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The impact of maturation on concentrations of key odour active compounds which determine the aroma of tequila

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ABSTRACT

Samples of non-mature and añejo (matured) tequila of the same brand/provenance were analysed using GC-MS and GC-O/AEDA to provide quantitative data on the most odour active compounds which contribute to the aroma of these spirits. Extracts of non-mature tequila was characterized by 26 odour-active regions, which included ethyl hexanoate, ethyl octanoate, 2-phenylethyl acetate- β-damascenone, isoamyl alcohol and octanoic acid as the most odour-active compounds (FD factor ≥ 6561). In contrast, extracts of the mature spirit showed 36 odour-active zones, where the compounds with the highest FD factors (6561) were ethyl hexanoate, ethyl octanoate, 2-phenylethyl acetate, isoamyl alcohol, phenethyl alcohol, guaiacol, 4-ethyl guaiacol, vanillin, cis/trans whisky lactones, β-damascenone and octanoic acid. The aromagram of mature tequila was thus differentiated from that of the non-mature spirit due to the presence of cask-extractive compounds and the increased FD factors of certain terpenes, higher alcohols and acetals. This study provides a comprehensive and quantitative understanding of changes in key odorants of tequila as a result of the maturation process and also reveals a further characterization of the likely impact of each compound on overall spirit flavour, in terms of odour activity values (OAVs).

KEYWORDS: *Agave tequilana;* volatile compounds; aroma extract dilution analysis: gas chromatography – mass spectrometry: tequila; non-mature; mature.

INTRODUCTION

Tequila is a distilled alcoholic beverage, with a unique flavour, produced from agave juice extract. Its production is strictly regulated such that only beverages produced from *A. tequilana* Weber blue variety cultivated in a protected region of Mexico can be labelled with the guarantee of origin (NOM-006-SCFI-2012). Tequila production involves multiple steps: (i) harvesting of the agave plant, (ii) steaming the head (core) to hydrolyzed fructans, (iii) milling the cooked agave heads to extract the juice, (iv) fermenting the extracted juice, (v) double distillation of the must to produce 'silver or white' tequila (blanco) and eventually (vi) ageing in white oak barrels to get 'rested' (reposado), product matured for a minimum of 2 months, 'añejo or extra aged' (añejo), product matured for at least 1 year and 'extra aged or ultra-aged' (extra añejo), product matured for at least 3 years, respectively (*1*).

Tequila flavour is well-known to be affected by multiple factors, such as the raw material, distillation conditions and ageing process (*2, 3*). However, fermentation is often viewed as the critical stage in tequila flavour formation (*4, 5, 6*). In some tequila distilleries fermentation is completed mainly by an inoculum of *Saccharomyces cerevisiae*, whereas in others the fermentation occurs spontaneously by a succession of different yeasts strains, which collectively contribute to the development of spirit flavour (*7*). Tequila flavour is complex, due to the great amount of volatile compounds present and the potential for interactions between these odourants, or for aroma to be moderated by physicochemical effects of the product matrix. A variety of chemical compounds, including acetals, aldehydes, ketones, alcohols, esters, terpenes and lactones are known to contribute to the complex flavour of tequila (*8*). Recently, Prado-Jaramillo et al (*9*) identified more than 327 compounds in 8 stages of Tequila's production, amongst which fermentation and distillation processes were the steps in which a higher number of volatile compounds were produced. Undoubtedly many of these compounds combine to define the flavour of the final product

and therefore the main characteristics that consumers associate with quality. A product with colour, flavour and more complex sensory characters is more likely to be the desired option of the consumer (*10*).

Although some studies have evaluated the chemical composition of tequila flavour in different stages of its production (*3*,*5*,*9*) few reports have focused on identifying which of these many compounds are most significant to the perceived flavour of tequila. In this context, gas chromatography olfactometry (GC-O) and aroma extract dilution analysis (AEDA) are significant techniques because they enable the odours experienced by a panellist to be traced to compounds eluting at the times aroma is experienced (*11*). GC-O thus enables the identification of odour-active volatiles from the bulk of odourless volatiles and AEDA then determines the relative odour potency of compounds present in a sample extract (by successively diluting the extract and identifying which aromas are detected orthonasally at the highest dilution factors) (*12*,*13*).

Maturation of spirits is known to change their flavour relative to fresh distillates, indeed that is one of the major objectives of the process. Key changes which occur during ageing include those in colour and flavour of the maturing spirit and a decline in both the volume and the alcoholic content (*14*). These changes are caused mainly by direct extraction of wood compounds, chemical reactions such as oxidation and hydrolysis, and evaporation of volatile compounds (*15*).

The aroma of most alcoholic beverages consists of hundreds of volatile compounds, however only a small proportion of the aroma compounds contribute significantly to the spirit flavour (*16*). These are the so-called key odourants, which maybe be characterized by GC-Olfactometry. Benn and Peppard (*9*) identified a total of 175 compounds in tequila, however only 60 odorants were considered to influence tequila flavour. Of these compounds five were determined to be the most powerful odorants of tequila: 3-methylbutanal, 3-methylbutanol,

β–damascenone, 2-phenylethanol, and vanillin. Lopez M. and Dufour J. (*17*) applied GC-O/CHARM to extracts of different classes of tequilas (blanco, reposado and añejo) obtained by LLE and confirmed the importance of phenylethanol, phenylethyl acetate, vanillin and an unknown compound in the overall aroma of these types of tequilas.

Whilst previous papers have reported flavour dilution factors from GC-O to identify the key odorants of different tequila samples, there have been no quantitative studies on the impact of maturation on the concentrations of the key components in tequila. Therefore, the main objective of this study was to use GC-O/AEDA and GC-MS to identify and quantify the key aroma compounds in extracts of non-mature and mature tequila of the same brand and provenance. This enables the chemistry of the maturation process and its impact on the aroma of aged tequila to be better understood. A further aim of the study was to evaluate the use of Solid Phase Extraction (SPE; *18*) alongside the more traditional liquid-liquid extraction (LLE) to see if this offered selectivity, or better sensitivity for particular groups of compounds. There are no prior reports in the literature of the application of SPE to study tequila flavour.

MATERIALS AND METHODS

Samples

Two commercial tequila samples (non-mature and mature) from the same batch were used to carry out each experiment and were sourced by the Scotch Whisky Research Institute (SWRI). The mature version corresponding to an 'añejo' tequila had been matured for 29 months (100% agave, 40% ethanol v/v) in barrels of American white oak of 53 gallon capacity.

Reagents and Chemicals

Standard aroma compounds (supplementary table 1) were supplied by Sigma-Aldrich (Poole, Dorset, UK), VWR International (Lutterworth, Leicestershire, UK), Fisher (Loughborough, Leicestershire, UK) or Merck (Merck KGaA, Darmstadt, Germany). All the other chemicals and regents used were of analytical grade.

Gas Chromatography analysis of spirit samples

Direct injection method

When performing GC analysis of extracts in dichloromethane, the solvent front of the chromatogram always obscures some highly volatile compounds in the sample. To analyse these compounds, such as methanol and acetaldehyde, we used a direct injection GC method, without prior extraction and concentration. This technique enabled the analysis of the major volatile compounds of spirit samples which include acetaldehyde, ethyl acetate, acetal, methanol, *n*-propanol, isobutanol, isoamyl acetate, *n*-butanol, amyl and isoamyl alcohols, ethyl lactate, acetic acid, and furfural. These compounds were analyzed by gas chromatography (GC) using a Bruker Scion 456-GC gas chromatograph, coupled to a flame ionization detector (FID). Spirit sample (0.5 μL) was injected into the chromatograph in split mode. Separations were performed using a ZB-Wax capillary column (60m× 0.25mm i.d., 1.0 μm film thickness; Phenomenex, Macclesfield, UK). Operating conditions were as

follows: carrier gas (helium) at 1.5 mL min^{-1}; initial oven temperature was 35ºC, then the temperature was raised at 6ºC/min to 120ºC and held for 0 min. Finally the temperature was increased at 100°C /min to 220°C and held for 4 min. Injector and detector temperatures were maintained at 200ºC and 210ºC, respectively. Quantification was achieved following normalization to the internal standard (250 μg mL^{-1}, *n*-pentanol) of eight diluted solutions in the range of 5 - 1250 μg mL^{-1}. Calibration curves reported a correlation coefficient (R^2) greater than or equal to 0.99 for each compound.

