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Cocoa quality: Chemical relationship of cocoa beans and liquors in origin identitation

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ARTICLE INFO

ABSTRACT

In this study, HS-SPME-GC–MS was applied in combination with machine learning tools to the identitation of a set of cocoa samples of different origins. Untargeted fingerprinting and profiling approaches were tested for their informative, discriminative and classification ability provided by the volatilome of the raw beans and liquors inbound at the factory in search of robust tools exploitable for long-time studies. The ability to distinguish the country of origin on both beans and liquors is not so obvious due to processing steps accompanying the transformation of the beans, but this capacity is of particular interest to the chocolate industry as both beans and liquors can enter indifferently into the processing of chocolate. Both fingerprinting (untargeted) and profiling (targeted) strategies enable to decipher of the information contained in the complex dataset and the cross-validation of the results, affording to discriminate between the origins with effective classification models.

1. Introduction

Cacao (*Theobroma cacao* L.) is a perennial tropical crop economically important for the countries growing it. It is also a raw material of great relevance for different fields including confectionary and functional food and beverages (cocoa and chocolate derivatives) that cover >60 % of the market (Research and Markets, 2021). The flavour is one of the main features linked to chocolate product quality besides brand and cost (CABISCO/ECA/FCC, 2015). Cocoa beans and the post-harvest treatments are uniquely responsible for the flavour and nutritional components of cocoa derivatives, which implies that the cocoa chain must be supplied with products with consistent standards of quality, i.e. uniform raw materials with specific quality parameters from the country of origin. This is a serious issue for chocolate manufacturers since they need bulks of raw or semi-finished cocoa products of consistent quality to respond to the demand.

However, standardisation of quality over time is difficult to achieve as cocoa beans are mostly produced in non-EU countries by a large number of independent farmers, resulting in remarkable fragmentation and heterogeneity of batches where socio-political instability and the impact of climate change negatively affect cocoa production and farm survival (Boeckx, Bauters, & Dewettinck, 2020; Danezis, Tsagkaris,

Brusic, & Georgiou, 2016; Lahive, Hadley, & Daymond, 2019; Medina, Perestrelo, Silva, Pereira, & Câmara, 2019; Somarriba et al., 2021). Objective and robust tools to trace the authenticity and the quality stability of cocoa products are therefore necessary to support the continuity year-to-year in an ever-increasing global demand at the industrial level.

Different instrumental analytical methods have so far been applied to sustain cocoa-origin authentication based on bioactive components or flavour-related compounds (Febrianto & Zhu, 2022; Kumar et al., 2022; Magagna et al., 2017; Marseglia, Musci, Rinaldi, Palla, & Caligiani, 2020; Johanningsmeier et al., 2016). Spectroscopic and spectrometric-based methods have recently been reported for cocoa, and more in general, for food and fingerprinting analysis (Gutiérrez, 2017; Medina, Perestrelo, et al., 2019; Scavarda et al., 2021; Acierno, Alewijn, Zomer, & van Ruth, 2018). In food authentication fingerprinting and profiling strategies are becoming widely used and accepted to monitor food integrity (Ballin & Laursen, 2019; Cavanna, Righetti, Elliott, & Suman, 2018; Cuadros-Rodríguez, Ortega-Gavilán, Martín-Torres, Arroyo-Cerezo, & Jiménez-Carvelo, 2021; Medina, Pereira, Silva, Perestrelo, & Câmara, 2019; Suman, Cavanna, Sammarco, Lambertini, & Loffi, 2021). Indeed, food authentication is often based on determining the degree of similarity of the fingerprints of some diagnostic chemical

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characteristics of an unknown sample compared to a representative reference sample (Cuadros-Rodríguez, Ruiz-Samblás, Valverde-Som, Pérez-Castaño, & Casado, 2016). Consequently, fingerprinting and profiling approaches require a suitable number of pure and authentic samples to establish a representative database of the "genuine" food population (Danezis et al., 2016). This step is known as food 'Identitation' and, if correctly carried out, affords a reliable food authentication (Cuadros-Rodríguez et al., 2016). Furthermore, flavour quality identitation requires analytical methods able to provide diagnostic detailed profiles correlated with the sensory features that can be monitored and quantified for an objective assessment in quality control (QC) (Bressanello et al., 2018, 2021; Magagna et al., 2017; Perotti et al., 2020; Stilo et al., 2021). Chromatographic hyphenated platforms combined with artificial intelligence are the tools of choice to obtain significant information encrypted in complex data sets to delineate significant trends and/or data structures. In addition, they are of crucial importance in reliably assessing associations and/or correlations between the chemical composition of foodstuffs and food extracts to determine compliance with quality and legal standards for the authentication of samples (Bressanello et al., 2017, 2018; Rodionova & Pomerantsev, 2020).

