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The use of chemometrics to study multifunctional indole alkaloids from *Psychotria nemorosa* (*Palicourea comb. nov.*). Part I: Extraction and fractionation optimization based on metabolic profiling

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ABSTRACT

Extraction methods evaluation to access plants metabolome is usually performed visually, lacking a truthful method of data handling. In the present study the major aim was developing reliable time- and solvent-saving extraction and fractionation methods to access alkaloid profiling of *Psychotria nemorosa* leaves. Ultrasound assisted extraction was selected as extraction method. Determined from a Fractional Factorial Design (FFD) approach, yield, sum of peak areas, and peak numbers were rather meaningless responses. However, Euclidean distance calculations between the UPLC-DAD metabolic profiles and the blank injection evidenced the extracts are highly diverse. Coupled with the calculation and plotting of effects *per* time point, it was possible to indicate thermolabile peaks. After screening, time and temperature were selected for optimization, while plant:solvent ratio was set at 1:50 (m/v), number of extractions at one and particle size at $\leq$180 μm. From Central Composite Design (CCD) results modeling heights of important peaks, previously indicated by the FFD metabolic profile analysis, time was set at 65 min and temperature at 45 °C, thus avoiding degradation. For the fractionation step, a solid phase extraction method was optimized by a Box-Behnken Design (BBD) approach using the sum of peak areas as response. Sample concentration was consequently set at 150 mg/mL, % acetonitrile in dichloromethane at 40% as eluting solvent, and eluting volume at 30 mL. Summarized, the Euclidean distance and the metabolite profiles provided significant responses for accessing *P. nemorosa* alkaloids, allowing developing reliable extraction and fractionation methods, avoiding degradation and decreasing the required time and solvent volume.

1. Introduction

Metabolomics has become an important field in plant sciences. It aims identifying and quantifying all metabolites in a biological system. However, this is a very bold goal when the chemical diversity of plants is considered. The metabolites present in an extract can vary in terms of polarity, chemical behavior, structure, stability and concentration, making the analyses extremely challenging [1,2]. Therefore, metabolite profiling combined with multivariate data analysis is being used, allowing sample classification without the need for metabolite identification and quantification [2,3]. Metabolite profiles can be obtained by different techniques. Spectroscopic techniques (*e.g.* IR, MS and NMR) are widely used because of their simplicity and good reproducibility [4]. However, spectroscopic methods have several limitations, such as low sensitivity and signal overlap since compounds are not separated [1]. For this reason, separation techniques (*e.g.* HPTLC, HPLC and UHPLC) are used aiming to better represent sample complexity [5]. Finally, the association of separation and spectroscopic techniques, i.e. hyphenated techniques, provides efficient separation of metabolites and, concurrently, valuable structural information [6].

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Prior to chemical analysis and data handling, plant collection and extraction steps must be overcome. As stated by Mushtaq et al. [7], researchers usually collect samples properly, but do not pay enough attention to the extraction step. In fact, a truthful snapshot of the metabolome demands proper extraction methods. Recently, Choi and Verpoorte [8] called the attention to this issue by editing a journal volume under the title: "Metabolome: what you see is what you extract". Some efforts were made in this sense, evaluating different extraction solvents, techniques and conditions (reviewed in [7]). However, some pertinent points should be stressed in this crusade. The solvent choice, extraction technique and its parameters must be selected based on the sample type, the metabolites one is focusing on, the main aims of the study, and the analytical technique used to access the metabolome. A wide range of options is nowadays available and many different combinations can be explored. Actually, to propose a *Panacea* to overcome these difficulties for all plants and metabolites seems to be utopic.

Another question to be addressed before the extraction optimization of the plant material is which responses are going to be evaluated. Once again, the goals of the study should be taken into account. Some usual responses include biological activity [9], yield [10] and amount of specific compounds [11] or of a class of metabolites [12]. However, for metabolome analysis, none of these responses is able to provide enough representative information. In fact, the main focus of current efforts in metabolome extraction optimization relies on the number of extracted metabolites since, in theory, this would give the best metabolome overview [1,7,13].

In order to access chemical diversity, different approaches have been proposed [14–19]. In most cases, visual inspection of the metabolic profile is used for quality evaluation. However, this is not a reliable response for extract optimization. Additionally, when examining factors that afford extracts with very similar profiles, visual inspection would not be able to give any good information about them. In some studies [18], orthogonal partial least squares-discriminant analysis (OPLS-DA) or principal component analysis (PCA) are used to detect cluster formation between similar profiles. Although additional information can be obtained, the extraction strategy is driven mainly by the metabolic profile or the group of profiles that is considered interesting to analyze.

