METABOLOMICS APPROACHES POWERFUL TOOLS FOR ANALYSING THE QUALITY OF RAW MATERIALS VETIVER ESSENTIAL OIL A CASE STUDY

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INTRODUCTION

Vetiver Essential oil (EO) *Chrysopogon zizanioides*, is one of the major ingredients used in fragrance industry. Its particular heavy, earthy fragrance with woody, grapefruit and smoky-earthy notes (2), make it a raw ingredient of choice for perfumers.

Figure 1. Picture of a Vetiver (chrysopogon zizanioides) plant

Originating from India, vetiver is widely cultivated in tropical regions including Haiti, Indonesia and Paraguay (1). Vetiver is mainly cultivated for the fragrant essential oil distilled from its roots which are extracted and used for cosmetics and aromatherapy. The different origins present variable chemical compositions resulting in different organoleptic properties.

The compositional analysis of essential oils by GCMS is a well-established method. It is possible to perform precise and accurate quantitative analyses, and to monitor the oil's quality and stability, based on few specific chemical markers. Nevertheless, the recent evolution of the regulations based on the Natural Complexes Substances (NSC), obliges suppliers to have a better knowledge of the phytochemistry of their products.

In this way, the combination of UPLC and UPC² analyses coupled with HRMS can be used for a more comprehensive overview of the raw ingredients using an untargeted metabolomic approach (3,4,5). A variety of Ionisation sources (ESI, APCI, ASAP), ionisation modes (positive/ negative) and capillary or corona voltages (low and high) were used to be as exhaustive as possible. In this study, 10 distinct vetiver oil samples of different botanical origins were analysed to analyse their chemical constituent make-up and asses their qualities.

METHODS

Vetiver essential oils from Haiti (x4), Indonesia (x4) and Paraguay (x2) origins were obtained from trade suppliers. Oil sample were subjected to dilution 1/10 in a mix of MeOH/ACN (50:50). **UPLC**: analyses were carried out on a Waters ACQUITY® UPLC with a CORTECS® , C18, 1.8 µm, 2.1 x 100 mm column. Mobile phases were: 5 mM ammonium formate (AF) solution (pH=3.8) and B: methanol (MeOH)/acetonitrile (ACN) (50:50) with 5 mM AF 0.1 % formic acid (FA). Gradient was 1 % B (2 min) to 90 % of B in 10 min, then to 99 % of B in 3min stay at 99% of B for 3 min; 1 µL injections were made. **UPC²**:analyses were carried out on a Waters ACQUITY® UPC² system. Chromatographic separation was achieved on a UPC² C18 HSS SB column, 1.8 µm, 3 x 100 mm maintained at 55°C. Mobile phase A was supercritical CO_2. Mobile phase B was MeOH/ACN (50:50). The gradient employed was isocratic for 3 min at 100 % A then to 1 % B in 2 min, remaining at 1% of B in isocratic mode for 3 min then ramping to 40 % B in 6 min, remain at 40% B for 4 min all with a flow rate of 1.2 ml/min. Injection volume was 1 µL. A splitter and a solvent makeup of MeOH:H_2O (99:1) with 0.1% FA was introduced at 0.45ml/min in order to improve the ionisation in positive mode and 5mM of AF in negative mode. All mass spectrometric analyses were performed on a Waters XEVO® G2 ToF mass spectrometer in MS^E, data independent analysis, mode. For **ESI mode analyses:** capillary voltages of 1 and 2.6 KV in positive and negative ionisation modes were employed. For **APCI mode analyses** corona currents of 5 and 20 µA in positive and negative ionisation modes were employed. For **ASAP mode analyses** a corona current of 20 µA was employed in positive and negative ionisation modes.The acquisitions were carried out in MS^E mode using argon as collision gas to obtain analyte fragmentation.

