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Untargeted LC–HRMS profiling followed by targeted fractionation to discover new taste-active compounds in spirits

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ABSTRACT

Taste is a key driver of food and beverage acceptability due to its role in consumers' pleasure. The great interest that natural food and beverages now arouse lies notably in the complexity of their taste, which in turn is related to a wide range of taste-active compounds. Going beyond the classic divide between targeted and untargeted strategies, an integrative methodology to spirits was applied. Untargeted profiling of several cognac spirits was implemented by LC–HRMS to identify compounds of interest among hundreds of ions. A targeted fractionation protocol was then developed. By using HRMS and NMR, dihydrodehydrodiconiferyl alcohol was identified and described for the first time in spirits and oak wood. It was characterized as sweet at 2 mg/L in two matrices and was quantified in spirits up to 4 mg/L. These findings demonstrated how this methodology is relevant and effective to discover new taste-active compounds.

Key words: untargeted approach; metabolomic; LC–HRMS; taste-active compounds; sweetness; quantitation.

1. Introduction

Beyond satisfying physiological needs, the consumption of food and beverages can provide pleasure to consumers thanks to their smell, taste and texture. For instance, enjoying a glass of spirits can be one of the delights of life, especially when it is old. It is well established that the value and the quality of cognac, whisky or rum significantly increase with their age. From a molecular point of view, such sensory enjoyment depends on a wide range of volatile and non-volatile compounds. Deciphering the molecules responsible for the taste of natural food and beverages has been a great challenge for scientists not only in their quest for knowledge but also for commercial reasons. A better understanding of the origin of taste in food products can result in the more accurate monitoring of their production and consequently better preservation of their identity and diversity.

For several years, the search for taste-active compounds has been based on the bio-guided purifications commonly used in pharmacognosy (Peters et al., 2010). In such studies, sensory analysis was used to perform the fractionation of various foods and drinks (Frank et al., 2005; Hufnagel & Hofmann, 2008; Spreng & Hofmann, 2018). For over 10 years, this approach has been implemented in our laboratory to isolate taste-active compounds from oak wood extracts, seeds and wine (Cretin et al., 2019; Marchal et al., 2011; Sindt et al., 2016). The success of this inductive approach is based on the choice of raw extract, the reliability of the sensory test, and on the access to various complementary analytical techniques. However, wine and spirits are complex matrices containing thousands of compounds, so separation efficiency combined with potential sensory interactions during the tasting of fractions are limitations of this targeted strategy.

Untargeted analysis has been the subject of numerous developments for studying natural products (Hoffmann et al., 2014). Metabolomics is an emerging and developing field for the chemistry of natural substances. High-performance liquid chromatography–mass

spectrometry (HPLC–MS) and nuclear magnetic resonance (NMR) are the main techniques used to investigate non-volatile molecules in these approaches (Wolfender et al., 2019). More particularly, high-resolution mass spectrometry (HRMS) is very efficient thanks to its sensitivity, its resolution and its wide dynamic range. Since these techniques generate a large amount of data related to all the compounds detected in a sample, bioinformatics tools are required (Blaženović et al., 2018). The objective is to highlight the significant information contained in the data collected.

Untargeted differential analysis has already been applied in enology to grapes (Son et al., 2009), oak wood extracts (Gougeon et al., 2009), wines (Gil et al., 2020) and spirits (Kew et al., 2017). While high-performance liquid chromatography has proven to be an effective method for the analysis of spirits and has contributed to a better understanding of their chemical composition (Collins et al., 2014; Kew et al., 2017), metabolomic approaches have recently shown great potential for their study (Garcia et al., 2013; Stupak et al., 2018). Most of these analyses were carried out to discriminate one or several cooperage parameters in order to authenticate spirits or simply to study the matrix (Møller et al., 2005; Roullier-Gall et al., 2020). While global analysis can produce conclusions only of a descriptive nature, its use coupled with tools allowing screening appears promising for the discovery of new active substances (Cabral et al., 2016; Nothias et al., 2018). Moreover, an exclusively untargeted metabolomic approach has limitations since it does not take sensory aspects into consideration.

Cognac is produced by distilling fermented grape juices mainly of the Ugni-blanc variety (*Vitis vinifera Trebbiano*), then leaving the spirit to age in oak barrels for a period of at least two years to several decades. This period of maturation is particularly important for spirits since they are devoid of non-volatile molecules at the beginning of aging. With aging the product becomes unique, gaining in flavor and revealing its color (Piggott, 1983). Thus,

aging in oak barrels plays a key role in the sensory perception of spirits (Cantagrel et al., 1992). Several phenomena can induce physicochemical and sensory modifications (Piggott, 1983). In particular, extractables are released from oak wood and insoluble macromolecular compounds such as lignin are degraded, followed by the release of the degradation products of lignin. In addition, spirits undergo many chemical reactions and some extractables lead to the formation of new chemical species. A recent study showed that the diversity of compounds in aged spirits is greater than in the wood itself (Roullier-Gall et al., 2018). This finding suggests that in spirits aged in barrels, various compounds natively present in the wood co-exist with molecules newly formed during aging. Several studies have focused on the composition of non-volatiles present in spirits but only a few have focused on taste-active compounds belonging in particular to the family of lignans, triterpenes and coumarins (Gammacurta et al., 2019, 2020; Winstel et al., 2020; Winstel & Marchal, 2019). Better knowledge of the composition of spirits would help to explain why the taste of cognac improves with aging in barrels. The combination of non-targeted and targeted approaches would throw light on this issue.

The present study attempted to provide a molecular explanation for the increase in sweetness observed in old spirits and, in doing so, to propose a new methodology for the discovery of taste-active compounds. For this purpose, untargeted metabolomic profiling by HRMS was carried out on several "eau-de-vie" of Cognac of different vintages. Then, statistical analyses (principal component analysis (PCA), k-means clustering and Pearson correlation) were performed to assess the overall structure of the metabolomic data and to select compounds of interest. Their combined use with separative techniques such as liquid–liquid extraction, centrifugal partition chromatography (CPC) and preparative-HPLC aimed to isolate new taste-active compounds that can be further identified, characterized for their sensory properties and quantitated in spirits.

2. Material and methods

2.1. Chemicals

Ultrapure water (Milli-Q purification system, Millipore, France) and HPLC-grade solvent (acetonitrile, ethanol, ethyl acetate, heptane, methanol and 1-butanol from VWR International, Pessac, France) were used for sample preparation and compound purification. Methyl *tert*-butyl ether (MTBE) was purchased from Scharlab S.L (Barcelona, Spain). HPLC–HRMS chromatographic separations were performed with LC–MS grade acetonitrile, deionized ultrapure water and formic acid (Optima, Fisher Chemical, Illkirch, France).