Extraction of volatile compounds from tequila

Volatile compounds were extracted from tequila samples using two different methods, to compare their efficiency and selectivity for different groups of compounds. These were: liquid-liquid extraction and solid-phase extraction, following methods previously described by Boothroyd et al. (*18*)

***Liquid-liquid extraction (LLE)*:** spirit samples (100 mL) were spiked with an internal standard (2-acetylthiazole; 10 μg mL^{-1}), diluted with 400 mL water and subsequently extracted with two successive aliquots of dichloromethane (200 mL) in a 1 L separating funnel. The two dichloromethane extracts were combined and dried with anhydrous magnesium sulphate before the concentration step. The solvent was then decanted into a conical flask, which was heated in a water bath at 37 °C. Finally, DCM extracts were concentrated down to 1 mL under a stream of nitrogen and transferred to a glass vial ready for GC analysis.

Solid-phase extraction (SPE): spirit samples (5 mL) were diluted with water (25 mL). An internal standard was added to the samples to achieve a final concentration of 10 μg mL^{-1} of 2-acetylthiazole, and then mixed and allowed to equilibrate for a period of 4 h. LiChrolut EN SPE columns (Merck KGaA, Darmstandt, Germany; sorbent bed 500 mg) were placed on a SPE vacuum manifold, conditioned with 8 mL methanol and equilibrated with 8 mL aqueous

ethanol (12 % ABV). Spirit samples were loaded onto individual columns and allowed to fully saturate the sorbent bed for 1 min before a vacuum was applied. Once the samples had been loaded, care was taken not to allow the bed to run dry until after the washing step, during which water (5 mL) was run through the cartridge. The sorbent bed was dried by applying a vacuum (10 kPa) for 30 min. Aroma compounds were eluted from the cartridge using dichloromethane (6 mL). Each spirit sample was extracted in triplicate in a randomized order. Dichloromethane extracts were dried with anhydrous magnesium sulphate (Sigma Aldrich) and concentrated to a final volume of 1 mL under a stream of nitrogen prior to GC analysis. Each spirit sample was extracted in triplicate using both extraction processes.

Gas Chromatography analysis of spirit extracts

Aroma extracts from tequila samples were analyzed by gas chromatography employing two methods of detection: i) mass spectrometry (MS): and simultaneous MS and ii) odour port evaluation using the technique of aroma extract dilution analysis (AEDA) (*19*).

Gas chromatography-Mass Spectrometry (GC-MS)

Analysis was performed following the conditions used by Boothroyd et al.(*18*) and included analysis of spirit extracts in dichloromethane using a ThermoScientific TraceGC Ultra with a DSQ II mass spectrometer and an AS 3000 Autosampler (Thermo Electron Corporation). Compounds were separated on a Zebron ZB-WAX column (30m× 0.25mm i.d., 1.0 μm film thickness; Phenomenex, Macclesfield, UK) starting at an oven temperature of 40 °C (1 min hold) followed by a ramp to 250 °C at 4 °C min^{-1}. The helium carrier gas flow rate was 1.6 mL min^{-1} and injection (1 μL; temperature 240 °C) was splitless. The transfer line from the oven to the mass spectrometer was maintained at 250 °C. The mass spectrometer was operated in full scan mode over the range *m/z* 35–250. Identification and quantitation of compounds was achieved using the Qual and Quan Browser applications of Xcalibur Software (Thermo Electron Corporation, Altrincham, Cheshire, UK). Identification was

based upon: (a) EI-MS library matching; (b) measurement and confirmation against literature sources of the linear retention index (LRI) against alkanes (C8 to C22); and, when possible, (c) confirmation of the retention time of authentic standards run under identical chromatographic conditions. Quantification was achieved following normalization to the internal standard (10 μg mL^{-1}, 2-acetylthiazole) of six diluted solutions in the range of 0.05 to 5 μg mL^{-1} containing the minor compounds listed in Table 1. However for major compounds such as isoamyl acetate, ethyl hexadecanoate, ethyl decanoate, 1-propanol, and 2-phenylethanol six diluted solutions in the range 2 to 64 μg mL^{-1} were prepared. Additionally for isobutanol six diluted solutions in the range of 20 to 640 μg mL^{-1} were prepared; and in the range of 50 to 1200 μg mL^{-1} for isoamyl alcohol respectively. Calibration curves reported a correlation coefficient (R^2) greater than or equal to 0.99 for each compound. Furthermore for those compounds that could not be quantified following internal standardization of the compounds listed in the supplementary table 1, the quantification was based upon: a) following normalization to the internal standard (10 μg mL^{-1} 2-acetylthiazole) and b) by using the calibration curve of the chemical compound with similar composition belonging to the same family of compounds.

Gas chromatography-Mass Spectrometry/Olfactometry (GC-MS/O).

GC-MS and odour port evaluation were carried out following the above conditions for GC-MS analysis. For odour port evaluation a splitter was fitted to the end of the ZB-Wax column (30m× 0.25mm i.d., 1.0 μm film thickness; Phenomenex, Macclesfield, UK), such that approximately half of the flow was diverted to an 'odour sniffing port' via a fused silica capillary passing within a heated transfer line, set at a temperature of 200°C. A panel of four panellists (3 female and 1 male between 24 and 30 years) were used to carry out the GC-O work. During each GC run, a panellist placed his/her nose close to and above the top of the sniffing port and evaluated the odour of the chromatographic effluent and recorded the time

at which they perceived an odour and gave an appropriated odour descriptor. As the GC runs were 52 min long, two assessors were used to sniff each chromatogram, swapping over half-way, in order to avoid fatigue. The GC-O analysis was performed following the aroma extract dilution analysis (AEDA) approach, for which spirit extracts were stepwise diluted using dichloromethane as the solvent to obtain dilutions of 1:3, 1:9, 1:27, 1:81, 1:243, 1:729; 1:2187 and 1:6561 of the original extract (*19*). Sniffing of each dilution was performed in triplicate until no odorant was perceived and then each odorant was assigned a flavour dilution factor (FD factor). A preliminary training session with the panellists was done by GC-O employing a mixture solution containing some of the important compounds of spirit flavour (*19)*. A further GC-O analysis was done to confirm the influence of the highly volatile, early eluting compounds of spirit samples (which are obscured by the solvent front in DCM extracts). The spirit direct injection method described above was replicated using the GC-MS/O set-up, such that a 20 minute run-time was enough to evaluate the influence of these compounds sensorially. To reduce the time of analysis a flavour dilution factor of 10-fold was implemented, such that only 4 dilutions per sample were analyzed (dilutions: 10, 100 and 1000). The analysis of each sample and dilution was duplicated.