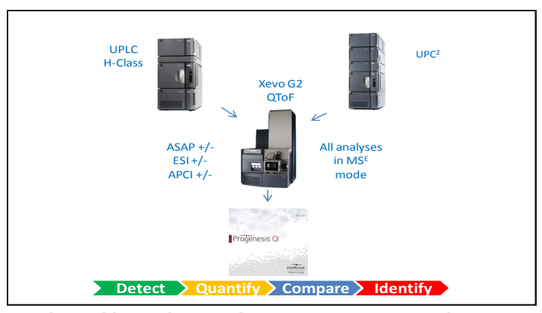

Figure 2. UPLC, UPC² and QToF MS system solution used for untargeted metabolomic experiments

The samples were investigated in replicate. QC samples comprising of a mixture of the essential oils were also included in the experiment. Data obtained were processed, searched (against a Robertet in-house vetiver database of over 400 compounds referenced in literature), and quantified with Progenesis QI v2.3. Features were filtered for statistical significance (CV <30%; ANOVA (p) <0.05, fold change >2). Data extracts with Progenesis were also submitted to statistical processing by Principal Component Analysis (PCA), Hierarchical clustering analysis (HCA), Partial Least Square-Discriminant Analysis and Orthogonal Partial Least Square-Discriminant analysis

RESULTS

A preliminary untargeted study, based on the fingerprinting obtained with the different devices used, showed the synergy and the complimentary nature of the data obtained with the different chromatographic techniques (UPLC & UPC²) but also with the different ionization modes (ESI and APCI)

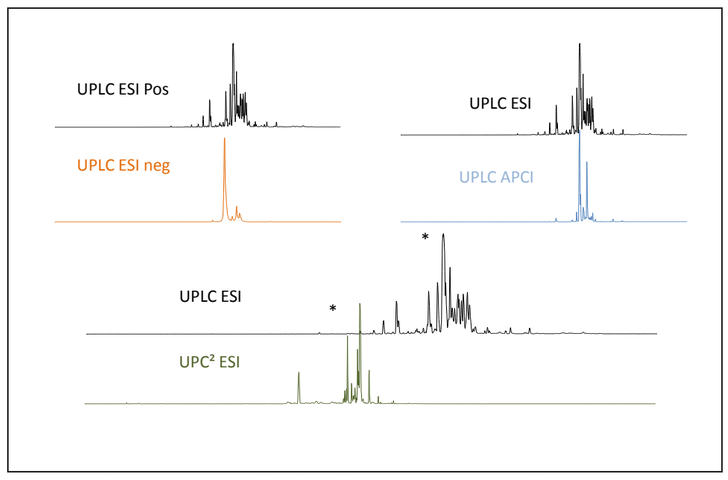

Figure 3. Complementary LC-MS profiles obtained using different techniques. Top left; Classical RP-UPLC analysis in ESI ionization in positive and negative modes. Top right RP-UPLC analysis in ESI and APCI ionisation modes Bottom; UPLC and UPC² analysis coupled with ESI ionization mode.

The modification of the probe voltages (low and high) allowed modification to the response of some compounds, the differences between the analyses obtained at different probe voltages were not significant. Nevertheless the profiles obtained (Figure 3) with different ionization interfaces, chromatographic techniques and ionization modes, gave additional complimentary chemical information and helped to characterize extracts in more detail.

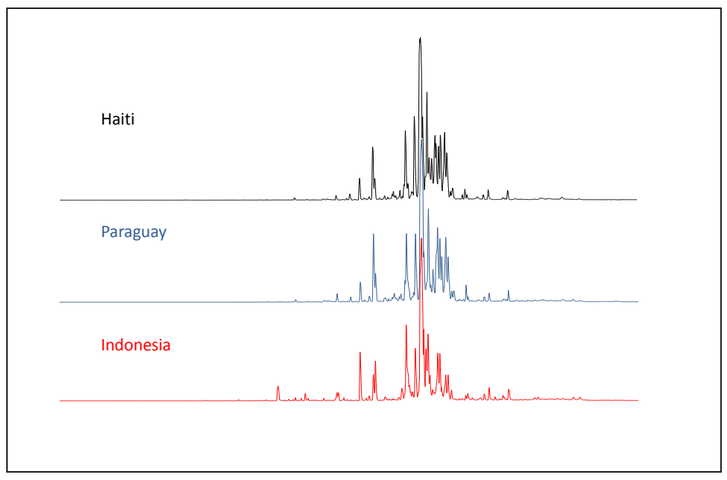

Figure 4. UPLC/TOF-MS profiles obtained in ESI positive ionisation modes of the samples from the three different geographical regions