In this study, HS-SPME-GC–MS combined with machine learning tools has been applied to the identitation of a set of cocoa samples of different origins. Untargeted fingerprinting capability and at the same time the potentiality of profiling in identitation of origin were tested as well as the chemical information they can provide from the volatilome of cocoa beans and liquors. This information is important on an industrial level for the development of an artificial smelling machine to check the quality of incoming beans and liquors and to fulfil the flavour reference driver in product design. Knowledge of the origin-key chemical markers, their quantification and determination of their odours impact correlating with specific sensory qualities are the basis for modelling artificial intelligent tools that support/complement sensory evaluation in the screening of incoming cocoa batches (Nicolotti, Mall, & Schieberle, 2019; Squara, Caratt, Fina, Liberto, Spigolon, Genova, & Cordero, 2023). Therefore this study is the first step (point 1) of a wider project that aims 1) to investigate the flavour profiles of different origins and define their chemical-sensory identity card; 2) to guarantee consistent supplies from different origins over time independently on climate change and geo-political conflicts; and 3) to ensure final standard quality of the cocoa products from a sensorial and qualitative point of view by blending different origins through objective assessments using chemical analysis and machine learning when supply difficulties may occur.

2. Experimental

2.1. Cocoa samples and reference compounds

A total of 160 samples of unroasted beans (n = 85) and liquors (n = 75) (*Theobroma cacao* L.) of cocoa were analysed. Samples were provided by Soremartec Italia s.r.l. (Alba, Italy) and were from 4 origins: Colombia (COL), Cameron (CAM) and West Africa (WA, a blend of Ghana, Ivory Coast and Nigeria) all from *Forastero* variety, Ecuador (ECU) from CCN51 Clone, harvested in 2015 and 2016 by different local regional farmers. Cocoa was of commercial grade (beans size "standard" based on counting test under guidelines of the Federation of cocoa commerce) (FCC, 2018). Liquors are obtained from fermented and drying beans that undergo to debacterization stage followed by an industrial roasting process, winnowing and grinding, and finally pressing to have cocoa mass or cocoa liquor. Samples were ground in liquid nitrogen to obtain a homogeneous powder and then stored at −80 °C until analysis. Pure reference standards for identity confirmation (key-aroma compounds (§) and informative volatiles) as reported in Table A1, normal alkanes (*n*-alkanes *n*-C9 to *n*-C25) for Linear Retention Index (I^T_S) determination and α-thujone as internal standard (ISTD) were from

Merk (Milan, Italy). An α-thujone solution (ISTD) at a concentration of 1000 mg/L was prepared in degassed sunflower seed oil and stored in a sealed vial at −18 °C.

2.2. Automated head space solid phase micro extraction: Sampling device and analysis conditions

Automated Headspace Solid Phase Microextraction (auto-HS-SPME) was performed using a Combi-PAL AOC 5000 (Shimadzu, Milan, Italy) online integrated with a Shimadzu QP2010 GC–MS system provided with Shimadzu GC–MS Solution 2.51 software (Shimadzu, Milan, Italy). SPME fibre: Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) df 50/30 μm – 2 cm length from Millipore (Bellefonte, PA, USA). Fibres were conditioned before use as recommended by the manufacturer. The standard-in-fibre procedure was adopted to pre-load the ISTD (α-thujone) onto the fibre before sampling (Wang, O'Reilly, Chen, & Pawliszyn, 2005). 5.0 μL of ISTD solution were placed in a 20 mL glass vial and submitted to HS-SPME at 50 °C for 20 min, stirring speed 350 rpm. Cocoa powder (1.00 g) was weighed in the headspace glass vials (20 mL) and submitted to automated HS-SPME sampling. After ISTD loading on the fibre, the SPME device was exposed to the headspace of cocoa for 40 min at 50° at a shaking speed of 350 rpm.