Data treatment and statistical analysis

Chromatograms obtained from the GC-MS analysis were integrated and the area ratio of each compound against its internal standard recorded. Analysis of variance (ANOVA) and Fisher´s Least Significant Difference (LSD) tests were performed using Statgraphics plus software Version 16.1.11. ANOVA and LSD analysis were carried out to establish which compound concentrations were significantly different among the samples according to both provenance (non-mature v mature) and extraction method (LLE v SPE). Finally Principal component analysis (PCA) was carried out using Simca software – P7.01. PCA was performed to depict

variability in the compound concentration data set as related to the sample provenance and the extraction technique used.

RESULTS AND DISCUSSION

Analysis of volatile compounds in non-mature and mature tequila samples

Data for the 'major' volatile compounds analysed by direct injection of tequila samples (GC-FID) is reported in Table 1, whereas that for the GC-MS analysis of tequila extracts, is reported in Table 3

A total of 39 volatile compounds were quantified in the LLE and SPE extracts of non-mature and mature tequila samples (Table 3). The compounds were drawn from a variety of chemical classes including acetals, acids, alcohols, esters, furans, ketones, phenols and terpenes. These compound classes have been reported previously as important contributors to Tequila flavour (*8, 17*). There were significant differences in the concentrations of all quantified compounds between the non-mature and mature tequila samples ($P < 0.05$).

Wood-derived compounds (oak lactones/whisky lactones) and volatile polyphenols (such as eugenol, guaiacol, 4-ethyl guaiacol, and vanillin) were volatile markers of maturation, identified only in the mature spirit (Table 3). These compounds are strong indicators of oak maturation, which influence the taste and aroma of maturing spirits such as tequila (*14*). Particularly important are the sensory effects caused by acids, aldehydes, and phenolic compounds including, whisky lactones, eugenol, and vanillin (*10,14,36*). Some of these are used as markers or aging indicators, since their quantification during the aging process can be used to estimate the time required to age a distilled beverage (*37*). Lignin hydrolysis is the major chemical process which occurs and it is through this that several phenolic compounds are extracted. Oxidation of these compounds yields aldehydes, acids, vanillin, and syringaldehyde (38). Furanic aldehydes are also important contributors of the aging character; however, other conditions affect their concentrations such that they cannot be taken as aging markers. *39*). Their presence has been attributed to physicochemical reactions that arise

238 during maturation, including the extraction of wood components, evaporation of volatile
239 compounds and interactions between wood and distillate components (*14,15*).
240 The presence of terpene compounds such as α-terpineol, linalool and citronellol is
241 characteristic of tequila. Concentrations of these compounds were greatly increased through
242 maturation (Table 2). The concentrations of terpene compounds are determined both
243 biochemically (via raw materials and fermentation) and chemically (through distillation and
244 aging) (*9, 20, 21*). In wine, terpene compounds from grapes have been reported to be sensitive
245 to acidic conditions and to increase with maturation temperature and storage time (*21*).
246 Some acetals have been reported to appear after fermentation and others after distillation
247 where they are concentrated (*9*). Their formation in spirits depends on the raw material and
248 normally is by addition of an alcohol to the carbonyl group of an aldehyde (*22*). Isobutanal
249 diethyl acetal and β-Ethoxypropionaldehyde diethyl acetal were two of the acetals, which
250 were only detected in mature tequila samples, and were therefore produced during the
251 maturation process (Table 2).
252 Table 3 summarises the analysed concentrations of volatile compounds in the aroma extracts
253 by chemical class. Extracts of the mature tequila sample in general contained higher
254 concentrations of the majority of volatile compounds detected, as compared to the
255 corresponding extracts of non-mature tequila (Tables 1 & 2). The most abundant classes of
256 aroma compounds analysed were alcohols, and esters and each increased significantly in
257 concentration in the extracts of mature tequila (Table 3). Concentrations of higher alcohols
258 and esters in tequila are regulated by Mexican law, (20-500 and 2-270 mg/100 mL anhydrous
259 alcohol respectively), to assurance consistency of production between factories (NOM-006-
260 SCFI-2012, 2012). Not surprisingly, analysed values for the present extracts of commercial
261 samples (using either LLE or SPE extraction) fell within the ranges specified (ester content

was in the range of 4.46 - 11.3 and higher alcohols in the range of 20.50 - 39.97 mg/100 mL anhydrous alcohol respectively).

Ethyl octanoate and ethyl decanoate were the esters present in the highest concentrations in the extracts of mature tequila (Table 2). Esters are produced by yeast during fermentation by condensation between Acyl-CoA and higher alcohols catalyzed by intracellular enzymes (*23*). Nevertheless, according to our results an increased ester content was observed in the extracts of mature tequilas, possibly due to esterification reactions during the maturation process (*20*). These results are in accordance with Vallejo-Cordoba et al *(34)*, who reported increased ethyl ester contents in extra-aged tequilas mainly because of fatty acid esterification in the presence of high ethanol concentrations. Furthermore, esters are well known for conferring pleasant 'fruity-notes' to alcoholic beverages (*24*).

Of the higher alcohols, isoamyl alcohol and isobutanol were present at highest concentrations, particularly in mature tequila samples (Table 2). Higher alcohols are secondary yeast metabolites, and their presence can have a positive or negative influence on aroma and flavour of alcoholic beverages (*23*). They confer a strong pungent taste and odour to alcoholic drinks. At concentrations less than 300 mg/L, they contribute to desired complexity but if they are present in concentrations greater than 400 mg/L they may confer negative attributes to spirit aroma (*5, 23*). The concentration of higher alcohols depends on several factors, including the type of yeast strain, fermentation temperature, pH, and amino acid composition of the culture medium (*23*, *27*).

Overall (Table 3), analytical data for the various chemical classes were quite similar across the two extraction techniques used. However, the asterisked compound groups in Table 3 (acids and ketones) are those for which the method of extraction caused a significant difference in recovery from the same sample. For organic acids it is apparent that LLE was a superior method of extraction, recovering greater amounts of these compounds. The acids are

secondary yeast metabolites, which can have both negative and positive impacts on aroma and flavour, depending on their concentration in the final spirit (*23*).

In the case of ketones, the extraction methods were broadly equivalent in the mature sample, but LLE was apparently superior in extracting the range of ketones present in non-mature tequila.

Principal component analysis (PCA) of compound concentration data

PCA was conducted on the analytical data for mature and non-mature tequila samples extracted by both SPE and LLE techniques. A bi-plot for PC1 and PC2 (Figure 1) accounted for over 88% of variation in the data set. Furthermore, PC1, which accounted for the majority of the variation, represented the separation between non-mature and mature samples, with compounds that significantly increased due to maturation having positive loadings on PC1. The concentrations of 2-phenyethyl acetate, isovaleraldehyde diethyl acetal, diacetyl, and ethyl 4-ethoxybenzoate were negatively correlated with PC1, indicating that these compounds were more prevalent in the non-mature spirit. PC2 was driven by differences in concentration due to the extraction technique. The fact that the majority of the compounds are located in the upper half of Figure 1 indicates the all-round superiority of LLE in terms of extraction efficiency; however, compounds such as eugenol, ethyl hexadecanoate, ethyl tetradecanoate, ethyl decanoate, isobutanol and eugenol were extracted more efficiently from mature samples by SPE.

Our data indicate that whilst LLE was better in terms of the extraction of a broad cross-section of tequila volatiles, SPE can be a useful complementary technique for the analysis of certain compounds. Both the SPE phase and extraction protocol employed were based on earlier studies by Boothroyd et al. in malt whisky and further optimisation for tequila was not carried out. Therefore, by choosing the appropriate SPE column and optimizing the

conditions to suit the elution of the groups of analytes required, SPE can be a successful method of extraction, especially for the recovery of semi-volatiles (*18*).

Identification of Odour-Active Compounds in tequila extracts using GC-O/AEDA.