2.2. Samples

For the metabolomic approach, a set of spirits (Table S1, Supplementary Data), supplied by Rémy-Martin, was used. Ten different vintages (1970, 1973, 1990, 1993, 1995, 2000, 2005, 2008, 2010 and 2015) of "eau-de-vie" of cognac from the same distillery and having undergone similar aging conditions were tested. The samples were not commercial cognac but "eau-de-vie" still in barrels. They were matured in used barrels (350-L coarse-grain oak barrels). For each year, a sample was collected from five different barrels (except for 1970 and 1973 for which only four replicates were available).

An oak wood extract (25 g) was macerated in 250 mL of a hydroethanolic solution (50:50, *v/v*) at room temperature for three days in order to perform targeted LC–HRMS screening. The spirit sample C-32 (Table S1, Supplementary Data), and a white wine aged 8 months in oak barrels (Rhône Valley, 2010, 14% vol. alc.) were also used.

Oak wood used to isolate targeted compounds was supplied by the cooperage company Seguin-Moreau (Merpins, France). It was sampled in April 2017 from a batch of staves used to make barrels. The botanical species was assigned to *Quercus petraea* according to the method described by Marchal et al (2016). The staves were air-dried for two years according

to the cooperage process. They were then reduced to sawdust by the barrel manufacturer (Seguin Moreau, Merpins, France).

For quantitative analysis, Alc-diHDDC was assayed in the same spirits as those described above for the metabolomic approach. The second set of spirits consisted of 28 commercial spirits aged in oak wood (with 12 cognacs, 5 whiskies, 4 grape brandies, 4 bourbons and 3 rums). The spirit samples were reduced to approx. 8% alcohol and then filtered at 0.45 µm. The dilution factor applied was taken into account when calculating the final concentration.

2.3. HPLC Analysis

2.3.1. *Metabolomic approach*

For the non-targeted profiling approach, the HPLC appliance consisted of a Waters Acquity I-Class UPLC system (Waters, Guyancourt, France). For liquid chromatography separation, a C18 column was used as the stationary phase (BEH C18, 2.1 mm × 100 mm, 1.7 µm particle size; Waters). The mobile phases were water containing 0.1% formic acid (Eluent A) and acetonitrile with 0.1% formic acid (Eluent B). The flow rate was set at 400 µL/min and the injection volume was 2 µL. Eluent B varied as follows: 0 min, 5% (curve: initial); 0.5 min, 5% (curve: 6); 10.5 min, 100% (curve: 6); 14 min, 0% (curve: 11); 16 min, 0% (curve: 11).

2.3.2. *Targeted fractionation and quantitation*

For the screening and quantitative analysis, the HPLC appliance consisted of an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and an Accela U-HPLC system with quaternary pumps.

Liquid chromatography separation was performed on a C18 column (Hypersil Gold 2.1 mm × 100 mm, 1.9 µm particle size; Thermo Fisher Scientific), with water containing 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) as mobile phases for screening analysis. The flow rate was 600 µL/min and eluent B varied as follows: 0 min, 10%; 1.0 min, 10%; 5.0

min, 50%; 5.3 min, 98%; 6.0 min, 98%; 6.15 min, 10%; 7 min, 10%. For the quantitative analysis, the column, the flow rate and the solvents used were the same as for screening analysis. However, eluent B varied as follows: 0 min, 5%; 1.6 min, 5%; 6.3 min, 50%; 7.0 min, 98%; 8.0 min, 98%; 8.1 min, 5%; 9.3 min, 5%. The injection volume was 5 µL for each method.

2.4. HRMS

For the metabolomic approach, a Q-Exactive Plus mass spectrometer equipped with a heated electrospray ionization (HESI-II) probe (both from Thermo Fisher Scientific, San Jose, CA) was used. For the screening and quantitative analysis, an Exactive Orbitrap mass spectrometer was equipped with a heated electrospray ionization (HESI-II) probe (both from Thermo Fisher Scientific). Each mass analyzer was calibrated with Pierce®ESI Positive Ion Calibration solutions each week (Thermo Fisher Scientific).

The spectrometric and ionization parameters were not similar for the three types of analysis and are summarized in Table 1. Optimization of voltages, gas values, and temperatures applied for ion transfer and ionization was performed in positive mode.

For the non-targeted profiling approach, the data-dependent MS^2 events were performed on the four most intense ions detected by full-scan MS.

For quantitative and screening analysis, detection of Alc-diHDDC (compound A) was based on the theoretical exact mass of the most intense ion, the fragment ion at *m/z* 331.1531, and its retention time at 5.57 min. Peak areas were determined by automatic integration of extracted ion chromatograms built in a 3-ppm window around the exact mass of the $C_{19}H_{23}O_5^+$ ion.

Quan and Qual Browser applications of Xcalibur (version 3.0, Thermo Fisher Scientific) were used to process all the data.

2.5. MS Data Processing

After analyzing the spirit samples on the Q-Exactive Plus, Thermo RAW files were exported to the open source software package MZmine 2 (2.38 version) for data processing (Pluskal et al., 2010). The protocol implemented includes five major steps: peak detection (mass detection), chromatogram builder, peak deconvolution, isotope suppression and peak list alignment. Each chromatogram was deconvoluted into individual chromatographic peaks with the "baseline cut-off" algorithm, which recognizes each chromatographic peak that has an intensity above a given minimum level (2.10^3) and spans over a given minimum time range (0.01–1.00 min). Chromatograms were deisotoped using the isotopic peaks grouper algorithm with a *m/z* tolerance of 10 ppm and a RT tolerance of 0.1 min. Peak alignment was performed using the Join aligner method. The last step was a "gap-filling" procedure, which reduces the number of missing values. All the parameters are described in Supplementary Data (Table S2). The generated peak lists were exported as comma-separated values (CSV) files to Excel 2016.

2.6. Statistical analyses

All the statistical analyses were carried out using the open-source software R Statistical (Foundation for Statistical Computing, Vienna, Austria). Results were interpreted by one-way analysis of variance (ANOVA), using *vintage* as factor. The values were previously transformed into logarithms in order to improve the homogeneity of the variances, a condition for applying the ANOVA. The normal distribution of the data was tested using the Shapiro-Wilk test. PCA was performed on normally distributed data. K-means clustering was also established for data classification (package *ClassDiscovery*). Pearson correlations were then carried out ($\alpha < 0.05$, correlation coefficient $r > 0.8$) and allowed the creation of groups of

compounds with a similar evolution depending on the vintages. The Pearson coefficient is an index reflecting a linear relationship between two continuous variables. A positive correlation indicates that the two variables vary together in the same direction. It was calculated according to the following formula:

$$r = \frac{\sum_{i=1}^{n}(X_i - \bar{X})(Y_i - \bar{Y})}{\sqrt{\sum_{i=1}^{n}(X_i - \bar{X})^2}\sqrt{\sum_{i=1}^{n}(Y_i - \bar{Y})^2}}$$

All *p*-values were corrected for multiple testing using the Benjamini-Hochberg correction (False Discovery Rate).

