What about the herb? A new metabolomics approach for synthetic cannabinoid drug testing

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Abstract

Background and aim Synthetic cannabinoids (SCs) are consumed as legal alternative to cannabis and often allow passing drug-screening tests. Their rapid transience on the drug scene, combined with their mostly unknown metabolic profiles, creates a scenario with constantly moving analytical targets, making their monitoring and identification challenging. The development of fast screening strategies for SCs, not directly focused on their chemical structure, as an alternative to the commonly applied target acquisition methods, would be highly appreciated in forensic and public health laboratories. **Methods** An innovative untargeted metabolomics approach, focused on herbal components commonly used for `spice´ products, was applied. Saliva samples of healthy volunteers were collected at pre-dose and after smoking herbal components and analyzed by high-resolution mass spectrometry. **Results** The data obtained, combined with appropriate statistical analysis, allowed to highlight and elucidate two markers (Scopoletin and N,N-bis(2-hydroxyethyl)dodecylamine), which ratio permitted to differentiate herbal smokers from non-smokers. **Conclusions** The proposed strategy will allow discriminating potential positives, on the basis of the analysis of two markers identified in the herbal blends. This work is presented as a step forward in SC drug testing, promoting a smart first-line screening approach, which will allow reducing the number of samples to be further investigated by more sophisticated HRMS methods.

Keywords

Spice; Herbal components; Synthetic cannabinoids; Saliva Biomarkers; Metabolomics; High-resolution mass spectrometry

Introduction

New synthetic cannabinoids (SCs) are introduced each year into the international `market´ as legal alternative to cannabis (1,2). Although they are structurally unrelated, SCs act functionally similar to $\Delta^9$-tetrahydrocannabinol (THC) *i.e.* the principal active component of cannabis. Branded herbal or `spice´ products, containing SCs, are easily purchased through online vendors and smart shops, where they are sold with misleading information about their effects and safety. Hence, SCs are considered a growing problem and are associated with severe negative effects in consumer's health (3–5).

The impetus of smoking herbal products, adulterated with SCs, is often not only to get intoxicated legally, but it also enables users to pass drug-screening tests. The latter is an important element for any setting where drug abstinence control is obligatory e.g. specific psychiatry or prison settings, driving liability testing or employment drug testing procedures (1).

The detection and identification of SCs is an analytical challenge due to their rapid transience on the drug scene (6,7). This fact combined with the mostly unknown and extensive metabolic pathways has created a scenario, where a high number and constantly moving analytical targets need to be considered for a comprehensive investigation of SCs consumption. Around 170 SCs have been reported until 2017 to the Early Warning System of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), a number that will surely increase in the next years. In addition, analytical reference standards are not always available or it takes much time to purchase them. This has triggered analytical chemists to develop comprehensive screening strategies mostly based on high-resolution mass spectrometry (HRMS), employing suspect and non-targeted approaches (8,9). HRMS has shown strong potential to screen for large lists of target compounds, as well as unknown substances, including in a retrospective manner (8,10). Its strong potential for identification and elucidation of compounds relies on accurate-mass full-spectrum data, but HRMS-based methods also have limitations in capacity and sensitivity (11), and data processing is very laborious and time consuming, which makes it increasingly challenging and costly when numerous samples need to be analysed.

The development of alternative, fast and generic screening methods, not directly focused on the analyte chemical structure, is an attractive approach that would facilitate the complex analytical scenario and provide fast response on potential SC consumption. Recently, Canneart et al. developed an assay that allowed activity profiling of SCs and their metabolites (11). This activity-based assay might serve as a first-line screening tool of urine, complementing conventional targeted and untargeted analytical methods.

This strategy does not allow the direct identification of SCs, but is neither limited to a given list of compounds, oppositely to the target approaches, which may lead to reporting false negatives when the compound (parent drug or metabolite) is not included in the candidate list.

In this work, we apply an innovative untargeted metabolomics approach for the rapid monitoring of SCs consumption. The strategy applied is focused on the main natural herbal components used for `spice´ products. Thus, instead of screening for target SCs or unknown substances, identified markers of herbs can be monitored to flag suspect samples, since these herbs are commonly used as the herbal base for the active synthetic chemical ingredients (12). We present the proof-of-principle of this original strategy directed towards saliva samples instead of urine. The collection of a saliva samples is fast, easy and non-invasive, it is also less prone to fraud with respect to urine samples, especially essential in obligatory drug control settings.