A Preliminary study of the UPLC/TOF-MS profiles of the samples from different geographical origins showed only minor differences. The main variations between samples concerned the different abundances of some components (markers). The fingerprints of the extracts were very similar.

Statistical evaluation of the vetiver datasets is shown in figure 5. On the left we can see that the unsupervised PCA analysis shows clear clustering of the 3 different botanical types of vetiver confirmed by the HCA analysis on the top right. The PCA score plots also shows separation between groups of low and high ionisation modes, and revealed outliers in the Indonesian group. The supervised PLS-DA analysis, displays better separation between the groups, decreases dispersion within the groups, and again highlights outliers in the Indonesian group. OPLS-DA was then applied, in order to extract the best discriminant markers for each geographical origins. From the OPLS-DA analysis we then created S-plots and were able to ascertain the features which have the highest confidence and contribution to the differences between the vetivers of differing botanical origin.

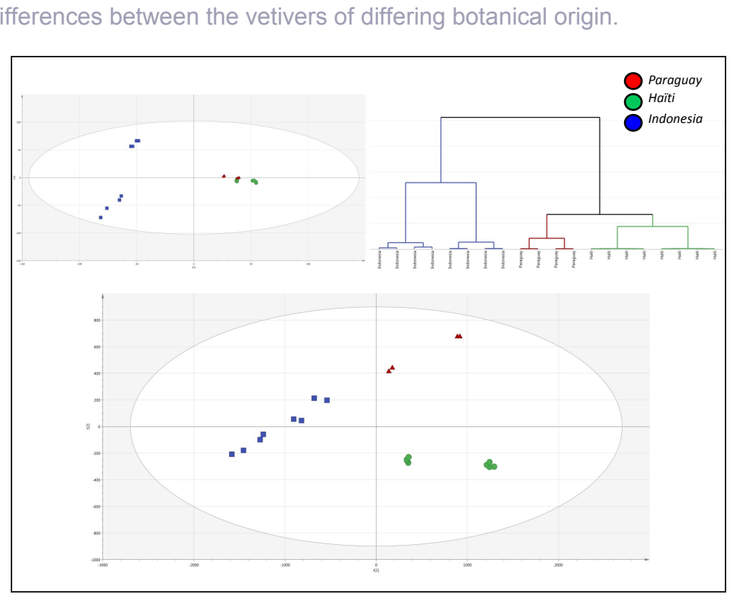

Figure 5. Top left: PCA score plots resulting from the analysis of vetiver oils of different botanical origins. Top right: Hierarchical clustering analysis of vetiver oils of different botanical origins. Bottom: OPLS-DA score plots resulting of the statistical analysis of the different geographical origins

The same strategy could be used in order to highlight the differences between samples of a same groups, as the observation made in the Indonesia group.

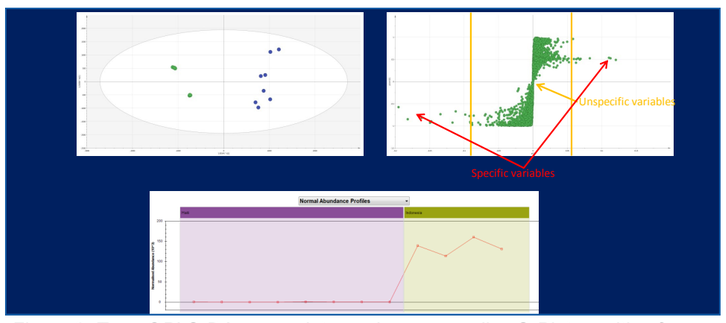

Figure 6. Top : OPLS-DA score plots and corresponding S-Plot resulting from the comparison between Haiti and Indonesia groups
Bottom :relative abundance profile of compound (RT:11.43 min ; 298.2502 Da) between Haiti and Indonesia groups

