Investigating aroma diversity combining purge-and-trap, comprehensive two-dimensional gas chromatography, and mass spectrometry

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Running title: Aroma analysis using P&T-GC×GC-ToF MS

Abbreviations: ^1D/^2D: first/second dimension; FOO: frequency of observation; GC×GC: comprehensive two-dimensional gas chromatography; HCA: hierarchical clustering analysis; PCA: principal component analysis; P_M: modulation period; P&T: purge and trap; RI: retention index; VOC: volatile organic compounds.

Abstract

Headspace GC is frequently used for aroma profiling thanks to its ability to naturally exploit the volatility of aroma compounds, and also to provide chemical information on sample composition. Its main advantages rely on simplicity, no use of solvent, amenability to automation, and the cleanliness

of the extract. In the present contribution, the most effective sampling (dynamic extraction), separation (multidimensional gas chromatography) and detection (mass spectrometry) techniques for untargeted analysis are exploited in combination, showing their potential in unraveling aroma profiles in fruit beers. To complete the overall analytical process, a neat workflow for data analysis is discussed and used for the successful characterization and identification of 5 different beer flavors (berries, cherry, banana, apple, and peach). From the technical viewpoint, the coupling of purge-and-trap, comprehensive two-dimensional gas chromatography, and mass spectrometry makes the global methodology unique, and it is for the first time discussed. A (low-)flow modulation approach allowed for the full transfer into the second dimension with MS-compatible flow (< 7 mL/min), avoiding the need of splitting before detection and making the overall method sensitive (1.2-5.2 fold higher S/N compared to unmodulated GC conditions) and selective.

1. Introduction

The aroma of food and beverage is of primary importance for the final products' sensory properties. Therefore, high is the interest in the analysis of volatile constituents, which represents one of the main tools employed to study the industrial processes involved in food products. It has been demonstrated that the profile of the volatile organic compounds in food carries concrete information which can be used to study, improve or monitor the stages of the manufacturing process, the raw materials used, the fermentation types, the shelf-life, the storage conditions, and to discover eventual defects or unwanted byproducts [1–6]. In terms of composition, fatty acid esters are the largest class of flavor compounds, and thanks to the lower odor threshold, they are key-components to the aroma of the alcoholic beverages. Beside the esters, also terpenoids, aldehydes, and ketones with characteristic odors have been detected and reported to contribute to the complex interplay of beer flavors [7–9]. From the manufacturing point of view, the presence of esters in alcoholic beverages is affected by the different processes involved in their production, for example, the fruit addition, fermentation or maturation [10,11]. It is also important to identify possible flaws in the process which may lead to the development of off-flavors, which are critical issues during beer production [12]. Therefore, the study and monitoring of the aroma could contribute to improve the knowledge of the brewing process.

The most commonly used aroma analyses involve techniques based on headspace enrichment, which consists in the pre-concentration on a sorbent material of the vapor phase released from solid or liquid samples. These non-destructive methods are considered green sample preparation techniques, as they avoid the use of solvents, allowing the isolation of VOCs in their natural form and are characterized by minimum sample preparation [13]. Two principles of headspace analysis exist depending on the equilibrium status between the sample (solid or liquid) and the headspace (gaseous), namely static and dynamic [14]. Compared to other extraction methods, the headspace techniques relate better to sensory properties, as the concentration of the aroma compounds in the gas phase depends on the interaction of the volatiles with the food matrix.

In static headspace sampling, the vapor phase is in equilibrium with the sample, so that these methods are especially appropriate for volatile compounds highly concentrated in the headspace. In dynamic headspace sampling, the equilibrium between the phases is continuously displaced in favor of the headspace. In the different dynamic approach, the continuous removal of volatiles from the matrix is obtained by flowing an inert gas over the headspace (dynamic headspace or DHS) or through it (purge and trap) [14].

The bubbling of inert gas through the sample in the purge and trap method greatly helps the stripping of volatile and semi-volatile compounds from the sample, which are subsequently concentrated on a sorbent trap. There are three main adsorbent types with different selectivities which can be used alone or in combination, namely the porous polymers (*e.g.*, Tenax®), graphitized carbon blacks (*e.g.*, Carbopack™) and carbon molecular sieves (*e.g.*, Carboxen®). Generally, graphitized carbon black and carbon molecular sieve materials are considered strong adsorbents because of their capacity in retaining low-boiling point compounds. On the other side, the porous hydrophobic polymers show better extraction recovery compared to the previous sorbents in retaining analytes with higher boiling points (in the range of C_6-C_{26}). The readers are directed to the literature for a deeper description of the sorbent material characteristics [15].