Due to the broader cross-section of compounds, which were extracted efficiently by liquid-liquid extraction (LLE), this method was selected to perform the Gas chromatography-olfactometry (GC-O) and Aroma Extract Dilution Analysis (AEDA) analysis of the Tequila samples. Furthermore the aroma impact of the major volatile compounds (Table 2) was also assayed using the GC-O/AEDA approach with direct injection of spirit samples.

GC-O identified 43 odour-active regions in the chromatograms of non-mature or mature tequila samples, taken across both the extract and direct spirit injection GC-O analyses. Table 4 presents data for each of these odour active regions, sorted by the flavour dilution factor obtained from AEDA analysis of the mature tequila sample. In theory this orders the compounds according to their likely impact on the aroma of mature tequila. As with all GC-O studies it must be noted that since odorants are sniffed individually during GC-O, this technique takes no account of potential interactions (e.g. synergy or masking) between odourants, which can influence perceived aroma. It is also not possible to account for factors such as sub-threshold enhancement or modification of aroma, whereby the perceived quality or intensity of an aroma can be modified by compounds which individually are present beneath their odour threshold. However, GC-O/AEDA remains a popular approach because it highlights compounds, which are likely to play a major part in determining the overall perception; namely those present in substantial excess of their sensory threshold, such that they are still sensed even at the highest dilution factors.

LLE extracts of non-mature tequila were characterized by 26 odour-active regions with flavour dilution factors (FD) $\geq$ 27. These regions are depicted on a ‘flavour dilution chromatogram’ (Figure 2), which indicates where the most potent odorants appeared during

gas chromatography, with bars sized according to the maximum FD factor at which each odour was detected. The compounds with the highest FD factors (6561) were ethyl hexanoate, ethyl octanoate, 2-phenethyl acetate, phenethyl alcohol, octanoic acid and β-damascenone. The individual aroma descriptors associated with these compounds (Table 4) include qualities such as fruity, rose-like, flowery, or cheese-like. A second important group of components (FD of 2187) consisted of isoamyl alcohol, the combined contribution of two co-eluting esters (ethyl benzoate/diethyl succinate), linalool and 2-acetylfuran. Since non-mature tequila is a freshly distilled product, the most potent odorants detected in the AEDA study are important markers of the cooking, fermentation and/or distillation steps of tequila production.

LLE extracts of mature tequila were characterized by the presence of 36 odour-active regions with flavour dilution factors ≥ 27. Figure 3 illustrates these regions on a 'flavour dilution chromatogram'. Comparison of Figures 2 and 3 reveals the increased complexity of mature tequila aroma, resulting both from the presence of maturation-derived components with high FD factors and from the increase in concentration of many other components, as already noted, across maturation. The compounds with the highest FD factors (6561) were isoamyl alcohol, phenethyl alcohol, ethyl hexanoate, ethyl octanoate, 2-phenylethyl acetate, β-damascenone, guaiacol, 4-ethyl-guaiacol, vanillin, cis & trans-whisky lactone, and octanoic acid. A further group of odorants (FD factor of 2187) consisted of the combined contribution of two co-eluting esters (ethyl benzoate, diethyl succinate), 2-acetylfuran, isobutanol, linalool, and citronellol.

The value of including direct injection of spirit samples in the GC-O work was demonstrated by the high FD factors (1000) of several low-boiling compounds in Table 4. Prior studies of the chemical mechanisms involved in the maturation of whiskey showed that the formation of acetaldehyde, acetic acid, and ethyl acetate originates in the distillate, whilst some acetic acid

is produced by interactions between the distillate and wood components (*26*). Furthermore, López-Ramírez et al (*35*) described physicochemical changes that arise as a consequence of tequila barrel maturation; among the parameters evaluated they observed a considerable increase in higher alcohols, methanol, ester, acetaldehyde, and furan-2-carboxaldehyde (furfural) content in the first weeks of maturation, thereby confirming the influence of the aging process over tequila flavour. Our results are in accordance with these findings, since increases in the concentrations of these major compounds were observed in the matured tequila (Table 1).

Odour activity values (OAVs) for key odour-active constituents of tequila

Besides FD factors, a further way to consider the likely impact of individual compounds to the overall aroma of a system is to consider 'dose over threshold'. In this approach the analysed concentration of the compound is divided by its published odour threshold (where available) to produce an odour activity value (OAV; Table 4). As can be seen from Tables 1 and 2, 27 components were present at concentrations higher than their reported odour thresholds, across both tequila samples. According to these OAVs, the most important odorants in the non-mature and mature tequila samples (OAV>20) were diacetyl, *cis*-linalool oxide, isoamyl acetate, *n*-propanol, 2-methyl-1-butanol, ethanol, acetal, ethyl acetate, linalool, β-damascenone and ethyl octanoate. Furthermore, α-terpineol, vanillin and *cis*-whisky lactone presented OAVs higher than 20 only in mature tequila (Table 4).

Overall, the authors prefer to rank the significance of odorants in terms of the AEDA FD factors (Table 4), because this is consistent with the panellists and samples used in this study. Whereas, the calculation of odour thresholds is subject to a number of factors including the sensory methodology adopted, the number and identity of the panellists used in the study and how/ in which matrix samples are presented. Hence reported odour thresholds can vary substantially according to source; this is probably the major reason why the ranking

according to OAV in Table 4 would be very different to that which is presented according to FD factor. Having said that, within a particular FD band (Table 4), the OAV provides further evidence of the likely significance of a particular odorant – in particular at the upper end of the study, where there is no information in the FD value over and above the fact that compounds were detected at the 6561-fold dilution factor. Amongst such compounds, β-damascenone was noteworthy as being present at very high OAV's, particularly in the non-mature tequila.

Conclusions

The aromagram of mature tequila was differentiated from that of the non-mature spirit due to the presence of cask-extractive compounds and the increased FD factors of certain terpenes, higher alcohols and acetals. Since several wood-derived compounds (*cis/trans* whisky lactones, guaiacol, 4-ethyl guaiacol, and vanillin) were present in mature tequila at the highest FD factors, the impact of maturation on the flavour profile of the añejo tequila was clearly evident. However, other odour-active compounds, such as ethyl hexanoate, ethyl octanoate, *cis*-linalool oxide, furfural, 2-acetyl furan, linalool, 5-methyl furfural, and ethyl decanoate, (Table 4), showed no impact of maturation on the flavour dilution factor, indicating the significance of other important steps of tequila production (raw material, cooking, fermentation, distillation) to tequila aroma (*9*).

The results presented here highlight many of the compounds identified in earlier studies as key components of tequila flavour (*8, 17*). Some differences in FD/CHARM values between such studies are to be expected, due both to the complexity of tequila flavour and the individual brands selected for analysis in each case. The present study provides a comprehensive and quantitative understanding of changes in key odorants of this brand of tequila as a result of the its unique maturation process and also reveals a further characterization of the likely impact of each compound on overall spirit flavour, in terms of

411 odour activity values (OAVs). Nonetheless, the fact that the present study relates to just one
412 brand of tequila needs to be borne in mind. Whilst the extent of agreement with prior
413 published studies confirms the validity of our data, it is to be expected that the nuances of
414 tequila flavour, and hence the underlying congener concentrations, would vary according to
415 the unique processes that characterize each tequila factory.
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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

GC, gas chromatography; GC-MS, gas chromatography-mass spectrometric; GC-O, gas chromatography-olfactometry; MS, mass spectrometry; AEDA, aroma extract dilution analysis; LLE, liquid-liquid extraction; SPE: solid-phase extraction; DCM, dichloromethane; EI-MS: electronic impact-mass spectrometry; GC-MS/OPA, gas chromatography-mass spectrometry/odour port evaluation; FD, flavour dilution factor; ANOVA, analysis of variance; LSD, fisher´s least significant differences; PCA, principal component analysis.