2.7. Purification protocol and structural elucidation

2.7.1. Extraction of oak wood

The oak wood material (600 g) was macerated in 6 L of H_2O/EtOH solution (50:50; *v/v*) at room temperature for 2 weeks, under an inert atmosphere and protected from light. Filtration (0.45 µm) was used to remove wood sawdust and particles. Then, it was pressed for 15 minutes at a pressure of 6 bar using a pneumatic press. The solution containing soluble wood compounds (5.5 L) was concentrated *in vacuo* by evaporation of ethanol and, partly, water. The aqueous extract (900 mL) was washed twice with 450 mL of *n*-heptane. This aqueous layer was then extracted with MTBE (6×500 mL). The combined organic layers were evaporated *in vacuo*, suspended in water, and freeze-dried twice to obtain brownish powders of MTBE (1.8 g) and aqueous (20.1 g) pre-purified extracts. They were stored under air- and light-protective conditions.

2.7.2. Centrifugal partition chromatography fractionation

CPC was performed on a Spot prep II LC coupled with an SCPC-100+1000 (Armen Instrument, Saint-Avé, France), both controlled by Armen Glider Prep V5.0 software. A 100-mL rotor (11 discs, 90 twin cells of 0.101 mL per disc) was used to fractionate samples

weighing less than 1 g and a 1-L rotor (21 discs, 72 twin cells of 0.555 mL per disc) was employed for samples weighing more than 1 g. The solvent was pumped into the column by a 4-way quaternary high-pressure gradient pump. The samples were introduced into the CPC column *via* an automatic high-pressure injection valve. All the experiments were conducted at room temperature, with UV detection at 254 and 280 nm.

The choice of an appropriate biphasic system of solvents was based on the study of the partition of extract compounds in both phases according to the procedure described by Marchal et al. (2011). The partition coefficient, Kd, was calculated as the ratio of the solute area in each phase. On this basis, various systems were tested, and the MTBE extract was fractionated using the Arizona solvent systems H (*n*-heptane/EtOAc/MeOH/H_2O, 1:3:1:3 *v/v/v/v*) and then by setting up a gradient on the Arizona-G system (*n*-heptane/EtOAc/MeOH/H_2O, 1:4:1:4 *v/v/v/v*). Separation was performed by one CPC run of 1.8 g injection. Experiment was carried out in ascending mode at 1200 rpm with a flow rate of 30 mL/min for 145 min for the elution phase and 50 mL/min for 45 min for the extrusion. The Spot Prep fraction collector was set to 25 mL/min. Every 10 CPC tubes, an aliquot (200 µL) was taken, evaporated, dissolved in 1 mL of H_2O/MeOH 95:5 and analyzed by LC–HRMS. Ten fractions F-I to F-X were constituted on the basis of their similar chromatographic profile, after being combined, evaporated *in vacuo*, suspended in water and freeze-dried.

The CPC fraction F-II (333.8 mg) was rich in targeted compound **A**. A second CPC experiment was carried out on this fraction using the Arizona-K system (*n*-heptane/EtOAc/MeOH/H_2O, 1:2:1:2 *v/v/v/v*). It was performed in ascending mode at 2300 rpm with a flow rate of 5 mL/min for 70 min for the elution phase and 10 mL/min for 30 min for the extrusion. Seven fractions were obtained (F-II_1 – F-II_7).

2.7.3. Preparative liquid chromatography

Preparative HPLC analyses were performed using a Waters Prep 150 LC including a 2545 Quaternary Gradient Module, a 2489 UV/Visible detector and a 2424 ELSD detector (Waters, Guyancourt, France). Final purification of the targeted compound **A**, which was present in the CPC fraction F-II_5 (54.3 mg), was achieved by preparative HPLC using columns chosen after LC–HRMS tests. Separation was performed using a SunFire Prep C18 OBD (19 mm × 250 mm, 5 µm particle size; Waters) equipped with a SunFire preparative C18 guard cartridge (20 × 19 mm, 5 µm particle size; Waters). The mobile phase was a mixture of ultrapure water containing 0.1% formic acid (Eluent A) and acetonitrile with 0.1% formic acid (Eluent B). The flow rate was set to 20 mL/min. Eluent B varied as follows: 0 min, 20%; 7.4 min, 20%; 37 min, 25%; 39 min, 98%; 44.2 min, 98%; 45.3 min, 20%; 52 min, 20%.

Aliquots (20 mg) of CPC fractions were dissolved in 400 µL of H_2O/MeOH 60:40, 0.45 µm-filtered and introduced manually into the system. UV detection was carried out at 280 nm and chromatographic peaks were collected manually just after the detector. The pure compound solution was evaporated *in vacuo* to remove acetonitrile and freeze-dried to obtain a white amorphous powder (3.1 mg).

Dihydrodehydrodiconiferyl alcohol (**A**, Alc-diHDDC): white amorphous powder; $[\alpha]_D^{25} - 2.6$ (c = 0.1, MeOH); HRMS *m/z* 361.1491 $[M + H]^+$ ($C_{20}H_{25}O_6^+$, 1.3 ppm); ^{1}H NMR (methanol-d_4, 600 MHz) and ^{13}C NMR (methanol-d_4, 150 MHz), see Table S3, Supplementary Data.

2.7.4. NMR experiments

NMR experiments were conducted on a Bruker UltraShield® System Avance 600 NMR, spectrometer (^{1}H at 600.27 MHz and ^{13}C at 150.95 MHz) equipped with a 5 mm TXI probe. All 1D (proton) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC) spectra were acquired at 300 K in methanol-d_4, which gave as reference the solvent signal (^{1}H δ 3.31; ^{13}C δ 49.00). Data analysis was performed with the NMRnotebook software (NMRTEC, Illkirch-Graffenstaden, France).

2.7.5. Polarimetry

Optical rotation was measured with a JASCO P-2000 polarimeter. The sodium emission wavelength was set at 589 nm and the temperature at 25 °C. The pure compound was dissolved in MeOH, and the final value resulted from a mean of 10 measurements of 10 s each.

2.8. Sensory characterization

Gustatory analysis was performed in a dedicated room, under normal daylight, and at room temperature (around 20°C). Pure compounds were tasted by five experts in winetasting, at 2 mg/L in water (eau de source de Montagne, Laqueuille, France), as well as in a non-oaked "eau-de-vie" adjusted to 40% *v/v*. Experts described the gustatory perception (bitterness, sourness, sweetness) of the targeted compound using the vocabulary of winetasting and were asked to evaluate the intensity on a scale from 0 (not detectable) to 5 (strongly detectable). The tasters were informed of the nature and risks of the present study and were asked to give their consent to participate in the sensory analyses. Even though the compound was purified from commercial oak wood used for cooperage and observed in spirits, the panelists were advised not to swallow but to spit out the samples after tasting.