Material and Methods

Chemicals and reagents

HPLC-grade water was obtained by purifying demineralized water in a Mili-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade acetonitrile (ACN), methanol (MeOH), and ammonium acetate (NH_4Ac) were acquired from Scharlab S.L. (Barcelona, Spain). Leucine-enkephalin, formic acid (HCOOH, 98 - 100 %), Scopoletin (99% purity) and N,N-bis(2-hydroxyethyl)dodecylamine (99% purity) were purchased from Sigma-Aldrich (Augsburg, Germany).

Six natural herbal components *Canavalia maritima, Leonurus sibiricus, Althaea officinalis, Turnera diffusa, Verbascum thapsus, Calendula officinalis* were purchased from Worldherbals (Vlaardingen, the Netherlands). Tobacco from different trademarks (Domingo, Fortuna and Camel) was purchased from a local tobacconist.

Sample collection and treatment

Hand-rolled cigarettes containing 0.5 g of tobacco or mixtures of 0.25 g herb with 0.25 g of tobacco (50:50, w/w), were prepared. Each mixture contained tobacco and solely one herbal component. Three healthy volunteers smoked the tobacco cigarette and six herbal mixtures, leaving three days between experiments. Saliva samples were collected in an Eppendorf tube before (at pre-dose, t = 0) and after smoking (t = 30 minutes), and immediately stored in the dark at −20 °C until analyses (within 1 week). The volunteers were informed, and were involved in the study protocol design, giving their consent.

Prior to analyses, saliva samples were thawed, and 0.5 mL sample was vortexed for 1 min. Subsequently, 1 mL of ACN was added, vortexed for 30 sec, sonicated for 1 min and centrifuged at 10.000 g for 10 min. Supernatant was led to dryness under vacuum and reconstituted in 50 µL of H_2O:ACN (90:10 v/v)(13). Quality control samples (QCs), consisting in a mix of all saliva extracts (blanks and smoked), as well as the sample extracts (10µL) were injected directly into the UHPLC-QTOF MS system.

Instrumentation

A Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration- ToF mass spectrometer (Xevo G2 QTof, Waters Corp., Manchester, UK) using a Z-spray electrospray ionization (ESI) interface operating in both positive and negative ionization modes. A

capillary voltage of 0.7 kV for ESI+ and 1.5 kV for ESI- mode, and a cone voltage of 20 V were used. MS^E data were acquired over the range m/z 50–1200. MS/MS experiments were performed applying a collision energy of 10, 20 and 30 eV.

The chromatographic separation was performed using a CORTECS® C_{18} fused core column (2.7 µm particle size, 2.1x100 mm) at a flowrate of 0.3 mL/min. The mobile phases used were A - MilliQ water and B - MeOH (both with 0.01% HCOOH). The total run time was 18 min.
Further details on instrument operating conditions both chromatographic and spectrometric can be found elsewhere (10).

Data processing

Data (*.raw) were converted to a machine independent format (*.cdf) using the DataBridge application within MassLynx™ (Waters Corp., Milford, MA, USA). The converted data was then processed using XCMS free R package. Peak picking was performed with *centWave*, an algorithm for feature selection, which integrates area, considering a peak width between 4-20 sec., ≥ 3 scans with more than 1000 counts, *s/n* ratio of 10, and a mass error of ≤ 15 ppm. Peaks were grouped in single features using the *retcor()* function based on their retention time and mass error (an initial variation < 15 seconds and < 10 ppm between samples along the batch was considered as the same feature). Features were labelled as M*XXX*T*YYY* where *XXX* corresponds to the nominal Mass of peak *XXX* while *YYY* matches the retention Time in seconds. Samples were log_2 transformed to reduce heteroscedasticity. Pareto scaling was applied before importing the data for statistical analysis.

Statistical analysis

Multivariate analysis was carried out using EZinfo 2.0 (Umetrics – Sartoris Stedim Biotech, Malmö, Sweden). First, Principal Component Analysis (PCA) was applied to ensure that the data was correctly acquired in both positive and negative ionization mode. Next, all data were merged in a single table for further statistical analysis. Partial Least Squares Discriminant Analysis (PLS-DA) was carried out to ensure that samples can be differentiated. Orthogonal Partial Least Squares – Discriminant Analysis (OPLS-DA) was performed in order to extract a small group of markers to differentiate between herb and tobacco.

