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Resolution of co-eluting compounds of *Cannabis Sativa* in comprehensive two-dimensional gas chromatography/mass spectrometry detection with Multivariate Curve Resolution-Alternating Least Squares

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ABSTRACT

Comprehensive Two Dimensional Gas Chromatography – Mass Spectrometry (GC × GC/qMS) analysis of *Cannabis sativa* extracts shows a high complexity due to the large variety of terpenes and cannabinoids and to the fact that the complete resolution of the peaks is not straightforwardly achieved. In order to support the resolution of the co-eluted peaks in the sesquiterpene and the cannabinoid chromatographic region the combination of Multivariate Curve Resolution and Alternating Least Squares algorithms was satisfactorily applied. As a result, four co-eluting areas were totally resolved in the sesquiterpene region and one in the cannabinoid region in different samples of *Cannabis sativa*. The comparison of the mass spectral profiles obtained for each resolved peak with theoretical mass spectra allowed the identification of some of the co-eluted peaks. Finally, the classification of the studied samples was achieved based on the relative concentrations of the resolved peaks.

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1. Introduction

Comprehensive two-dimensional gas chromatography (GC × GC) provides remarkable features in the understanding of the qualitative and quantitative composition of complex samples. According to the literature, GC × GC offers some advantages in comparison to the classical one-dimensional chromatography such as signal enhancement (higher signal to noise ratio), higher separation capacity and structure-retention dimensionality [1,2]. However, it requires specific approaches to analyze experimental data and to extract the chemical information [3].

The combination of high order instrumental data and efficient data treatment methodologies widens significantly the possibilities of chemical fingerprinting of the targeted and non-targeted analysis in sensitive fields such as bioactive compounds [4] or metabolomics [5]. It is precisely in the assessment of natural lead compounds in drug development where those two fields merge and the needs for sample clustering, classification and chemical fingerprinting are especially highlighted [6].

Among the plant extracts, the analysis of cannabinoids is gaining interest not only due to its extended recreational use but also because *Cannabis sativa* is a strong candidate for new drug source [7]. As a matter of fact, in these extracts more than 400 different compounds can be found, which contain more than 100 terpenoids and around 65 cannabinoids [8,9].

GC–MS based methods for the analysis of *Cannabis sativa* extracts are the most extended, either with derivatization (silylation or methylation of carboxylic groups) or directly without any derivatization step (i.e. chemical fingerprints) [9,10]. In this sense, the development of GC × GC methods would improve the separation and identification drawbacks of one dimension chromatographic techniques.

Many works on GC × GC/qMS emphasize that the technique can be used to analyze complex mixtures in unsurpassed detail [2] but it requires fast detectors that generate large data sets [11]. As a consequence, pre-processing steps such as baseline correction, noise reduction or retention time alignment are usually applied to the raw data in order to reduce the variations occurred during the data collection [12]. Once these artifacts have been corrected and/or

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reduced the resulting 2D chromatograms are treated to carry out identification and quantification tasks [1], and to do so, both proprietary licenses and open source programs are used following different data treatment approaches [3,13,14].

The use of chemometric techniques has been proved to be efficient to obtain the essential information from chromatographic data. As a consequence, several techniques such as Parallel Factor Analysis- PARAFAC-, PARAFAC2 or multivariate curve resolution – MCR have been successfully applied in diverse research fields due to the ability to handle multidimensional data [15,16]. In this sense, MCR and PARAFAC2 have demonstrated to provide an excellent resolution of the overlapped peaks even in the presence of retention time shifts or irregular baseline drifts [17].

Recently, the combined use of multivariate curve resolution with alternating least squares algorithm (MCR-ALS) has gained attention because it provides a bilinear description of the data under chemical constrains, as reviewed by Ruckebusch et al. [18–20]. These features provide a promising alternative for resolution of overlapping peaks in two dimensions avoiding previous peak alignment steps [21–23]. The MCR-ALS analysis of a single 2D chromatogram can be extended to all the samples gaining the robustness of a cumulative analysis [24].

The aim of this work is to analyze different co-elution regions of the GC × GC/qMS chromatograms from *Cannabis sativa* extracts by means of MCR-ALS. In this particular approach, instead of using a high resolution mass spectrometer (e.g. TOF), we wanted to combine the GC × GC-MS and multivariate approaches and test their capabilities to resolve complex mixtures. In addition to this aim, the identification of the resolved peaks was attempted by means of the recovered mass spectral profile (MCR-ALS) and their retention times. Moreover, the ability of the MCR-ALS approach to differentiate the studied *Cannabis* sativa species was compared to the one offered by commercially available software packages.