2.9. Quantitation of purified compound in spirits

2.9.1. Preparation of calibration solution

A stock solution of Alc-diHDDC (1 g/L) was prepared in ethanol. Successive dilutions of this solution were performed with ultrapure water to provide calibration samples (10 mg/L, 5

mg/L, 2 mg/L, 1 mg/L, 500 µg/L, 200 µg/L, 100 µg/L, 50 µg/L, 20 µg/L, 10 µg/L, 5 µg/L, 2 µg/L and 1 µg/L).

2.9.2. Validation method for quantitating Alc-diHDDC

The method for quantitating Alc-diHDDC in spirits was validated by studying linearity, intraday repeatability, sensitivity, trueness and specificity. The sensitivity of the LC–HRMS method was determined according to the approach described by De Paepe et al. (2013). The limit of detection (LOD) of a molecule is defined as the lowest concentration at which a reproducible and reliable signal is observed. Moreover, it had to be different from a blank performed under the same conditions. The five lowest calibration points (1, 2, 5, 10 and 20 µg/L) were injected five times. Limit of quantitation (LOQ) corresponded to the lowest concentration of the molecule that can be quantitatively determined by the method, with an accuracy (recovery of back-calculated concentrations) higher than, e.g., 90% and a precision lower than, e.g., 10%. A calibration curve was created by plotting the areas for each concentration level *versus* the nominal concentration. Linearity was assessed by deviations of each back-calculated standard concentration from the nominal value and by correlation coefficient (R^2). Three intermediate calibration solutions (100 µg/L, 1 mg/L and 10 mg/L) were injected into five replicates to define intraday precision. The calculation of relative standard deviation (RSD%) was carried out. Trueness was verified by calculating the recovery ratio (between expected and measured areas) from two analyzed samples of "eau-de-vie" (EDV-1; EDV-2). They were spiked with calibration solution (addition of 10 µg/L, 200 µg/L and 10 mg/L) of Alc-diHDDC. Injections of the same two samples (10 µg/L and 10 mg/L) for five consecutive days were performed to estimate interday repeatability. Specificity was evaluated by assessing retention time repeatability and mass accuracy.

3. Results and discussion

3.1. Metabolomic Fingerprinting of cognac spirits

An untargeted analysis by HRMS was performed on a series of “eau-de-vie” of cognac of 10 different vintages from 2015 to 1970. The 48 samples, which were collected in the same distillery and aged in similar conditions, were not commercial cognac but “eau-de-vie” still aging in barrels (Table S1, Supplementary Data). For each vintage, a sample was collected from four or five different casks to limit variations due to cask effect.

The main objective of this untargeted profiling was to observe potential differences in composition between the young and the old vintages of "eau-de-vie" of cognac by a global analysis. To this end, the chromatographic conditions were set up in order to optimize the separation of the compounds present in these spirits. The spectrometric parameters were also adapted to enhance the sensitivity of a maximum of non-volatile molecules. After the HPLC–HRMS analysis, the data were processed according to the metabolomic strategies implemented for the research of natural products (Wolfender et al., 2019), with the use of MZmine 2 software. Thanks to these treatments, a peak list of 47,243 positive ions was obtained between *m/z* 100 and 1500, then filtered into a peak list of 1,293 ions having an associated data-dependent MS^2 spectrum. These results revealed the chemical diversity and complexity of cognac, which is consistent with previous studies on different spirits (Kew et al., 2017; Roullier-Gall et al., 2020). The rest of this work was carried out on this set of ions. It is important to note that the data reduction essential for statistical analyses is also likely to cause data loss, which could constitute a major limitation to this type of approach.

A metabolomic study sometimes involves a differential analysis of fingerprints, with semi-quantitative expression of the results (Boudah et al., 2013). Here, a PCA of the data was carried out using the peak areas of the 1,293 positive ions highlighted by the ANOVA. The two factorial axes of the positive mode explained 79.7% of the data set (Figure 1, A). The

vintage effect was clearly significant on the first axis (that explained 71.5% of the total variation of the data set). Moreover, ANOVA showed that the vintage effect was significant ($p < 0.05$) for 1,036 compounds out of 1,293 (80%). K-means clustering followed by Pearson correlations allowed us to divide the compounds into different groups showing similar trend to evolve according to the vintages. Of the 1,036 compounds detected in positive mode and significantly influenced by the vintage, 854 were assigned to a group among the five created (Figure 1, B). Groups 2 and 3 represented 82% of the compounds whose concentrations were significantly influenced by the vintage, which were more abundant in older vintages. Group 5 was composed of 80 compounds whose contents increased during 23 years of aging and then slowly decreased. Group 1 contained 10 compounds present in higher concentration in young "eau-de-vie" of cognac and then less abundant during aging. Group 4 was composed of 60 molecules whose contents were relatively constant over the first 30 years of aging and decreased significantly thereafter. The group evolutions (Figure 1, B) suggested that most of the compounds (703/854) were present at higher concentrations in old spirits and that there were more compounds in old vintages. This observation was consistent with the dry extract contents obtained for the 10 cognac vintages: higher in the old vintages than in the young ones (Table S1, supplementary data).

The creation of statistical groups revealed the presence of a wide variety of compounds in these "eau-de-vie" of cognac. For most of them, the concentrations were higher in aged spirits, while the opposite was observed for less than 20% of the compounds. Two hypotheses could explain these observations: either the compounds were released from wood to spirits continuously during aging, or the compounds were neoformed by chemical reactions in the spirits.

3.2. Untargeted metabolomic analysis to select and purify molecules of interest

In general, the last step in a metabolomic analysis aims at tentatively or formally identifying the compounds associated with the detected signals. This step is the most hazardous because ESI ionization often produces more than one ion for a given metabolite. These ions can correspond to fragments produced in the source, pseudo-dimers, adducts or their isotopic derivatives that have passed through the filters of the treatment. Moreover, identification of the compounds generally requires the combined use of several analytical techniques such as mass spectrometry, NMR, UV or IR. During an LC–MS approach, formal identification is generally based on the similarity of the chromatographic retention time, the exact mass and the MS^n spectra between the studied compound and a standard or a compound present in spectral libraries. If some of these criteria are not validated, the characterization of the compound is not complete and the identification remains putative. Therefore, this tool cannot lead to the unambiguous identification of a new compound.

The identification of all the metabolites present in these "eau-de-vie" of cognac by dereplication was not undertaken because it is time-consuming and only a few non-volatile compounds are known to this day. Instead, the peak list obtained by the MZmine analysis and the data from the statistical analysis were used to target new natural products. We focused particularly on compounds that were formed during the aging of the spirits, since they were more likely to contribute to taste modifications and tried to purify them.