Results and Discussion

Six natural herbal components commonly used as the herbal base for 'spice' products were selected (1,14). The SCs are typically blended with or sprayed onto these herbs (12).

Hand-rolled herb cigarettes were prepared as a mixture of tobacco and herb (50:50, w/w). Although differences between "only herb and only tobacco" saliva samples would possibly be more pronounced, tobacco could mask the markers for herbs, as cannabis (and very likely SCs as well) is often mixed with tobacco or consumed in parallel (15). Thus, a strategy focused on discriminating between saliva after smoking tobacco and herb-tobacco mixtures would allow a first screening of potential positive samples containing SCs.

Saliva samples were collected using the same material of the same batch to avoid misinterpretations on assignment of potential markers. During experimental days, the volunteers did not have a strict diet. Since all samples collected from the three volunteers were processed at the same time, differences in diet were compensated and the selected markers could therefore be linked to herb and/or tobacco smoking.

Metabolomics enables to discover and highlight differences between subject groups based on the powerful analytical capabilities of HRMS and bioinformatics (16–18). Hence, in this study, an untargeted metabolomics approach has been applied as a data-mining tool, to distinguish between tobacco and herb-tobacco mixtures administration. HRMS data was processed and grouped by multiple peak picking functions and statistical tools, considering retention time and mass error. PCA analysis allowed to eliminate possible outliers (Hotelling's T^2 Ellipse (0.95)) and control the possible instrumental drift along the time. The latter could be done by injecting QCs in the initial part of the batch and after every 10 samples. The QCs (n= 6) were grouped in the center of the PCA plot, indicating the correct acquisition of the data. Further statistical analysis by means of PLS-DA modelling showed differences in saliva samples at pre-dose (blank) and after smoking herbs or tobacco (72 % of variance for the first 2 components) (**figure 1A**). An S-Plot from OPLS-DA, representing all features, permitted to highlight the best two markers (**figure 1B**).

The ratio between the peak area of these two features was evaluated achieving the discrimination between the blank and/or tobacco saliva samples versus the six different herb samples (**figure 2**). A threshold value of 0.02 could be set (figure 2, insert), meaning that samples with a ratio > 0.02 can be flagged as suspects.

The elemental compositions of the two most significant markers, retrieved from OPLS-DA, were calculated based on their accurate masses. Subsequently, tandem mass spectrometry (MS/MS) QTOF experiments were performed in order to obtain accurate-mass product-ion spectra. This information was used to search for plausible structures in available mass spectra libraries (Metlin and Massbank), chemical databases (Chemspider), and in-silico fragmentation web sources (MetFrag). **Figure 3** shows the accurate-mass product-ion spectra acquired at a collision energy of 30 eV. The accurate mass of the protonated molecules (mass error < 3 ppm) and of the fragment ions allowed the tentative identification of Scopoletin (marker 1; figure 3 top) and N,N-Bis(2-hydroxyethyl)dodecylamine (marker 2; figure 3 bottom), which were subsequently confirmed by the acquisition of the reference standards.

Conclusions and future perspectives

In this work, we present an innovative analytical strategy to obtain fast response on potential SC consumption. The application of an untargeted metabolic approach, based on the screening of saliva samples after consumption of tobacco and herbal components commonly used for spicing with SCs, has revealed two biomarkers (scopoletin and N,N-Bis(2-hydroxyethyl)dodecylamine). The ratio between these two biomarkers in all six herbs investigated permitted to discriminate between "tobacco and herb-tobacco mixture" samples. By monitoring their ratio in saliva samples, a value above 0.02 reveals the smoking of herbs, and therefore suggesting, indirectly, the potential consumption of SCs. In this way, samples can be pre-selected for further profound HRMS investigation in order to identify the consumed SCs.