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Table 1. Major volatile compounds of non-mature and mature tequila samples, analysed by the spirit direct injection GC-method.

Major compounds				**Non-mature Tequila**	**Mature Tequila**
Compound	**LRI (experimental)**	**LRI (literature)**	R^2	**Concentration (mg/L)**	
Acetaldehyde	781	718	0.9995	11.4±2.50	39.1±1.23
Ethyl acetate	888	898	0.9985	82.7±2.50	112±1.68
Acetal	895	900	0.9988	48.8±2.50	81.2±1.53
Methanol	909	907	0.9922	919±11.2	653±3.85
n-Propanol	1061	1037	0.9999	183±0.60	275±0.37
Isobutanol	1119	1099	0.9996	309±2.30	358±0.75
n-Butanol	1177	1151	0.9999	ND	ND
2-methyl-1-butanol	1229	1228	0.9998	329±14.4	551±14.9
Isoamyl alcohol	1234	1230	0.9972	482±18.6	768±19.6
Ethyl lactate	1382	1358	0.9981	10.6±0.3	16.3±0.26
Acetic acid	1481	1477	0.9963	94.9±18.3	281±10.4
Furfural	1504	1485	0.994	ND	3.27±0.08

LRI (experimental): Experimental Linear Retention Index.

LRI (literature): Linear Retention Index taken from literature (http://www.pherobase.com/database/kovats/kovats-index.php).

Compounds were identified by comparison of their retention times (R.I) against those of authentic standards and confirmation based on their linear retention index (LRI). Data represent the average of three independent injections into the GC-FID ± standard deviation. ND: not detected under the conditions of analysis.

Table 2. Analysed concentrations (mg/L) of volatile compounds in extracts of non-mature and mature tequila samples by GC-MS following either liquid-liquid extraction (LLE) or Solid Phase Extraction (SPE).

						Non-mature Tequila	Mature Tequila	Non-mature Tequila	Mature Tequila
Compound	**LRI (experimental) ZB-Wax**	**LRI (literature)**	**Ions (m/z)**	**R^2**	**Identity**	**LLE**		**SPE**	
Diacetyl*	993	984	43,83	0.997	A,B	0.75±0.02	0.15±0.021	0.14±0.03	0.15±0.02
n-Propanol*	1038	1037	31,42	0.999	A,B	9.02±2.18	17.10±4.65	3.93±0.87	6.91±1.28
Isovaleraldehyde diethyl acetal*	1083	NA	103,115	---	B	0.32±0.03	0.12±0.02	0.23±0.02	ND
Isobutanol*	1105	1099	41,43	0.999	A,B	120±21.3	169±33.6	151±14.6	190±14.5
Isoamyl acetate	1139	1117	43,70	0.997	A,B	2.40±0.29	3.12±0.21	2.38±0.04	2.65±0.14
n-Butanol	1158	1145	31,41	1.000	A,B	0.42±0.09	1.41±0.24	0.52±0.04	1.51±0.06
Isoamyl alcohol	1227	1230	55,70	0.998	A,B	381±43.9	801±40.9	431±12.7	796±24.4
Ethyl hexanoate	1251	1244	88,90	0.995	A,B	0.09±0.01	0.25±0.02	0.06±0.01	0.24±0.01
Isobutanal diethyl acetal	1274	NA	47,73	---	B	ND	2.75±0.03	ND	2.86±0.01
Dihydro-2-methyl-3(2H)-furanone	1291	NA	43,72	1.000	A,B	0.79±0.08	1.50±0.1	0.75±0.07	1.44±0.02
β-Ethoxypropionaldehyde diethyl acetal	1319	NA	47,59	1.000	A,B	ND	2.39±0.11	ND	2.43±0.01
Ethyl lactate	1367	1358	45	1.000	A,B	1.36±0.09	1.96±0.22	1.21±0.03	1.77±0.04
Ethyl octanoate	1452	1446	88,101	1.000	A,B	4.83±0.10	8.37±1.34	5.35±0.41	7.85±0.24
Cis Linalool oxide	1465	1449	59,94	1.000	A,B	0.36±0.02	0.84±0.01	0.30±0.01	0.79±0.01
Furfural	1497	1485	39,96	1.000	A,B	0.74±0.05	2.29±0.12	0.77±0.01	2.24±0.04
3-Ethyl-4-methyl-1-pentanol	1529	NA	48,41	---	B	0.90±0.01	2.28±0.21	0.91±0.05	2.15±0.04
2-Acetylfuran	1539	1534	95,110	0.999	A,B	0.26±20.0	0.75±0.05	0.23±0.03	0.77±0.01
Linalool	1566	1565	71,93	0.997	A,B	0.41±0.01	1.46±0.04	0.34±0.02	1.44±0.02
5-Methyl furfural	1609	1590	110	1.000	A,B	0.40±0.01	0.61±0.06	0.35±0.050	0.67±0.01
Ethyl decanoate*	1656	1636	88,101	0.998	A,B	2.06±0.13	6.32±0.80	1.35±0.12	10.1±0.16
Ethyl benzoate*	1690	1675	77,105	1.000	A,B	0.01±0.00	0.01±0.00	0.07±0.00	0.01±0.00
Diethyl succinate	1699	1705	101,129	1.000	A,B	0.10±0.01	0.41±0.06	0.08±0.01	0.42±0.01
α-Terpineol	1724	1720	59,93	0.997	A,B	2.44±0.16	6.28±0.40	2.20±0.12	6.48±0.06
Citronellol	1786	1762	41,55	0.995	A,B	0.27±0.04	0.53±0.10	0.23±0.02	0.48±0.04
2-Phenylethyl acetate	1852	1829	43,104	1.000	A,B	0.36±0.02	0.15±0.01	0.30±0.02	0.16±0.00
β-Damascenone*	1854	1836	69,121	0.996	A,B	0.33±0.09	0.03±0.004	0.03±0.01	0.07±0.01
Ethyl dodecanoate	1860	1852	88,101	0.996	A,B	0.42±0.11	0.68±0.12	0.42±0.05	4.73±0.19

Guaiacol	1899	1892	81,109	0.999	A,B	ND	0.01±0.001	ND	0.01±0.00
Trans-whisky lactone	1929	1977	99	0.999	A,B	ND	0.31±0.07	ND	0.33±0.01
Phenethyl alcohol	1951	1929	91,122	1.000	A,B	1.22±0.05	1.61±0.032	1.10±0.03	1.75±0.03
Cis-whisky lactone	2002	1985	99	0.999	A,B	ND	1.50±0.36	ND	1.66±0.01
Ethyl tetradecanoate*	2060	2072	88,101	0.995	A,B	ND	0.02±0.01	ND	0.16±0.01
4-Ethyl-guaiacol	2064	2054	85,137	0.999	A,B	ND	<0.01±0.00	ND	<0.01±0.00
Octanoic acid*	2088	2083	60,73	0.999	A,B	0.27±0.02	1.84±0.35	ND	0.57±0.08
Ethyl 4-ethoxybenzoate*	2187	NA	121,149	---	B	0.6±0.106	0.07±0.00	0.02±0.00	0.03±0.00
Eugenol*	2188	2186	164	0.996	A,B	ND	0.02±0.00	ND	0.07±0.02
Ethyl hexadecanoate*	2238	2250	88,101	0.997	A,B	0.025±0.00	0.08±0.01	ND	0.12±0.01
Decanoic acid*	2269	2296	60,73	0.992	A,B	0.70±0.13	6.14±1.45	ND	2.07±0.22
Vanillin	2516	2569	151	0.999	A,B	<0.01±0.00	0.88±0.04	<0.01±0.00	0.77±0.01

LRI (experimental): Experimental Linear Retention Index. LRI (literature):

Linear Retention Index taken from literature (http://www.pherobase.com/database/kovats/kovats-index.php).