From this strategy, a specific marker responsible for the outliers was extracted. This compound, with a retention time of 11.43 min and with a neutral mass of 298.2502 Da (Figure 6), as well as others responsible for sample discrimination, was subjected to database search of an in-house vetiver database. The database contained over 400 chemical compounds cited by literature to exist within vetiver and also common contaminants. Figure 7 shows that Metascope was able to identify the feature as ricinoleic acid using the high energy fragmentation information present in the MS^E analysis.

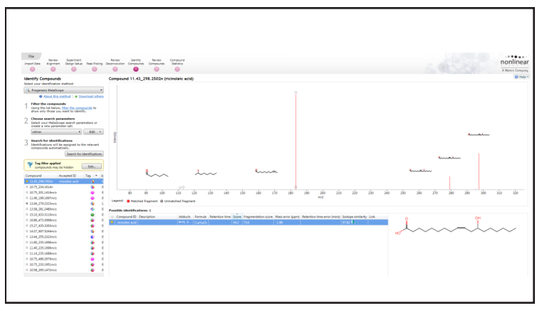

Figure 7. Progenesis QI browser window showing identification with Metascape of ricinoliec acid

This result shows the capacity of the proposed strategy to easily and quickly highlight contamination, and as a consequence, non-conformity of a sample. Contamination in the case of a classical GC analysis would go unnoticed.

Finally, these results could be easily obtained by using ASAP probe as a first step of screening (Figure 8). In fact a 5 min acquisition using ASAP probe allows to obtain a first fingerprint of samples. In which it is possible to identify contamination.

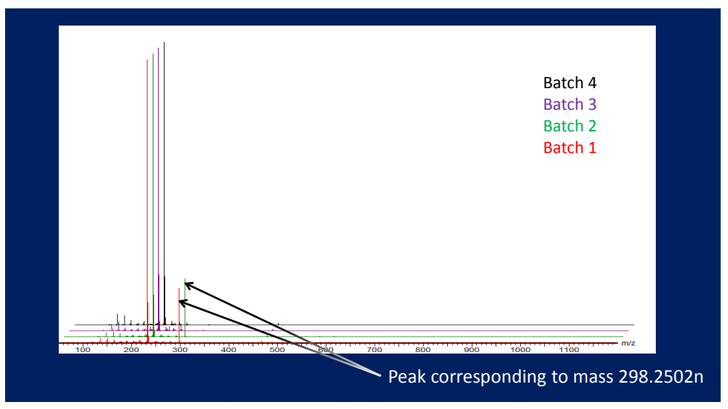

Figure 8. Mass spectra fingerprints of different batches of vetiver oil

CONCLUSION

The workflow employed in this study enabled the analysis of the LCMS data generated from a multitude of analyses of vetiver essential oil in a quick and easy way.

Complementary chemical information about the essential oil was obtained using the different Ionisation methods (UPLC, UPC² , ESI or APCI, positive and negative ion modes) and this was easily interrogated with Progenesis QI, allowing increased product knowledge.

The study showed that an untargeted metabolomic discovery strategy applied to vetiver essential oil is an efficient tool for discriminating samples of different botanical origin.

Unexpectedly non–conforming batches of essential oil were easily discriminated via unsupervised PCA analysis in Progenesis QI.

This method may be considered as a further complementary tool to the well-known and effective approaches based on volatile analysis, involving GC-FID and GC-MS techniques.

UPLC and UPC² MS techniques in combination with advanced statistical tools provided in Progenesis QI, can successfully be applied in quality control, and to authenticate natural raw ingredients.

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