We provide the proof-of-principle for this new approach, which can be considered as an important step forward towards a more generic SC drug test, not directly based on their structures, but on the herbal markers. However, in order to reach that goal future research is needed. This should be focused on the following issues: (i) to understand the significance of the selected biomarkers (already confirmed by their reference standards) and their link to the composition of the herbs investigated; (ii) full validation of the approach suggested, considering more volunteers and time/data points in order to evaluate more accurately the overall smoke/blood/saliva distribution of the markers; (iii) evaluation of the ratio for the identified markers after smoking other herbs also used as herbal base (i.e. *Nymphaea alba, Scutellaria lateriflora, Zornia latifolia, Nelumbo nucifera, Trifolium pratense, Leonotis leonurus, Astragallus root, Lamiaceae herbs* and *Rosa canina* (12)); (iv) development of target analytical methods for the identified biomarkers in saliva, e.g. based on LC-MS/MS with triple quadrupole.

The strategy suggested is the target analysis (e.g. by LC-MS/MS QqQ) of identified herb biomarkers i.e. indirect indicators of SC consumption, and the subsequent monitoring of their peak area ratio. This approach has two major advantages: i) only positive/suspect samples need further investigation to identify the SCs smoked, which is more rapid and cost-effective since time-consuming analysis and data processing of all samples is avoided. ii) fraudulence in drug-screening tests by SCs consumers becomes more difficult, because monitoring is not based on the chemical structures of individual target SCs, but on the herbs markers. Therefore, it bypasses the fast-changing nature of the SCs market, where the current applications of target analysis might lead to reporting false negatives. In addition, this untargeted metabolomics approach could be applied to other matrices such as breath, hair and urine (19,20) for a similar purpose. This might allow the detection of SC consumption over a longer period of time, opening new possibilities for the laboratories to face the complex issue of monitoring the SCs market.

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Figure captions

Figure 1: A) PLS-DA 3D score plot for the first three components. B) S-Plot from OPLS-DA where all features are represented

Figure 2: Boxplot representing ratios of the peak areas between marker 1 and marker 2 in the saliva samples *i.e.* blank and after tobacco and herb smoking (n= 24, 6, 18, respectively). Insert; zoom at ratios 0 to 0.15

Figure 3: Identification by QTOF operating in MS/MS acquisition mode of Scopoletin (top) and *N,N*-Bis(2-hydroxyethyl)dodecylamine (bottom) in saliva samples. (Inserts: structure, chemical formula and exact mass of the protonated molecule)