First, the creation of a table enabled us to classify the molecules according to their intensity in the spirits from 1970 to 2015 (data not shown). Subsequently, LC–HRMS screening of these molecules in this matrix and in oak wood extracts was investigated. Thanks to its mass measurement accuracy and separative performance, LC–HRMS is a powerful technique to screen complex matrices. The compounds of interest were selected according to two criteria: a significant abundance of the targeted compound and a strong increase in concentrations in old vintages. Despite the large number of chromatographic peaks, a

compound combining the previously defined criteria was targeted for the rest of the study: compound **A** with a nominal mass of 331. It was indeed classified among the compounds with the greatest intensity difference between the spirits of 1970 and 2015, with an increase of 3,142%, and it is one of the 475 compounds present in Group 2 (Figure 1, B). By LC–HRMS screening, compound **A** was also detected in an oak wood extract (Figure S4, Supplementary Data). Extracted ion chromatograms (XICs) were built by targeting the positive ion at *m/z* 331.1531 within a window of 5 ppm around its theoretical *m/z*. In addition, the HRMS/MS spectra revealed the same fragment signals in both matrices with retention times of 4.02 minutes (Figure S4, supplementary data). Therefore, compound **A** is present natively in wood. Its content in aged spirits may therefore be explained by its continuous release during maturation in barrels.

For reasons of availability of materials, its purification protocol was carried out using extracts of oak wood. Its sensory properties as well as its structural characterization were established.

3.3. Isolation and identification of compound A in spirits

*3.3.1. Extraction and purification of compound **A** from oak wood*

To reproduce cellar conditions, a hydroethanolic solution was used to perform the solid–liquid extraction of wood sawdust of *Quercus* petraea. Thus, the extracted molecules present in this solution had to be the same as those released in an "eau-de-vie" aged in barrels. After removal of ethanol, the first step consisted of sequential liquid–liquid extractions using MTBE to remove compound **A** from the aqueous solution. The chemical complexity of the enriched MTBE extract required a further fractionation by CPC. Systems containing relatively apolar solvents from the Arizona range were tested for this fractionation. A gradient from Arizona-H system to Arizona-G allowed the best partition of the MTBE extract between the two phases.

At the end of this experiment, the tubes were analyzed by LC–HRMS and pooled according to their composition to give fractions. After solvent evaporation and freeze-drying, 10 fractions (noted F-I to F-X) were obtained as powder in variable quantities. Fraction II (F-II) was rich in compound **A** so it was kept for the rest of the fractionation. It was still chemically complex and contained many compounds. Therefore, a second CPC experiment was performed. Different solvent systems from the Arizona range were tested in order to choose the one allowing optimal fractionation. The Arizona-K system was selected and seven new fractions were obtained (noted F-II_1 to F-II_7).

After freeze-drying, fraction F-II_5, which was richer in compound **A**, was submitted to preparative HPLC with UV detection. A preliminary injection of 5 mg showed that the chromatograms presented a refined profile with only a few peaks detected both in ELSD and in UV at 280 nm. Thus, an appropriate gradient was chosen and the entire fraction F-II_5 was fractionated by successive injections. The peak corresponding to compound **A** was collected manually just after the UV detector to give 3.1 mg of a white amorphous powder after acetonitrile removal and freeze-drying.

3.3.2. Structural elucidation of compound A

The HRMS spectrum of compound **A** exhibited the most abundant positive ion at *m/z* 331.1531 ($C_{19}H_{23}O_5^+$). This corresponded to a fragment linked to the quasi-molecular ion $[M+H]^+$, which was observed at a very low intensity whatever the ionization parameters. The protonated ion at *m/z* 361.1649 suggested an empirical formula of $C_{20}H_{24}O_6$ (with an accuracy of 0.3 ppm). This molecule must be fragmented in the ionization source. Thus, the most detected species corresponded to a neutral loss of 29.9961 compared to the ion at *m/z* 361, characteristic of a methoxy group (CH_2O). Moreover, the positive ion at *m/z* 313.1426 ($C_{19}H_{21}O_4^+$), could correspond to a dehydration in relation to the ion at *m/z* 331. The HRMS

spectrum also presented an ion at *m/z* 383.1463 corresponding to a sodium adduct $[M+Na]^+$ ($C_{20}H_{24}O_6Na^+$) (Figure S5, Supplementary Data).

By comparing the data obtained in LC–HRMS, the interpretation of ^1H and ^{13}C NMR spectra (Figure S6, Supplementary data) and the data from the literature, compound **A** was identified as dihydrodehydrodiconiferyl alcohol (Alc-diHDDC). The determination of its structure from NMR data is not detailed here since it has already been described in the literature (Hanawa et al., 1997). However, it was necessary to specify its relative configuration. It has been demonstrated that the coupling constant between H-7 and H-8 (equal to 6.5 Hz here) cannot unambiguously distinguish the *cis*/*trans* diastereoisomers with a skeleton of this type (García-Muñoz et al., 2006). The interpretation of the ^1H-^1H ROESY spectrum showed a correlation between H-7 (δ_H 5.50, *d*) and H-9 (δ_H 3.75, *m*). This observation indicates that the protons H-7 and H-8 were in the *trans* position, as detailed in the literature (García-Muñoz et al., 2006) (Figure 2).

This neolignan was highlighted for the first time in 1970 in *Silybum marianum* (Weinges et al., 1970) and has been identified in several natural species such as *Pinus massoniana* (Suga et al., 1993), but never in the *Quercus* genus. It has also been found in maple syrup and maple sugar (Liu et al., 2017) but no publication mentions its presence in spirits. Thus, this study allowed the first identification of Alc-diHDDC in oak wood and cognac. Moreover, a white wine aged in oak barrels was screened by LC–HRMS. The presence of Alc-diHDDC was also assessed in this matrix which appeared consistent with its release from oak wood (Figure S4, supplementary data).

3.3.3. Gustatory properties of targeted compound A

This study is the first to assess the gustatory properties of Alc-diHDDC. It was dissolved in water and in a non-oaked “eau-de-vie” at 2 mg/L, and the taste of each solution was characterized in comparison to the same water / “eau-de-vie” as a reference. QTT I was used

as a sweetness standard since its sensory properties have already been characterized (Marchal et al., 2011). In water, Alc-diHDDC exhibited a sweet taste. On a 0–5 scale representing relative sweetness intensity assessed as a consensus between the five panelists, Alc-diHDDC scored 4/5 and QTT I was assessed as 2/5. Alc-diHDDC was also dissolved in non-oaked "eau-de-vie" to study its influence on the taste balance of spirits. The control "eau-de-vie" was scored 0/5 for sweetness and 5/5 for the perception of burning. As a reference, "eau-de-vie" spiked with QTT I (2 mg/L) was described as sweeter (4/5) and less burning (1/5). Alc-diHDDC also modified the taste of the "eau-de-vie" by decreasing the perception of burning (2/5) and increasing that of sweetness (4/5).

The results suggested that Alc-diHDDC developed a sweet taste at 2 mg/L with an intensity close to that of QTT I, whose gustatory detection threshold is relatively low for non-volatile compounds (i.e. 590 µg/L in wine (Marchal, 2010)), which is much lower than that of glucose, i.e., 4 g/L (Ribéreau-Gayon et al., 2006).

