the negative ionisation mode except for the ion spray voltage, which was set at −4200 V.

In addition to the standard MS parameters, within a retention time window of 60 s for each BM, Analyst software 1.5.1. from ABSciex automatically optimised dwell times with the intelligent scheduled Multiple Reaction Monitoring (MRM) algorithm to maintain a scan cycle of 0.5 s.

For the identification of compounds, an Information Dependant Acquisition (IDA) was performed (Fig. 1). Each BM was targeted by one mass transition; when the signal of this transition exceeded 1500 counts per second (cps), three Enhanced Product Ions (EPIs) were generated at three different collision energies (CEs), namely low (20 eV), medium (35 eV) and high energy (50 eV). The scan range was between 70 and 1000 Th with a scan speed of 1000 Th/s, and the fill time of the trap was set at 50 ms.

The limit of identification (LOI) was determined for each BM, and represented the concentration at which the three EPI spectra were exploitable for comparison with the database.

During the data processing, each EPI was compared with the in-house-built library. Fits were automatically calculated using the Analyst software. Several criteria such as mass tolerance (±0.2 Th) and retention time (±1 min) were specified to limit the search of the database. To identify the compound, all EPIs must have a correlation higher than 0.6 with the spectra from the library. Two parameters, Fit and Reverse Fit (RevFit), were used to calculate the correlation. Fit value calculates matches between library spectra with an unknown spectrum, whereas RevFit compares peaks of an unknown spectrum with peaks from the library spectra. Ideally, Fit and RevFit will have a correlation of one.

For quantification, MS acquisition was based on a conventional MRM mode. Two mass transitions were targeted for each BM, one for quantification and one for confirmation. LC and source parameters were the same as those used for the qualitative approach. Limits of quantification (LOQs) were determined for each mass transition from the signal-to-noise ratio (S/N = 10) of the analysis of standard solutions.

### 2.5. Matrix effect assessments

To assess the matrix effect, four mixtures of five plants each (in equal weight proportions) were prepared. Mixture 1 was composed of *Cynara scolymus* L., *Echinacea* sp., *Filipendula ulmaria* L., *Fraxinus excelsior* L. and *Illicium verum* Hook.*f.*; Mixture 2 was

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