GC–MS analysis- Chromatographic conditions: analyses were run on a Shimadzu QP2010 GC–MS system, controlled by Shimadzu GC–MS Solution 2.5SU1 software (Shimadzu, Milan, Italy) Injector temperature: 240 °C, injection mode: splitless; carrier gas: helium, flow rate: 1 mL/min. Sampled analytes were recovered by thermal desorption into the split/splitless (S/SL) injection port of the GC system at 240 °C for 5 min. GC column: SolGelwax (100 % polyethene glycol) 30 m × 0.25 mm d_c × 0.25 μm d_f Trajan Analytical Science (Ringwood, Australia). Temperature program, from 40 °C (2 min) to 200 °C at 3,5°C/min, then to 240 °C (5 min) at 10 °C/min. MSD conditions: ionization mode: EI (70 eV); temperatures: ion source: 200 °C; quadrupole: 150 °C; transfer line: 260 °C; scan range: 35–350 amu. Each sample was analyzed in triplicate.

2.3. Analytes identification and data analysis

Untargeted fingerprinting data elaboration, Principal Component Analysis (PCA), Partial Least Square Discriminant Analysis (PLS-DA) and regression analysis, was carried out with Pirouette® (Comprehensive Chemometrics Modelling Software, version 4.5–2014) (Infometrix, Inc. Bothell, WA). Heat map and hierarchical clustering were obtained with Morpheus (https://software.broadinstitute.org/morpheus/). Targeted analysis was on 55 compounds identified by matching their EI-MS fragmentation patterns (NIST MS Search algorithm, version 2.0, National Institute of Standards and Technology, Gaithersburg, MD, USA, with Direct Matching Factor > 900) with those stored in commercial (NIST2014 and Wiley 7n) and in-house databases, and/or with reference standards available in the laboratory. Linear retention indices (I^T_S) were taken as a further parameter to support identification and experimental values were compared to tabulated values (Table A1 in Appendix A). Profiling data elaboration and the Kruskall-Wallis test were performed with XLSTAT version 2021.4.1 statistical and data analysis solution. (Addinsoft (2022), New York, USA. https://www.xlstat.com/en).

3. Results and discussion

3.1. Untargeted fingerprinting approach and origin identity

Untargeted fingerprinting approaches are time-saving and can be exploited in screening for authentication and very recently in detecting food fraud thanks to platforms with highly informative power (Greño, Plaza, Luisa Marina, & Castro Puyana, 2023; Suman et al., 2021). The increasing difficulties in supplying high-quality raw materials due to the climate and social instability crisis have prompted the large-scale industry to look for new sources or new strategies to maintain or improve

although no individualist markers have so far been detected in cocoa/-cocoa products (Dunkel et al., 2014). Moreover, several non-odorant compounds can significantly affect the sensory profiles through the so-called odour synaesthesia (Chambers & Koppel, 2013; Prescott, 2015). Therefore, gas chromatographic analysis of cocoa volatilome benefits from detailed profiling, which can then be used for correlations with sensory data and characterisation of the chemical odour code. From these points of view, it became interesting to evaluate the chemical information provided by beans and liquors, because, in general, the panel (s) taste the liquors to evaluate the cocoa flavour. Despite the further processing that the liquors undergo compared to the beans, the analytical patterns of volatiles are similar when describing the origin of samples, albeit to different quantitative extents, as shown in the heat map in Fig. 2. Fig. 2 shows the chromatographic profiles in plan view, where L and B indicate liquors and beans, and the volatile distribution depending on the origins. The clustering of the origins is based on the agglomerative average linkage method and the one-minus Pearson correlation distance on the zeta score-adjusted data matrix. The colour scale ranges from red (volatile substances in larger quantities) to blue (volatiles in smaller quantities). The representative abundance of the volatilome of both beans and liquors for the different origins under study is reported on the tree chart in Fig. A1 in Appendix A.

Normalized responses of the main volatiles in beans and liquors were transformed by logarithmic function and pre-processed by Pareto scaling before PCA analysis. Explorative data elaboration by PCA of the target volatiles displays similar power in the origin discrimination both for beans and liquors on the first 3 principal components (PCs) confirming an existing correlation between the two patterns of volatiles Fig. 3 a and b.