3.4. Quantitation of Alc-diHDDC in spirits by LC–HRMS

3.4.1. Method validation

Preliminary accuracy tests had shown that there was no significant matrix effect for this compound in spirits. Therefore, absolute quantitation was carried out by preparing calibration solutions of Alc-diHDDC in ultra-pure water. The limits of detection (LOD) and quantitation (LOQ) were established at 2 µg/L and 5 µg/L, respectively. For a range from 5 µg/L to 10 mg/L, a quadratic calibration curve (1/x statistical weight) with a good correlation coefficient (R^2 of 0.996) was obtained. Moreover, accuracy was established, since recovery of back-calculated concentrations was higher than 90% at each method calibration level. Intraday repeatability (RSD%) for each concentration was lower than 9.3%. Injections of two spirits spiked with stock solutions were also performed. Recovery ratios ranged from 85 to 95%, which remained in accordance with common specifications (Guidance for Industry, 2018).

Moreover, interday repeatability was < 10% at 10 µg/L and < 7% at 10 mg/L. Consequently, these results established the repeatability and the trueness of the method applied to spirits. Analysis of the above samples revealed small variations in retention time (< 0.04 min) and a mass deviation lower than 0.3 ppm at various concentrations, guaranteeing the specificity of the method. All these results validated the LC–HRMS method to quantitate Alc-diHDDC in spirits (Table 2).

3.4.2. Application of method to quantitate Alc-diHDDC in spirits

Alc-diHDDC was quantitated in the samples of "eau-de-vie" of cognac used for untargeted metabolomic analysis (Table S7, Supplementary data). The concentrations presented in Figure 3 correspond to the mean values of the five replicates (Figure 3, A). The contents measured for Alc-diHDDC were higher in old vintages, until reaching an average concentration of 3.3 mg/L for the 1970 vintage. Thus, long aging in barrels seemed to promote the extraction of this compound. For 2008 and 2005 vintages, a high standard deviation was observed because of a high value in the series. For all the barrels, the aging conditions were similar, which suggests that these differences were due to heterogeneity in oak wood composition. Such variations have already been observed for volatile and non-volatile compounds (Marchal et al., 2016). These results were consistent with its relative quantitation (Figure S8, Supplementary Data) obtained from the untargeted metabolomic approach, for which the same trend was observed. More generally, these results confirmed the effectiveness and relevance of this approach.

Twenty-eight commercial spirits were analyzed to assess the range of Alc-diHDDC amounts in some cognacs, but also whiskies, rums, and other brandies, using the previously validated LC–HRMS method (Table S9, Supplementary Data). Error bars indicated the repeatability of the method. Alc-diHDDC was detected in all samples at concentrations ranging from 0.4 to 4.0 mg/L, with a mean value of 1.7 mg/L (Figure 3, B). The contents also varied according to

the type of spirit, with higher values in rums and bourbons than in cognacs (except for sample C-12) and brandies. The higher Alc-diHDDC concentration in the C-12 sample was correlated with longer aging in barrels than for the other cognacs analyzed. The differences in concentration between the spirits could be due to the botanical origin of the wood used for aging. Indeed, cognacs and brandies are generally aged in French sessile or pedunculate oak barrels, while bourbons are aged in American oak barrels. The potential influence of aging conditions on Alc-diHDDC concentrations in spirits needs to be clarified in further studies. Moreover, 9 of the 28 commercial spirits had concentrations of Alc-diHDDC greater than 2 mg/L (value used for tasting), which could suggest its sensory contribution to the sweetness of these spirits.

4. Conclusion

The aim of this work was to propose an efficient methodology for discovering new taste-active compounds in oak wood and oaked spirits. A graphical summary of this approach is presented in Figure 4. An untargeted metabolomic profiling by HRMS was implemented on "eau-de-vie" of cognac from ten vintages. Firstly, a purely descriptive approach was undertaken. After statistical analysis, thousands of compounds were observed in these spirits. Two categories of molecules emerged: those extracted from oak wood in spirits and those formed by chemical reactions in spirits. Among them, compound **A** (*m/z* 331) was chosen on the basis of two criteria including its high abundance in old vintages. After the development of a fractionation protocol using CPC and preparative HPLC, dihydrodehydrodiconiferyl alcohol (Alc-diHDDC) was identified and purified from oak wood extracts. Its presence in this matrix and in spirits has never been described until now. Sensory analysis revealed that Alc-diHDDC exhibits a sweet taste at 2 mg/L in water and in a non-oaked spirit. The development of a quantitative HPLC–HRMS method showed that some commercial spirits contained Alc-diHDDC at concentrations above this value. Even if these results established

the sensory relevance of the newly identified compound, the determination of its gustatory detection threshold will establish its impact. Future work will clarify the cooperage parameters likely to affect its concentration in oak wood, wines and spirits.

These results demonstrate the relevance and the effectiveness of combining untargeted differential analysis to highlight the molecules significantly affected by barrel aging, and a targeted fractionation protocol to isolate them. This work offers promising perspectives for the search for new natural products and could also be coupled with the inductive approach, guided by the gustatometric analysis conventionally used in our laboratory.

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Competing interests statement

The authors declare no competing financial interest.

Author contributions

Delphine Winstel: Conceptualization, Methodology, Investigation, Validation, Writing – Original Draft

Delphine Bahammou: Methodology, Validation

Warren Albertin: Conceptualization, Formal analysis, Writing – Review and editing, Visualization

Pierre Waffo-Teguo: Validation, Resources, Writing – Review and editing

Axel Marchal: Conceptualization, Validation, Writing – Review and editing, Supervision, Funding acquisition