LLE: Liquid-Liquid Extraction; SPE: Solid Phase Extraction;

ND: not detected under the conditions of analysis. NA: information not available in the literature.

A, B: Compounds were identified by EI-MS library matching (NIST), comparison against authentic standards and confirmation of their linear retention index (LRI) against published values for a DB-Wax column. http://www.pherobase.com/database/kovats/kovats-index.php

B: Compounds were identified by EI-MS library matching (NIST), and confirmation of their linear retention index (LRI) against published values for a DB-Wax column (http://www.pherobase.com/database/kovats/kovats-index.php)

Data represent the average of three independent extractions ± standard deviation.

* Indicates statistically significant difference between results for the same sample extracted by LLE and SPE ($P<0.05$).

Table 3. Concentrations (mg/L) of volatile compounds by chemical class identified in the extracts of tequila samples

Chemical class	Non-mature Tequila	Mature Tequila	Non-mature Tequila	Mature Tequila
	LLE		SPE	
Acetals	0.32±0.03	4.89±0.24	0.23±0.02	4.86±0.03
Acids*	0.97±0.15	7.98±1.80	ND	2.64±0.30
Alcohols	513±67.5	993±79.9	589±28.3	999±40.3
Esters	12.3±1.19	21.4±2.81	11.2±0.71	28.2±0.81
Furans	1.39±0.06	3.64±0.22	1.35±0.09	3.66±0.06
Ketones*	1.87±0.40	3.50±0.55	0.92±0.11	3.64±0.06
Phenols	0.004±0.00	0.91±0.04	0.002±0.00	0.85±0.03
Terpenes	3.48±0.23	9.11±0.46	3.07±0.17	9.18±0.13

LLE: Liquid-Liquid Extraction; SPE: Solid Phase Extraction; nd: not detected.

Data represent the average of three independent extractions ± standard deviation.

* Indicates statistically significant difference between results for the same sample extracted by LLE and SPE ($P<0.05$).

ND: not detected under the conditions of analysis.

Table 4. The most odour-active (FD ≥ 27) volatile compounds identified in non-mature and mature tequilas, and their estimated odour-activity values (OAV). Data are sorted by FD factor in the mature tequila .

Generic description	Odorant	Identity	Flavour Dilution Factor (FD)		Odour threshold (mg/L)	OAV		Earlier reported as compound in tequila
			Imm. Teq	Mat. Teq		Imm. Teq	Mat. Teq	
Flowery, lactone-like	*Trans*-whisky lactone*	A, B	ND	6561	0.79[b]	ND	0.39	*(8, 9)*
Flowery, lactone-like	*Cis*-whisky lactone*	A, B	ND	6561	0.067[b]	ND	22.4	*(8, 9)*
Flowery	4-ethyl-guaiacol*	A, B	ND	6561	0.0069[b]	ND	1.45	*(8, 9)*
Fruity, sweet, anise, strawberry	Ethyl hexanoate	A, B	6561	6561	0.03[b]	3	8.33	*(8, 9)*
Fruity, green, leafy, mint	Ethyl octanoate	A, B	6561	6561	0.147[b]	33	56.6	*(8, 9)*
Rose-like, fruity	2-Phenylethyl acetate	A, B	6561	6561	0.108[b]	3	1.39	*(8, 9)*
Rose-like, fruity	β-Damascenone	A, B	6561	6561	0.0001[b]	3320	270	*(8, 9)*
Rose-like, flowery	Phenethyl alcohol	A, B	6561	6561	2.6[b]	0.47	0.62	*(8, 9)*
Cheese, fresh	Octanoic acid	B	6561	6561	10[e]	0.03	0.18	*-9*
Phenolic, smokey, flowery, green-like	Guaiacol*	A, B	729	6561	0.0092[b]	ND	1.2	*(8, 9)*
Caramel, vanillin, sweet, burnt sugar	Vanillin*	A, B	729	6561	0.022[a]	0.45	40	*(8, 9)*
Balsamic, flowery	2-Acetylfuran	B	2187	2187	10[a]	0.03	0.08	*-9*
Lavender, flowery	Linalool	B	2187	2187	0.006[a]	68	243	*(8, 9)*
Celery, potato baked	Ethyl benzoate	A, B	2187	2187	0.06[a]	0.18	0.24	NA
Celery, potato baked	Diethyl succinate	A, B	2187	2187	1.2[e]	0.08	0.34	*(8, 9)*
Rose-like, flowery, sweet	Citronellol*	B	729	2187	0.04[a]	6.5	13.25	*(8, 9)*
Pungent, sweet	Acetaldehyde[$]	A,B	1000	1000	19.2[h]	1	2	*(8, 9)*
Varnish, leafy	Ethyl acetate[$]	A,B	1000	1000	0.005-5[a]	16600-16.6	22340-22.3	*(8, 9)*
Earthy, solvent	Acetal[$]	A,B	1000	1000	0.719[h]	68	113	*(8, 9)*
Solvent-like	Methanol[$]	A,B	1000	1000	100[b]	20	26	*(8, 9)*
Ethanol, solvent-like, sweet	Ethanol[$]	A,B	1000	1000	24.9[b]	----	----	*(8, 9)*
Winey, sweet, solventy	Isobutanol[$]	A,B	1000	1000	40[b]	8	9	*(8, 9)*
Malty, solvent, sweet, fruity	2-methyl-1-butanol[$]	A,B	1000	1000	4[b]	82	138	*(8, 9)*
Malty, solvent, sweet, fruity	isoamyl alcohol[$]	A,B	1000	1000	56.1[b]	9	14	*(8, 9)*
Green-like, grass	*n*-Hexanol[$]	B	1000	1000	8[h]	0.1	0.1	*(8, 9)*
Green-like, grass	Ethyl lactate[$]	A,B	1000	1000	14[a]	0.8	1.2	*(8, 9)*
Green-like, grass	Acetic acid[$]	A,B	1000	1000	75.52[h]	1	4	*(8, 9)*
Solvent-like, fruity, sweet	*n*-Propanol*[$]	A,B	100	1000	9[b]	20	31	*(8, 9)*
Clove-like, anaesthesia, numb odour	Eugenol*	A, B	ND	729	0.0071[b]	ND	2.82	*(8, 9)*
Fruity-like	Isoamyl acetate	A, B	729	729	0.03[c]	80	104	*(8, 9)*
Sweet, waxy	Ethyl hexadecanoate	A, B	729	729	>2[a]	0.02	0.04	*(8, 9)*
Clean soap, sweet, fatty	Decanoic acid	A, B	729	729	6[e]	0.12	1.02	*(8, 9)*
Herbal, mint, green-like, anise	α-Terpineol*	B	243	729	0.25[f]	9.76	25.1	*(8, 9)*
Sweet, caramel	Dihydro-2-methyl-3 (2H)-furanone*	B	729	243	NA	NA	NA	*-8*
Green-like, fruity, leafy, sweet	*Cis*-Linalool oxide	B	243	243	0.006[a]	60	140	*(8, 9)*
Burnt sugar, caramel	5-Methyl furfural	A, B	243	243	16[d]	0.04	0.04	*(8, 9)*
Solvent-like, fresh, fatty	Ethyl 4-ethoxybenzoate*	B	81	ND	NA	NA	NA	NA
Fruity, fatty, herbaceous odour	Isovaleraldehyde, diethyl acetal*	B	ND	81	NA	NA	NA	*(-9)*
Solvent-like, varnish	β-Ethoxypropionaldehyde diethyl acetal*	B	ND	81	NA	NA	NA	NA