Beans from different countries of origin have different ratios between volatiles, which mainly reflect the origin and primary processing in the country of origin (mainly fermentation and drying), as we can see from the boxplots of the different chemical classes in Fig. 4. The content of

volatiles of cocoa beans from Colombia was somewhat lower than in the other countries of origin. Factors such as the system of storage of the pods and its duration influence the pH, titratable acidity and temperature reached during fermentation affecting enzyme activities and flavour development (Afoakwa, Paterson, Fowler, & Ryan, 2008; Aprotosoaie et al., 2016). Further steps in the processing of the beans (e. g. roasting, alkalisation, batch blending, milling) affect the overall profile, especially the abundance and ratios of volatiles. The relative content of volatiles was highest in cocoa liquors from Ecuador as also reported by Lie et al. (Liu et al., 2017). The processing of the beans leads to a quali-quantitative "flattening" of some volatiles between the origins, e.g. aromatics and aldehydes, while it increases the differences in esters, alcohols, terpenes, ketones and acids although with a different trend depending on the origins. For example, acids decrease more in COL and WA, but less in ECU and CAM liquors, and they change their distribution with the origins. Acids are generated in the fermentation process, in particular, acetic acid whose content is in strict relationship with the fermentation conditions (Schwan & Wheals, 2004). Esters are an important class of volatiles formed from amino acids in unroasted beans that impart fruity and floral aromas whose content is negatively influenced by roasting (Aprotosoaie et al., 2016). Liquors from Ecuador contain higher amounts of esters compared to other origins (Fig. 5).

However, supervised methods are needed to classify and predict origin based on chemical characteristics (Casale, Malegori, Oliveri, Liberto, Rubiolo, Bicchi, & Cordero, 2020). The main objective of supervised machine learning is to model data to obtain qualitative prediction models, and thanks to dedicated algorithms to define target compounds that are characteristic of the origin. The Variable Importance in Projection (VIP), for example, is a well-known method to find significant variables in complex data sets. It enables to select variables from the PLS model by calculating the VIP scores for each chemical variable and removing those with a VIP score below a predefined threshold (default = 1) (Medina, Perestrelo, et al., 2019).