As an example, P&T has been used for analyzing fruits and fruit juices and alcoholic beverages, followed by conventional GC analysis [16–18]. Indeed, particularly well-suited for gas (vapor) analysis, GC is the method of choice for the separation of the sorbent-trapped VOCs, after

their thermal desorption. Introduced nearly 3 decades ago, comprehensive 2D GC increases

enormously the separation and identification capability compared to conventional systems, especially

when coupled with mass spectrometry. Nowadays, GC×GC-MS represents the most powerful tools

for VOC and semi-VOC determination, especially in untargeted analysis [19–26]. In recent years,

thanks in part to a common intent to make the use of GC×GC less expensive and more accessible,

cryogenic-free or pneumatic modulators have gained attention [27,28]. Amongst the latter group,

recent studies have demonstrated that the combination of differential flow modulators with MS is

effective, maintaining the high sensitivity and selectivity typical of the GC×GC technique [29–31].

The reader is directed to the literature for detailed explanations on the modulation forms, with their

advantages and drawbacks [27,32].

A variety of sample enrichment techniques have started to be more commonly used in

combination to GC×GC, to exploit the wider potential of an integrated sample preparation-separation

approach [33]. In this context, even though P&T represents a sensitive technique and holds the

advantages of a dynamic extraction, no uses are reported in combination with comprehensive two-

dimensional gas chromatography.

In the current research, a method for the aroma determination and characterization of fruit

beers is presented, including I) the sampling method, II) the analytical measurements and III) the data

processing. The analytical method implicates the P&T extraction on trap tubes, which are

successively desorbed into a GC×GC-MS system equipped with a differential flow modulator. The

P&T and GC×GC techniques, which are coupled here for the first time, are evaluated on beer VOCs.

Throughout the paper, importance is given to the optimization of the flow modulation conditions,

which allowed satisfactory performances at MS-compatible flow, representing its first use in

combination with a ToF MS with a 100% modulation duty cycle.

2.0 Materials and Methods

2.1 Samples and standards

The C_{7-30} n-alkane mix and the single C_{9-12}, respectively used for retention index calculation

and for modulation optimization, were purchased from MilliporeSigma (Bellefonte, PA, USA). The

standard mix containing 37 compounds (Table S1), used for the GC×GC separation evaluation, was supplied by Restek Corporation (Bellefonte, PA, USA).

Five different types of commercial fruit beer, contained in glass bottles (330 mL), were purchased in a local store (Liège, Belgium). The main aroma characteristic of the five fruity beers, and reported on their respective labels were red berries (Ber), cherry (Che), banana (Ban), apple (App) and peach (Pea). Samples were degassed by sonication for 5 min and aliquots of 10 mL were transferred into a 20 mL headspace vial containing 1 g of NaCl. Four replicates were prepared for each beer type.

2.2 Purge and trap analysis

The Adsorbent Tube Injector System (ATIS^{TM}) from MilliporeSigma was used to either flash-vaporize standards into thermal desorption tubes and to perform the dynamic headspace purge and trap extraction, in an offline manner. For the evaluation of the GC×GC separation, 2 μL of a mix of 37 compounds (Restek Corp., the information in Supplementary Table S1), with a concentration of approx. 40 ppm, was injected in the heated (120 °C) ATIS^{TM} glassware, and the vapors were efficiently transferred in the thermal desorption tube using a 100 mL/min nitrogen flow for 2 minutes.

For beer analysis, 10 mL of samples were purged at room temperature for 5 minutes with a nitrogen flow of 150 mL/min, for a total sample volume of 750 mL. During the purge cycle, the trap was also maintained at room temperature. The trap consisted of thermal desorption tubes packed with Tenax® TA (Gerstel Inc., Linthicum Heights, USA). For each sampling, a different tube was used. After each cycle, the tubes were cleaned as advised by the manufacturer and verified to be blank. The tubes containing the sample were collected and analyzed off-line in the GC×GC-ToF MS within 24 h. The absence of sampling breakthrough was assessed by connecting two tubes in series during the sampling and ensuring the absence of signals from the second tube.

2.3 Thermal desorption and flow modulated GC×GC-ToF MS conditions

The system used for the analysis consisted of a Pegasus 4D (Leco Corporation, St. Joseph, MI) GC×GC-ToF MS instrument with an Agilent 7890 GC equipped with a thermal desorption unit, cooled injection system, and a MultiPurposeSampler autosampler (Gerstel K.K., Japan). The modulation on the GC×GC occurred by means of a differential flow modulator in symmetrical

configuration [31]. Briefly, the lab-made modulator was constructed by using two MXT Y-unions (Restek Corp.) and a 3-way solenoid valve (located outside GC), connected to an auxiliary pressure source. The two unions were bridged using a deactivated capillary of 20 cm × 0.51 mm id for the loop.