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Abbreviations

PCA: Principal component analysis

Alc-diHDDC: dihydrodehydrodiconiferyl alcohol

LOD: limit of detection

LOQ: limit of quantitation

FIGURES AND TABLES

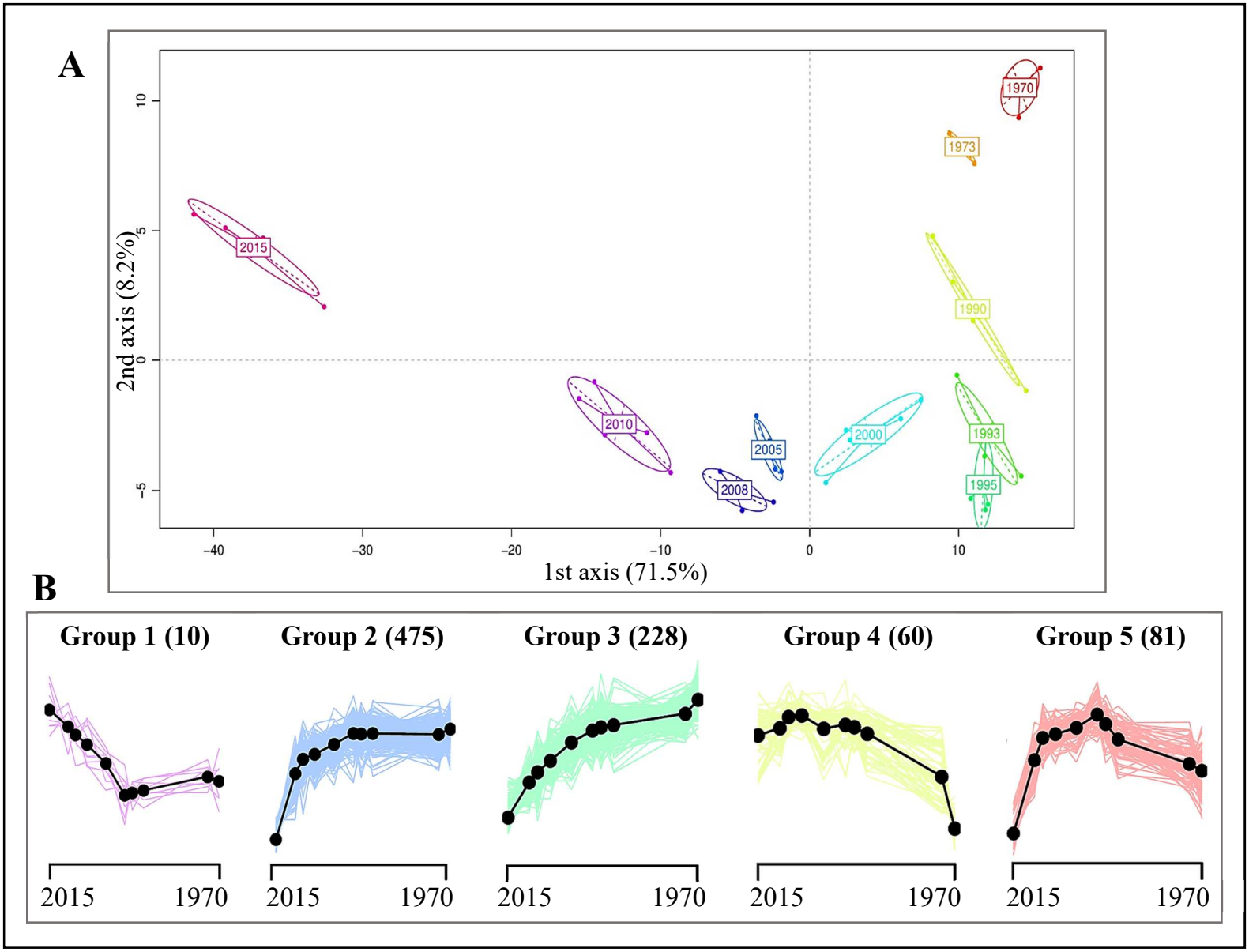

Figure 1: (A) PCA representation based on 48 "eaux-de-vie" of cognac (10 vintages, from 2015 to 1970) and (B) Representation of different groups of compounds according to their evolution in same samples.

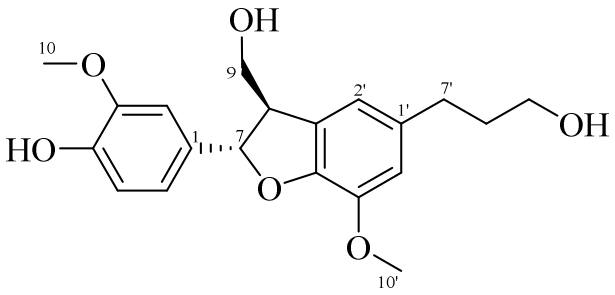

Figure 2: Chemical structure of compound A.

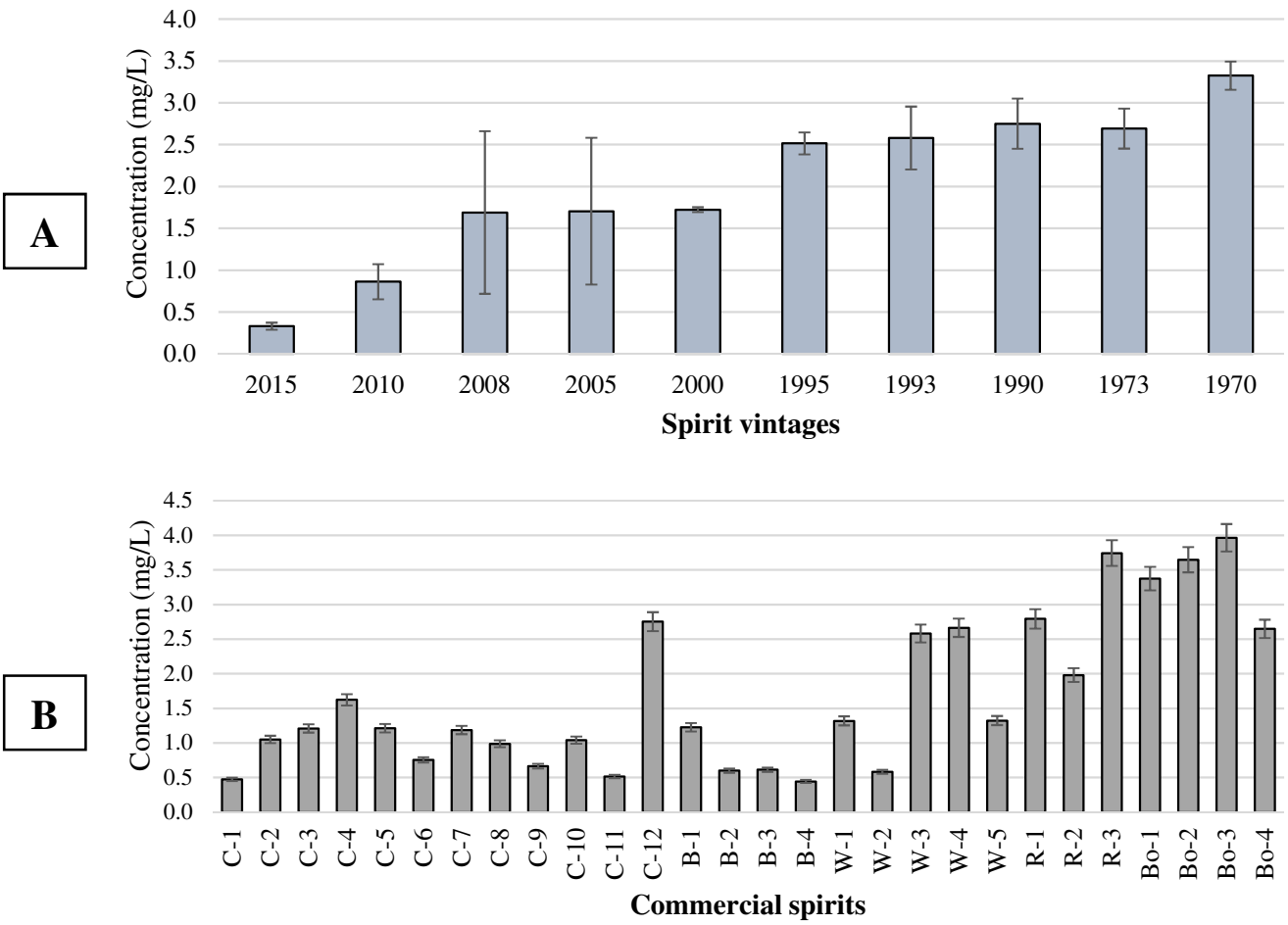

Figure 3: Concentrations of Alc-diHDDC in 10 vintages of "eau-de-vie" of cognac coming from same distillery (**A**) and in 28 commercial spirits (**B**).