Fig. 2. Heat-map of the target volatiles in describing the different origins; clustered data by Z score and Pearson correlation. Colour rendering from low (blue) to high amount (red). L: target liquor volatiles, B: target bean volatiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
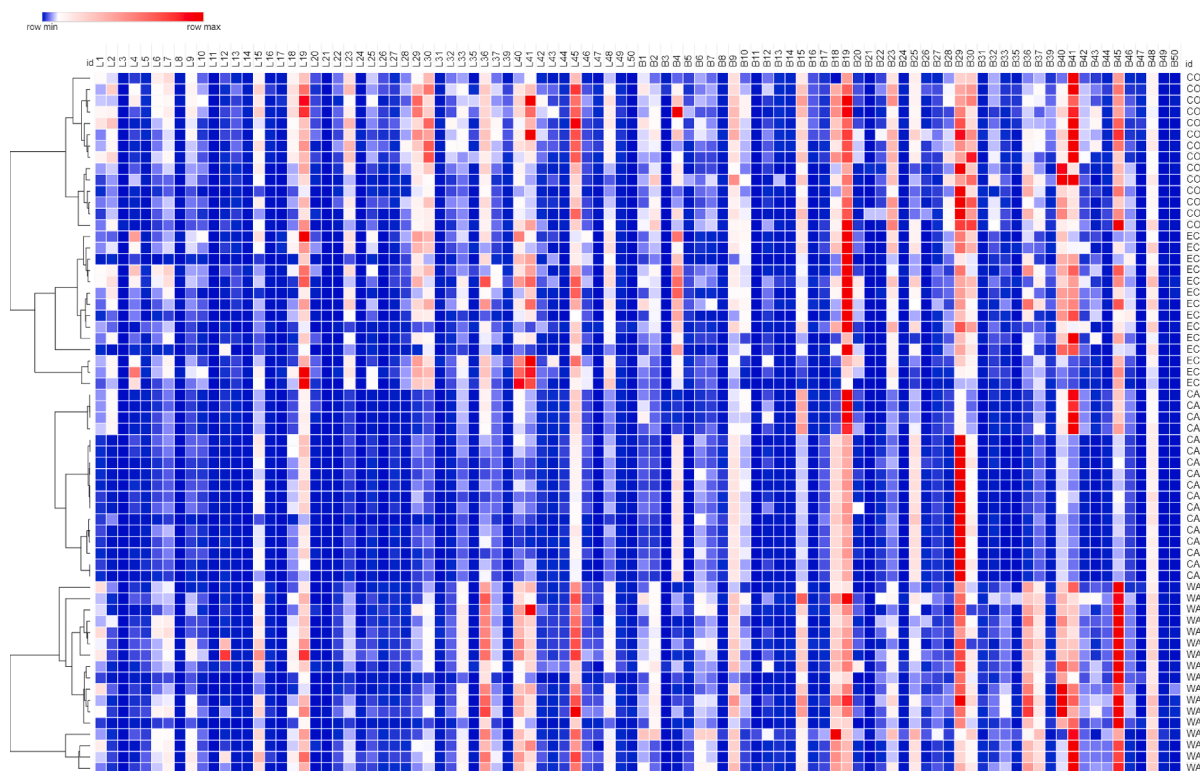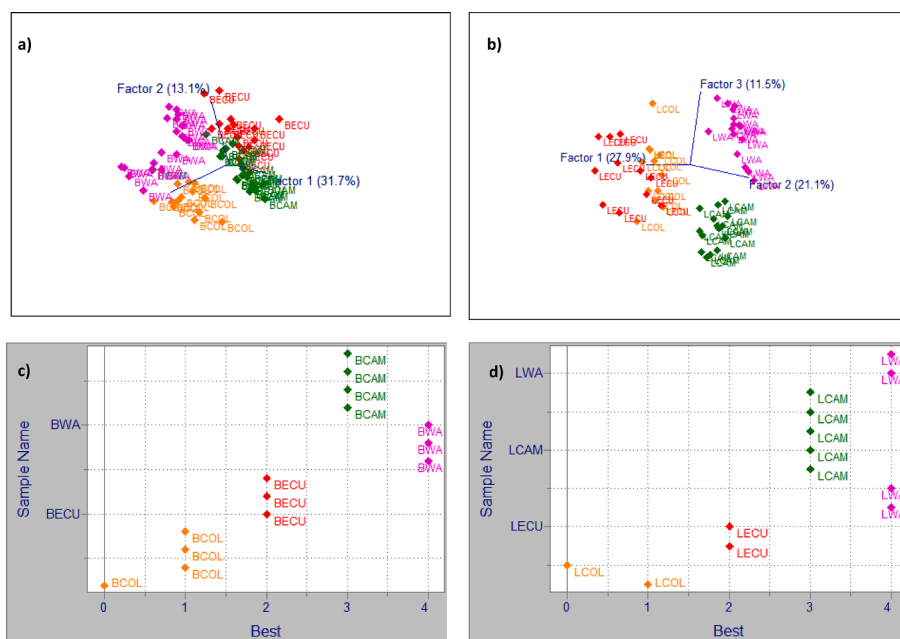
Fig. 3. Targeted metabolites profiles from PCA a) and b) scores plots of beans and liquors respectively, c) and d) are the results from the OPLS-DA Class Predicted plot for beans and liquors. Pre-process: autoscale. Class 1: COL, Class 2: ECU, Class 3: CAM, Class 4: WA.
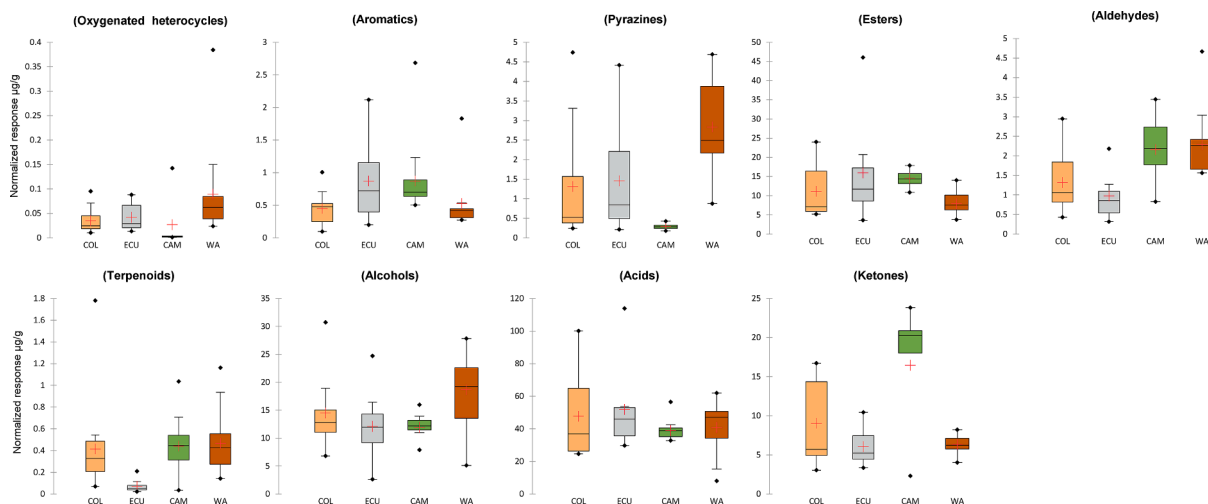