2. Materials and methods

2.1. Samples

The *Cannabis sativa* plants analyzed in this work were collected from local gardens. A total of 17 samples of five different kinds of plant buds (*AK*-47, *amnesia*, *somango*, 1024 and *critical*) were analyzed and whenever possible leaves were analyzed as well. The plants were cryogenically milled in a cryogenic grinder 6770 freezer/mill® (SPEX Sample Prep, Metuchen, New Jersey, USA). The pre-treatment took a previous cooling time of 5 minutes, a milling time of 4 min and 1 cycle. The obtained particle size was about few μm so the homogeneity of the analyzed samples was guaranteed. All milled samples were stored in amber vials at −20 °C until analysis.

2.2. Supercritical fluid extraction (SFE) of the plants

An amount of 50 mg of milled plant was homogeneously mixed with 150 mg of diatomaceous earth and accurately placed in a high pressure extraction vessel of 1 mL (EV-1 Jasco). The extraction conditions were previously optimized and are thoroughly described elsewhere [25]. Briefly, the extraction was performed using SC–CO_2 (Carburos Metálicos 99.9995%, Barcelona, Spain) at 100 bar, 35 °C and 1 mL min^{-1} without co-solvent for the extraction of monoterpenes and 20% of EtOH (purity, Lab-Scan, Spain) as co-solvent for the extraction of sesquiterpenes and cannabinoids. Once the extraction was over, the samples were stored in vials at 4 °C until analysis.

2.3. FID/MS analysis

All the extracts obtained by SFE were analyzed by means of GC × GC-Flame Ionization Detector-Mass Spectrometer (GC × GC-FID/MS). A GC7890A gas chromatograph (Agilent Technologies, PA, USA) equipped with a FID and 5975C MS detector and an Agilent G-3486 A capillary flow plate modulator was employed. The control of the second pressure source was handled with a pressure control module. A three-way solenoid, Fluid Automation System Valve, was used for flow switching. The column set for GC × GC-FID/MS analysis consisted of two columns connected by a valve modulator. The first dimension consisted of HP-5 MS capillary column (Agilent Technologies, 30 m × 250 μm i.d. × 0.25 μm film thickness) and the second dimension consisted of DB-17 MS (Agilent Technologies, 5 m × 250 μm i.d. × 0.25 μm film thickness). Two deactivated but not coated fused silica tubes (restrictor) were used in order to split the flow to the detectors: a 0.70 m 0.32 mm i.d. restrictor connected to the FID and a 0.45 m 0.10 mm i.d. connected to the MS.

The subsequent temperature programmed conditions were set as follows: from 60 °C to 102 °C at 4 °C · min^{-1}, from 102 °C to 165 °C at 12 °C · min^{-1} and from 165 °C to 300 °C at 6 °C · min^{-1} (hold 5 min). The GC was equipped with a split/splitless injector (290 °C). The injections were performed in the splitless mode injecting 2 μL of each sample into the GC using a 7683 Agilent autosampler. Modulation periods of 1.42 s, first column flow of 1.23 mL min^{-1} and second column flow of 17.55 mL min^{-1} were used. Hydrogen (> 99.9995%, AD-1020 Hydrogen Generator, Cinel Strumenti Scientifici, Padova, Italy) was employed as carrier gas taking into account all the safety issues necessary in the laboratory. The FID was operated at a data collection frequency of 100 Hz at 300 °C. The MS detector worked in full scan mode from m/z 50 to 350, at the faster electronic mode that assured a sampling rate of 12,500 amu/s, and temperatures of quadrupole and source were 150 °C and 230 °C respectively.

2.4. MCR-ALS analysis

The GCxGC/qMS chromatograms were exported from GC Image (v. 2.0, Zoex Corporation, Houston, USA) as * cdf files into MATLAB 7.0 (MathWorks, Natick, MA, USA, 2010R2). In order to reduce the amount of information and to facilitate the calculations, only the *regions of interest* (RoI) in the chromatograms were chosen and saved as new chromatograms.

The theory behind MCR-ALS has been discussed in previous works [18,26–28]. Herein we will briefly highlight the main features of MCR-ALS. MCR is a model-free method (this means, MCR does not require the pre-assumption of an empirical model for the peaks to be modeled) that focuses on describing the evolution of the experimental multicomponent measurements through their pure component contributions [26]. Each RoI **D** (*I*,*J*, *K*), composed by *I* elution times in the first chromatographic dimension, *J* elution times in the second chromatographic dimension and *K* *m*/*z* intensities in the third dimension, must be unfolded to adapt the three-way structure to a bidimensional matrix **D** (I^*J,*K*) as indicated in Fig. 1.

MCR-ALS looks for a bilinear data decomposition of the experimental matrix **D** (I^*J,*K*) for *N* components (*N* stands for the number of chemical components present in the RoI) using an iterative algorithm based on constrained linear least-squares steps based on Eq. (1).

$$D = CS^T + E \quad (1)$$

where **C** (I^*J, *N*) is the matrix of pure elution profiles, $\mathbf{S^T}$ (*N*, *K*) is the matrix of pure mass spectra and **E** (I^*J, *K*) is the residual matrix reflecting the experimental error unexplained by the resolution

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