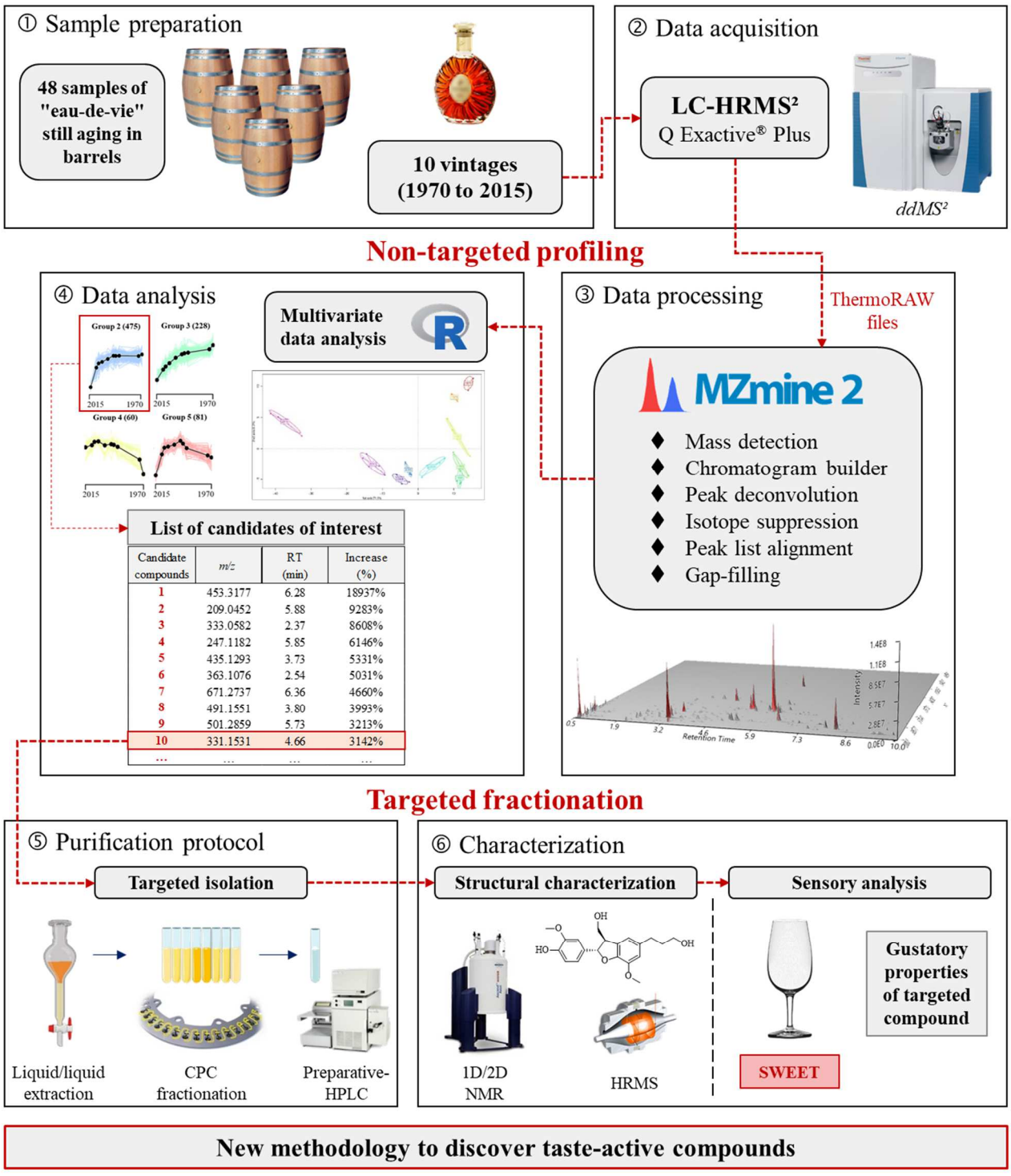

Figure 4: Schematic diagram of new methodology used for discovering taste-active compounds.

Table 1: Ionization and spectrometric conditions for HRMS analyses.

Ionization mode	**Positive**			
Mass Spectrometer	**Q-Exactive Plus**		**Exactive**	
Use	LC–MS^n Metabolomic approach		LC–HRMS Screening	LC–HRMS Quantitation
Mass scan	Full MS	dd-MS^2	Full MS	Full MS
Sheath gas flow [a]	48		70	60
Auxiliary gas flow [a]	11.25		15	15
Spare gas flow [a]	2.25		0	0
HESI probe temperature	400 °C		320 °C	300 °C
Capillary temperature	300 °C		350 °C	300 °C
Electrospray voltage	3.5 kV		3.5 kV	4.0 kV
S-lens RF level [b]	55		-	-
Capillary voltage	-		25 V	57.5 V
Tube lens voltage offset	-		120 V	140 V
Skimmer voltage	-		20 V	18 V
Mass range (in Th)	100–1500	200–2000	200–800	200–800
Resolution [c]	35,000	17,500	25,000	10,000
AGC value [d]	10^6 ions	10^5 ions	10^6 ions	3.10^6 ions
Maximum injection time	60 ms	50 ms	-	-
Fragmentation	-	28 eV	-	-

[a] Sheath gas, auxiliary gas and spare gas flows (all nitrogen) are expressed in arbitrary units

[b] S-lens RF level is expressed in arbitrary units

[c] Resolution $m/\Delta m$, fwhm at m/z 200 Th

[d] Automatic gain control

Table 2: Validation parameters for HRMS quantitation of Alc-diHDDC in spirits.

Parameters		**Matrix - Spirits**		
Sensitivity	**LOD (µg/L)**		**LOQ (µg/L)**	
	2		5	
Linearity and accuracy	**Working range**		R^2	
	5 µg/L–10 mg/L		0.996	
Specificity	t_R **variation**		**Mass accuracy**	
	0.04 min		0.3 ppm	
Repeatability and trueness		**Intraday repeatability**		
		10 µg/L	*200 µg/L*	*10 mg/L*
		9.3%	5.2%	5.3%
		Interday repeatability		
		10 µg/L	*10 mg/L*	
		9.8%	6.7%	
		Recovery		
		100 µg/L	*1 mg/L*	*10 mg/L*
	EDV-1	85%	95%	94%
	EDV-2	87%	91%	94%

Graphical abstract