Fig. 4. Box-plots of the comparison of the composition of the beans by representative chemical classes.

Orthogonal signal correction Partial Least Square Discriminant Analysis (OPLS-DA) is a supervised approach giving excellent results in the origin classification and identification of components potentially associated with both beans and liquors with a classification rate of over 92 % (i.e. 92.86 % for beans and 92.31 % for liquors) Fig. 3 c and 3 d. The 20 % of samples were selected by the Kennard-Stone algorithm within each sample set (i.e. beans and liquors) to create the external test sets. OPLS-DA was built on the training data sets (65 samples for beans and 60 samples for liquors), log10 transformed, autoscaled and cross-validated (10 CV). The models were then applied to the external test sets (i.e. 14 beans and 13 liquors) (Fig. 3 c and d). The results of origin classification were similar to the untargeted fingerprinting approach but with a better classification rate in particular for liquors.

Fig. 6 a and b reports the VIP graphics from the OPLS-DA both from beans and liquors profiling, the scale is from 0 and 1.8, but only those higher than 1 were selected as important for origin classification. Several VIP (VIP > 1) compounds are in common between beans and liquors: Acetophenone, Benzaldehyde, 3-Methylbutanoic acid, 2,3-

Butanediol, Tetramethylpyrazine, 2-Heptanone, Styrene, 4-Heptanol Fig. 6 a and b. These volatiles are important variables in the origin classification and share similar relative abundance in beans and liquors of the same sample (Fig. A2 in Appendix A). Benzaldehyde, Tetramethylpyrazine and 3-Methylbutanoic acid have been also evidenced as aroma-active compounds in liquors for WA and Cameroon besides other origins from