One response that can be used to optimize chemical diversity in a metabolic profile, measured according to an experimental design, is the feature counting. In a chromatogram, for example, it would correspond to the number of peaks detected [20,21]. However, in this response, the intensity information is lost. Thus, if two profiles have the same number of peaks, but with different intensities (concentrations), it would not be possible to define any preference from one extraction strategy. Martin et al. [18] used the correlation between time and m/z detected by LC–MS analysis for feature detection. However, once again, the intensity information was not taken into account.

Our research group has been working with *Psychotria* subg. *Heteropsychotria* Steyerm. species for a long time. Current studies [22,23] have been demonstrating that most *Psychotria* subg. *Heteropsychotria* species can be considered synonyms of *Palicourea* Aubl. In fact, several taxonomical analyses reclassify *Psychotria* subg. *Heteropsychotria* species to the *Palicourea* genus [22,23]. Following this trend and in accordance with Prof. Taylor's recommendation (personal communication), *Palicourea comb. nov.* was added to original *Psychotria* names to identify the current uncertainty about *Psychotria* subg. *Heteropsychotria* species. In addition to taxonomical similarities, chemotaxonomic proximity between *Palicourea* and *Psychotria* subg. *Heteropsychotria* is also observed. Different bioactive indole alkaloids (MIAs), mainly responsible for the modulation of enzymes related to neurodegeneration, have already been described for these species [24–26]. Recently, we evaluated the monoamine oxidases (MAOs), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory effects of different alkaloid fractions obtained from *Psychotria*, *Palicourea* and *Rudgea* species [27]. We observed that the most promising source of multifunctional alkaloids was *Psychotria nemorosa* Gardner (*Palicourea comb. nov.*), displaying the lowest IC_{50} for MAO-A and BChE [27]. Aiming to access alkaloid profiling of *P. nemorosa*, as well as to develop a reliable time- and solvent-saving extraction and fractionation method, different optimization strategies were studied and several responses evaluated, including yield (%), sum of peak areas, peak counting, the Euclidean distance, and the entire metabolic profile.

2. Material and methods

2.1. Chemicals

All HPLC gradient solvents were acquired from Tedia (Fairfield, CA, USA). The remaining chemicals were of analytical grade and purchased from F. Maia (Cotia, Brazil).

2.2. Plant material

The leaves of *P. nemorosa* were collected from forty three individuals distributed in five different places of Blumenau/SC, Brazil. The species was identified by the botanic A.L. Gasper. Vouchers of each collection were deposited in the Dr. Roberto Miguel Klein Herbarium under the numbers FURB 43759, 43756, 43758, 43755, 43580. Access authorization was given by CNPq/Brazil under the number 010772/2014-6. Plants were dried in an air oven (Lawes, Brazil) at 40 °C for 48 h and later ground using an analytical mill (IKA, Königswinter, Germany). For experiments, a pool of the forty three samples was prepared mixing all in the same proportion. All mass calculations were performed taking into account a moisture content of $11.33 \pm 0.08\%$ ($n = 3$). The ground material was passed through sieves with different openings (from 710 μm to 180 μm), using a mechanical shaker Retac 3D (RETSCH, Haan, Germany) in order to have vegetal material with different particle sizes: >710 μm, 501–710 μm, 356–500 μm, 181–355 μm, and ≤180 μm.

2.3. Extraction optimization

2.3.1. Preliminary extraction assays

In order to compare different extraction methods, the one-factor-at-a-time approach was chosen. For all methods, the particle size was kept at 356–500 μm, the plant:solvent ratio at 1:20 (m/v) and the solvent used was methanol. All experiments were performed in triplicate. The evaporation of the solvent was performed using a rotatory evaporator with a vaccum pump (V-710, BÜCHI, Flawil, Switzerland) at 40 °C. The samples were kept under vacuum in a desiccator containing activated silica gel beads for two weeks prior to liquid–liquid fractionation experiments.

2.3.1.1. Static maceration. Maceration was performed during 48 h. After this period, the extracts were filtered through previous by methanol-soaked filters and fresh methanol (in the same plant:solvent ratio) was poured into the remaining vegetal material. This procedure was repeated three times. All the extracts were combined and solvent evaporated.

2.3.1.2. Dynamic maceration. The extraction was performed during a period of 4 h using a multiposition magnetic stirring plate (IKA) at 3 g. Filtration was performed as described previously. The procedure was also repeated three times, with fresh methanol being used in each cycle. All the extracts were combined and solvent evaporated.