Solvent-like	Ethyl tetradecanoate*	B	ND	81	4[g]	NA	0.01	NA
Almond, sweet, earthy, woody, flowery	Furfural	A, B	81	81	15[d]	0.05	0.15	*(8, 9)*
Fruity, sweet, grape	Ethyl decanoate	A, B	81	81	0.5[e]	4.12	12.6	*(8, 9)*
Fruity-like, sweet, mango	Ethyl dodecanoate*	B	27	81	5.9[g]	0.07	0.12	*(8, 9)*
Green, fresh, solvent	*n*-Butanol*	B	ND	27	0.5[b]	0.84	2.82	*-9*
Solvent-like	Isobutanal diethyl acetal*	B	ND	27	NA	ND	NA	NA
Sweet, solvent-like	3-Ethyl-4-methyl-1-pentanol *	B	<27	27	NA	NA	NA	NA
Butter	Diacetyl	A,B	27	ND	0.007[h]	115	24	*(8, 9)*

ND: not detected under the condition of analysis. NA: Odour threshold data not available in the literature.

A, B: Compounds were identified by EI-MS library matching (NIST), comparison against authentic standards and confirmation of their linear retention index (LRI) against published values for a DB-Wax column.

B: Compounds were identified by EI-MS library matching (NIST), and confirmation of their linear retention index (LRI) against published values for a DB-Wax column

Odour threshold references: [a]: http://www.leffingwell.com/odorthre.htm; [b]: Schieberle et-al. 2008 (*27*); [c]: Ferreira et al.(*28*); [d]: Franco et al.(*29*); [e]: Peinado et al.(30); [f]: Lopez et al.(*31*) [g]: Pino et al (32). [h]: Uselmann et al. (33)

Odour thresholds from most of the references were determined in hydroalcoholic solutions (Ethanol 10 and 40%), with the exception of ref [a] which was determined in water.

* Compounds with a significant difference in odour potency between non-mature and mature tequila samples.

[$] Flavour dilution factors (FD) optimized for the analysis of the influence of major volatile compounds into the overall aroma of tequila flavour.

---: data not available since the concentration was not determined

Note: an average delay of 18 s was found between the detection of the compounds by GC-MS and panellist's nose during the GC-O analysis

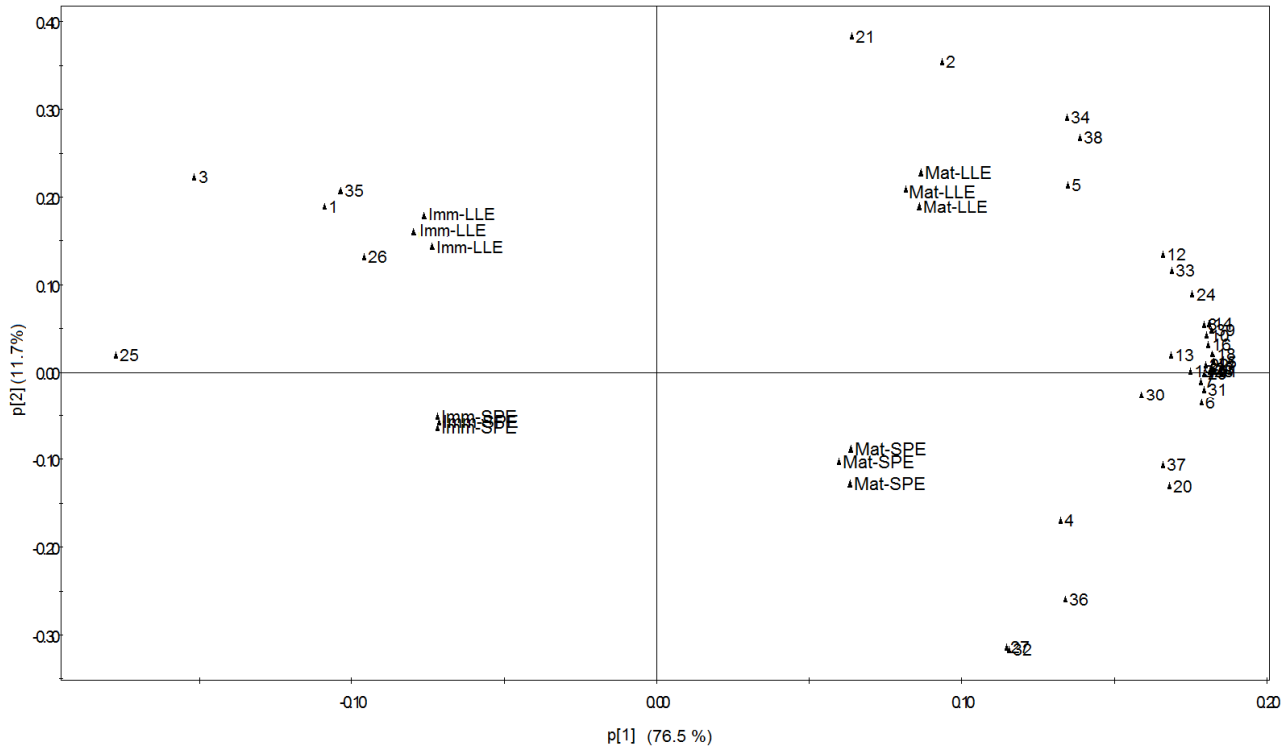

Figure 1. Principal Component analysis (PCA) bi-plot of the analytical data for volatile compound concentrations, with the spirit samples overlaid.

PC1 primarily separates compounds according to concentrations in non-mature versus mature tequila, whilst PC2 separates the compounds according to their extraction efficiency via LLE (positive loading on PC2) versus SPE (negative loadings on PC2).

1: diacetyl; 2: 1-propanol; 3: isovaleraldehyde diethyl acetal; 4: isobutanol; 5: isoamyl acetate; 6: 1-butanol; 7: isoamyl alcohol; 8: ethyl hexanoate; 9: isobutanal diethyl acetal; 10: dihydro-2-methyl-3 (2H)-furanone; 11: β-ethoxypropionaldehyde diethyl acetal; 12: ethyl lactate; 13: ethyl octanoate; 14: cis-linalool oxide. 15: furfural; 16: 3-ethyl-4-methyl-1-pentanol. 17: 2-acetylfuran; 18: linalool; 19: 5-methyl furfural; 20: ethyl decanoate; 21: ethyl benzoate; 22: diethyl succinate; 23: α–terpineol; 24: citronellol; 25: phenethyl acetate; 26: β-damascenone; 27: ethyl dodecanoate; 28: guaiacol; 29: trans-whisky lactone; 30: phenylethyl alcohol; 31: cis-whiskey lactone; 32: ethyl tetradecanoate; 33: 4-ethylguaiacol; 34: octanoic acid; 35: ethyl 4-ethoxybenzoate; 36: eugenol, 37: ethyl hexadecanoate; 38: decanoic acid; and 39:vanillin. Imm-LLE: Extract of non-mature tequila obtained by liquid-liquid extraction; Mat-LLE: Extract of mature tequila obtained by liquid-liquid extraction; Imm-SPE: Extract of non-mature tequila by solid phase extraction; Mat-SPE: Extract of mature tequila obtained by solid phase extraction..