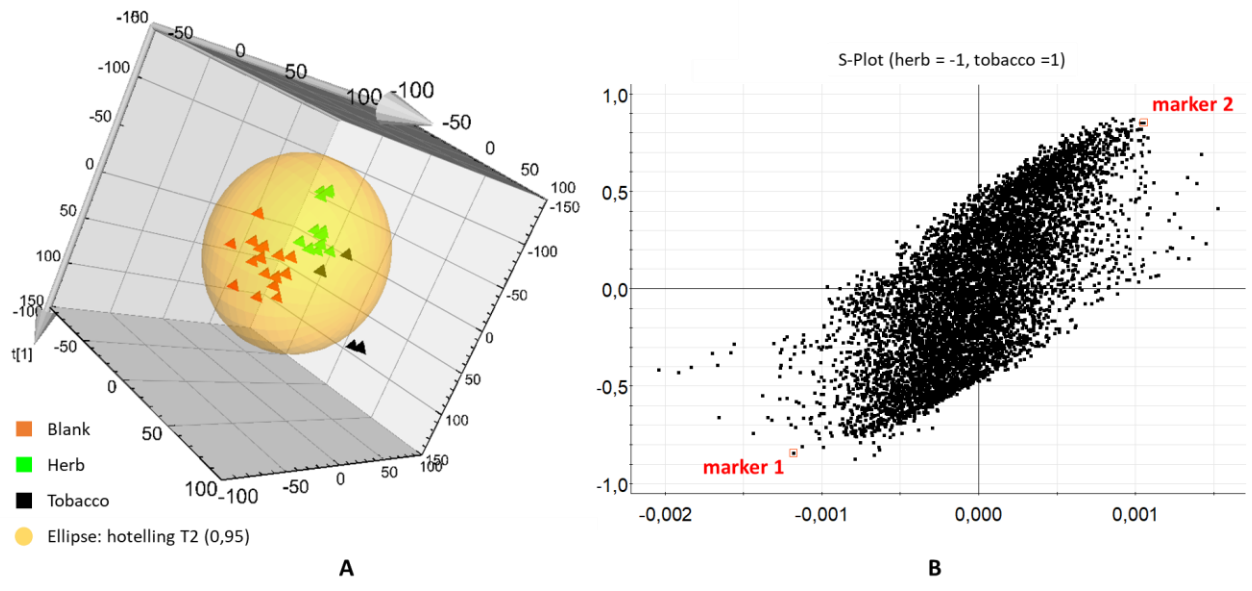


Figure 1

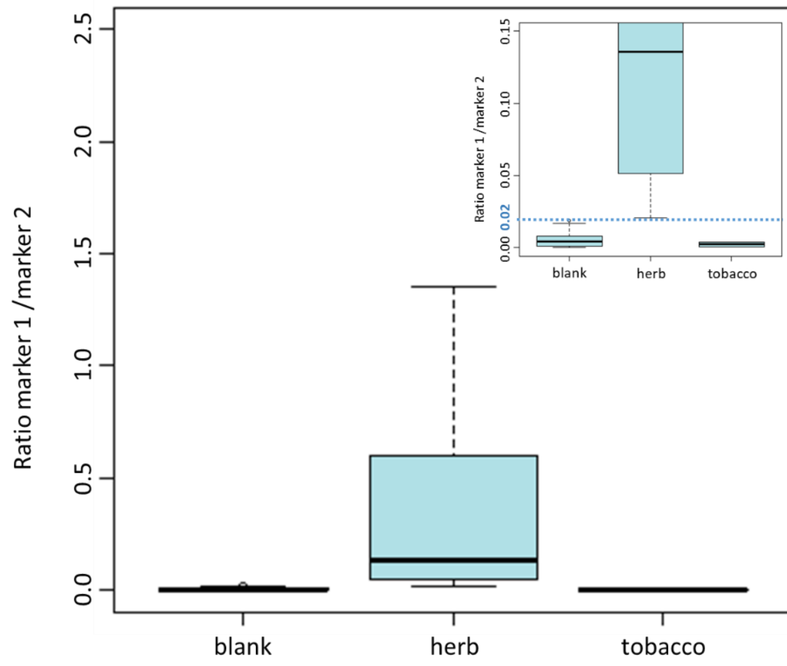
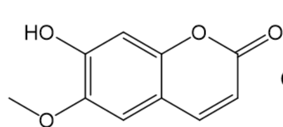


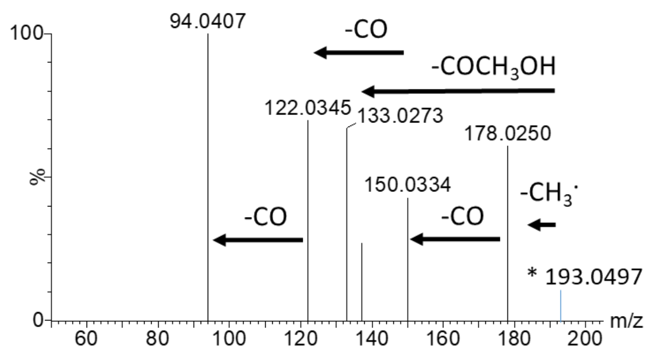
Figure 2

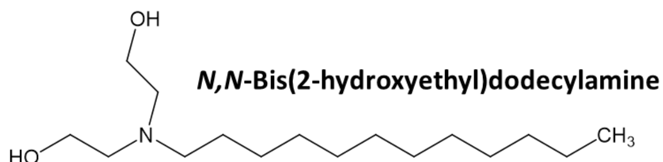


Scopoletin

Chemical formula: $C_{10}H_8O_4$
$[M+H]^+$: 193.0495

* Observed at collision energy 10eV





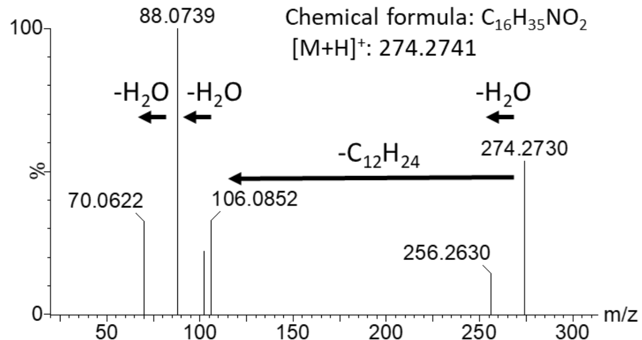


Figure 3