Liu et al., while Isobutyl acetate, Benzaldehyde, Trimetylpyrazine, Butanal, 2 and 3-methyl-Butanal, β-Myrcene and 2-heptanone were indicated by Marseglia et al. as characteristic compounds both of unroasted and roasted beans although at different extents, higher in roasted beans (Liu et al., 2017; Marseglia et al., 2020). Tetramethylpyrazine whose odour quality is described as *cocoa, chocolate - coffee*, comes from Maillard reactions that can also occur in the beans during fermentation and drying due to the temperatures reached in this stage. However, roasting is the main factor contributing to its formation in liquors. WA cocoas result richer in Tetramethylpyrazine compared to other origins both in beans and in liquors and generally higher in liquors (Fig. 6c). WA cocoa beans, and more in general from African regions,
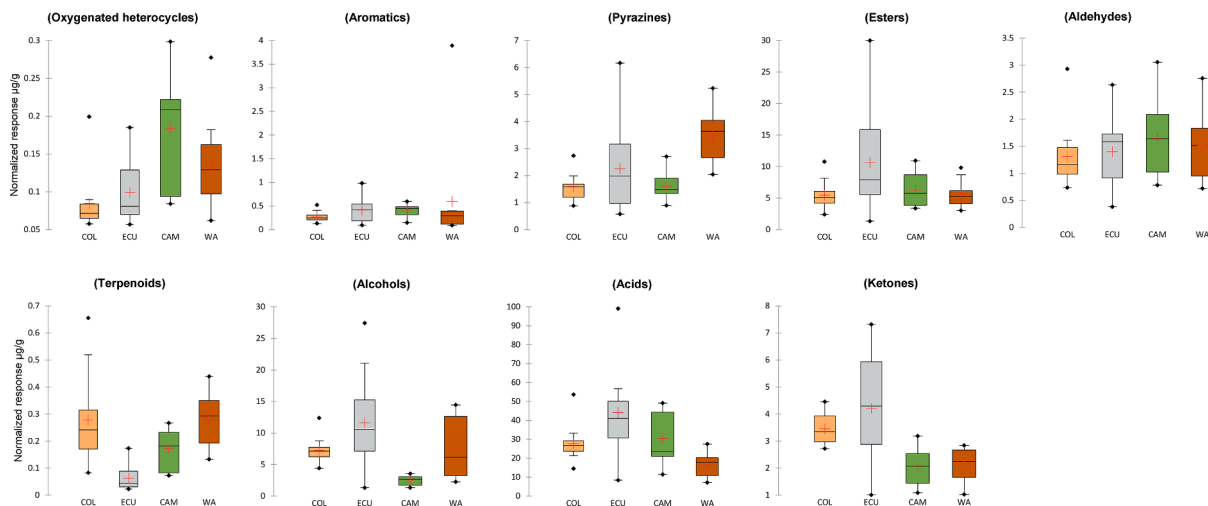
Fig. 5. Box-plots of the comparison of the composition of liquors according to representative chemical classes.
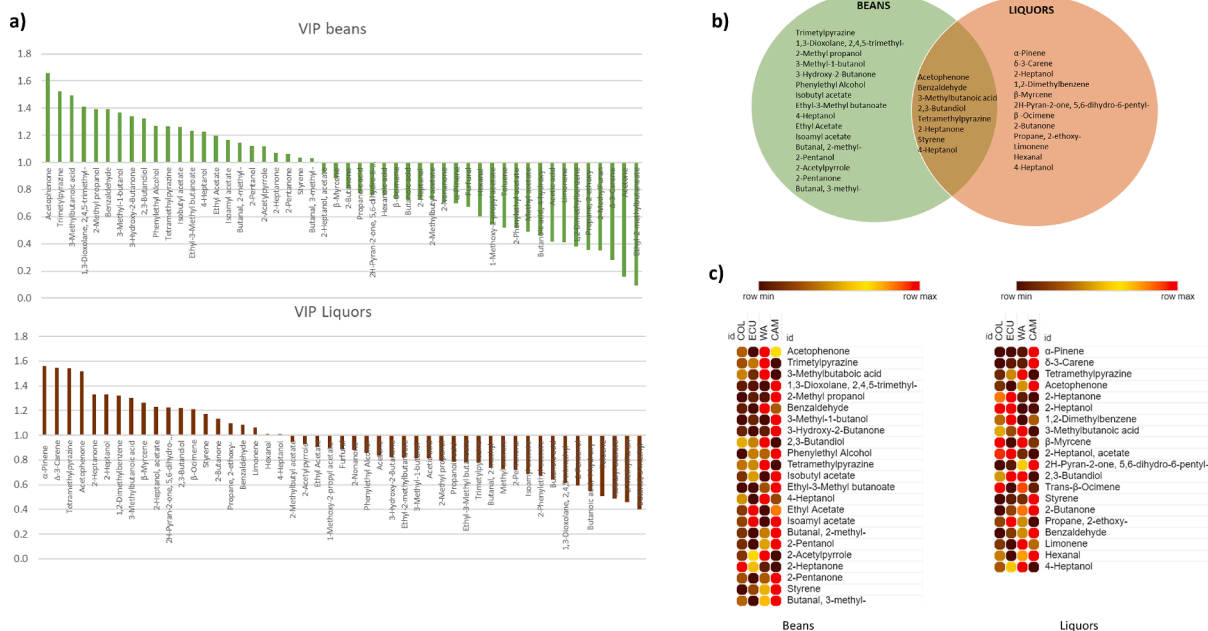

Fig. 6. VIP (higher than 1) from the OPLS-DA from the OPLS-DA on targeted profiling for beans and liquors respectively in a), in b) Venn diagram of volatiles describing the origins with VIP > 1 from the OPLS-DA of beans and liquors, c) heat map of the VIP distribution between the origins.

result richer in Acetophenone responsible for the *floral* and *sweet* notes among the ketones, and in 3-Methylbutanoic acid (*sweaty, rancid*), Trimethylpyrazine (*cocoa, roasted nutty*), Benzaldehyde (*bitter almond*), 4-Heptanol (*alcoholic*) (Fig. 6c), confirming the observation of Marseglia et al. although their relative distribution, compared to other origins, are not respected for all compounds in liquors (Marseglia et al., 2020; Tuenter et al., 2020).