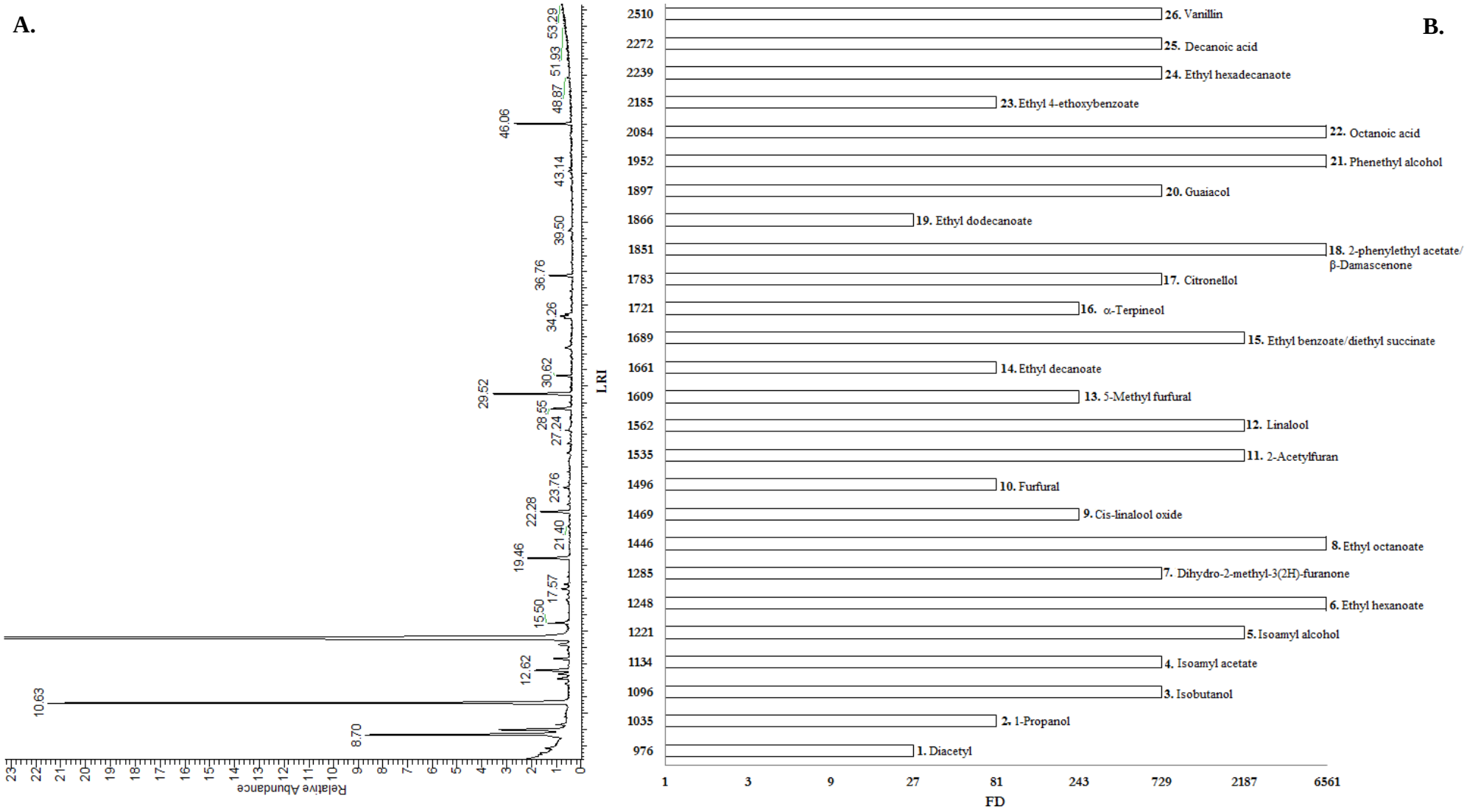

Fig. 2. Gas chromatogram (**A**) and flavour dilution (FD) chromatogram (**B**) of the volatile fraction extracted from non-mature Tequila.

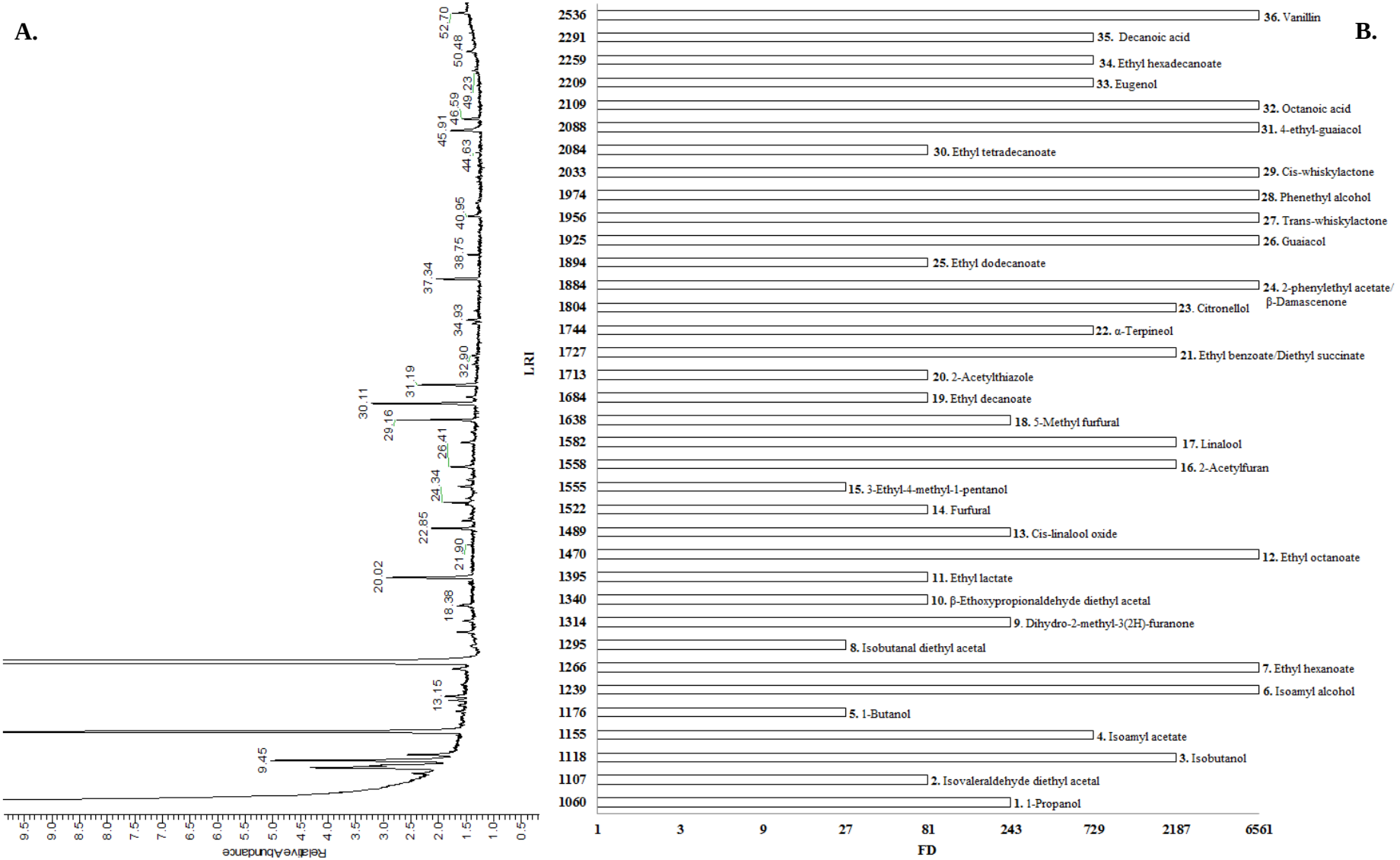

Fig. 3. Gas chromatogram (**A**) and flavour dilution (FD) chromatogram (**B**) of the volatile fraction extracted from mature Tequila.