For example, some 2-Heptanol (*citrus*), 2-Heptanone (*sweet, fruity*) and 2-Heptanol acetate (*fruity*) tend to be characteristics of liquors from South American countries (Fig. 6c), which agrees with Marseglia et al. but contrast with the findings of Tuenter et al. who compared West Africa bulk with Ecuadorian fine-flavour cocoas (Marseglia et al., 2020; Tuenter et al., 2020).

Besides these volatiles, a group of monoterpenoids contribute significantly to the definition of the origin of liquors, the main ones of them being α-pinene (*woody, resinous-piney*), δ-3-carene (*sweet citrus*), *trans*-β-ocimene (*floral*), β-myrcene (*balsamic, must, spicy, sweet*),

limonene (*citrus-like*) and linalool (a key-odorant of cocoa conferring a *floral, leafy, and tea-like* note) Fig. 6 and b. Although these volatiles are associated with odour description very far from the cocoa flavour and have a high odour threshold, they do affect its overall aroma (Liu et al., 2017; Ullrich et al., 2022). In addition, some of the above monoterpenoids are chiral compounds and their enantiomers can have different odour thresholds and/or sensory descriptions, and can differently influence the cocoa aroma, thus making of particular interest the determination of their enantiomeric distribution in cocoa products (Cagliero, Sgorbini, Cordero, Liberto, Rubiolo, & Bicchi, 2017). These components derive from the monoterpenoid biosynthetic pathway and, during fermentation, they are present in higher amounts in beans than in pulp (Chetschik et al., 2018). Monoterpenoids have not yet been studied in depth in cocoa, but they can play a marked role in defining/monitoring i) the origin through the liquor volatilome, ii) the different fermentation processes, and iii) more in general, the post-harvest processing in the countries of origin (Afoakwa et al., 2008; Aprotosoaie

et al., 2016). For instance, Calva-Estrada et al. have recently shown that limonene has an important role in the composition of the dark-chocolates aroma profile from Latin American cocoas (Calva-Estrada, Utrilla-Vázquez, Vallejo-Cardona, Roblero-Pérez, & Lugo-Cervantes, 2020).

4. Conclusions

The volatilome of cocoa beans and liquors is a very rich source of information to define the origin of incoming raw material. Both fingerprinting (untargeted) and profiling (targeted) strategies allow to decipher the information contained in the complex dataset resulting from HS-SPME-GC–MS analysis, and the cross-validation of the results enables to discriminate between the origins of both beans and liquors studied with suitable classification models. Fingerprinting and profiling approaches provided comparable classification performance. The untargeted approach can be used for rapid screening for quality control and for a preliminary evaluation of incoming lots, but it does not provide chemical information. On the other hand, profiling is more time-consuming but provides detailed chemical information on the samples, enabling the definition of the chemical marker(s) of origin and is very useful, in particular, if carried out in connection with the sensory profile. Targeted methods have allowed the identification of several origin-related key odorants in both bean and liquor samples. Furthermore, the targeted approach enables the establishment of correlations with the chemical pathways involved with the volatile formation. This information can be linked to the soil and climate characteristics, and in-situ processing of the world region under consideration before the next steps of the chocolate production chain. However, volatile fingerprinting to be reliable and to be used as a robust authentication tool for origin requires a large number of representative samples, including the variability of cocoa products, to build up a representative cocoa chemical identity card correlated to the sensory profile. The information provided by the untargeted approach can thereby be sufficient for the basic authentication of cocoa volatiles for routine control quality to standardize the quality year-to-year. On the other hand, the targeted approach is mandatory when a certification of origin is requested, as it requires an identity card detailing the chemical and sensory characteristics of the origin itself to fulfil the flavour reference driver in product design.

CRediT authorship contribution statement

Eloisa Bagnulo: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Camilla Scavarda:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Cristian Bortolini:** Conceptualization, Resources, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Chiara Cordero:** Methodology, Writing – review & editing, Funding acquisition. **Carlo Bicchi:** Writing – review & editing. **Erica Liberto:** Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Cristian Bortolini is presently employees of Soremartec Italia s.r.l Alba, Italy. Academic authors declare no conflict of interest.

Data availability

The authors do not have permission to share data.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113199.

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