The first dimension (^{1}D) column was a non-polar Rxi-5SilMS (1,4-bis(dimethylsiloxy)phenylene dimethyl polysiloxane, Restek Corp.) of 30 m × 0.25 mm id × 0.25 μm d_f. The second dimension (^{2}D) column was a mid-polar Rxi-17Sil MS (equivalent to a (50%-Phenyl)-methylpolysiloxane, Restek Corp.) of 5.0 m × 0.25 mm id × 0.25 μm d_f. The carrier gas was helium and the optimized column flow conditions were 0.4 and 7 mL/min, respectively in the ^{1}D and ^{2}D.

The initial temperature of the TDU was set at 30 °C then heated to 300 °C (held 1 min) at 700 °C/min. The interface temperature was kept at 275 °C. The VOCs were desorbed from the thermal desorption unit in splitless mode and were focused at 20 °C on a Tenax$^®$ glass liner. The injector was programmed from 20 °C to 300 °C at 12 °C/sec, and the injection was performed in split mode (1:10). The primary and secondary oven temperature program was the same and started at 50 °C (hold 2 min), then ramped to 260 °C with a rate of 3 °C/min. A final fast temperature ramp of 20 °C/min to 330 °C assured the column conditioning and cleaning for the successive run. The final P_M was 6.6 sec, consisting of an accumulation and reinjection time of 6 and 0.6 sec, respectively. A mass range of 40 to 400 m/z was collected at a rate of 150 spectra/sec. The ion source was maintained at 230 °C.

For the separation and sensitivity comparison, unmodulated GC-MS profiles were acquired switching off the solenoid valve and maintaining the same GC×GC-MS flow and temperature conditions.

Data acquisition, data alignment, and data processing were performed using ChromaTOF$^®$ (Leco Corp., v. 4.72). For peak detection, a S/N cutoff was set at 150, and detected peaks were tentatively identified by a forward search using the NIST 2017 library (70 % minimum similarity was required) and using retention index information (±20 RI was considered). The reference linear retention indices on the non-polar column and the compound odor characteristics were extrapolated from AromaOffice$^®$ (Gerstel K.K., v.4).

For the alignment of peaks across chromatograms, maximum ^{1}D and ^{2}D retention time deviations were set at ±12 sec and ±0.1 sec, respectively, and the inter-chromatogram spectral match threshold was set at 65%. Moreover, the search for peaks not found by the initial peak finding during the alignment was set to 50 *S/N*.

2.4 Statistical analysis

After assessing that the chromatographic signal for each chemical class was within the linear range, the area of unique masses was used for the entire data processing. A frequency of observation criterion was applied to use the most consistent features and consisted of a positive detection in 75% of the replicates within each sample type (3 out of 4). Statistical analyses and figures were performed using R (v. 3.3.0). The only data manipulation involved the auto-scaling for PCA and heatmap visualization. The R packages FactoMineR, MetaboAnalyst, and VennDiagram were used to generate PCAs, HCA, correlation/distance matrix and the Venn diagram.

3.0 Results and Discussion

3.1 Sampling and GC×GC-ToF MS optimization

The two main variables that account for extraction efficiency of analytes using the P&T technique are the total extraction volume (or purge volume) and sample extraction temperature. Considering the latter, although sample heating generally improves the VOCs extraction, temperatures higher than 30 °C can alter original characteristics of beers. Thus, the extraction temperature was set at room temperature (20 °C). The total purge volume was instead adjusted to 750 mL to avoid breakthrough and maintain a satisfactory sensitivity for the beer samples.

The aroma composition of the fruit beers is mainly characterized by the presence of esters, aldehydes, ketones, alcohols, and terpenoids [7,9]. Thus, the selection of the sorbent type for the extraction was driven by the selectivity of the trapping material, with the goal to retain as much as information as possible for the untargeted analysis, therefore maintaining the widest analyte coverage with acceptable sensitivity and good repeatability. In an analogous study, in which different sorbent materials were compared and discussed, the porous polymer Tenax® was confirmed to be optimal for dynamic headspace analysis. Specifically for high water content samples, Tenax® was reported more repeatable and sensitive than other sorbents over a wide range of analytes [34,35]. For these reasons,

tubes packed with Tenax® were used for sampling and as trapping material in the GC inlet liner and can be considered as a good first choice in case of untargeted profiling. In case of targeted applications, depending on the nature of the analytes of interest and the objective, more selective sorbents or a combination of them can be used for sampling. For example, if highly volatile compounds (*e.g.* ≤ C_6) are sought, the use of a stronger sorbent can give better extraction recoveries. However, in this case, more attention must be payed for the water management during the desorption and injection into the GC.

After setting-up the sampling parameters, a fine optimization of the GC×GC separation was realized. Particular attention was devoted to the flow modulation conditions to make possible both the unitary transfer into the ^{2}D and an efficient modulation. The flow modulation approach used is based on previous researches, where the accumulation and injection phases of modulation were reconsidered in terms of intra-loop chromatographic bands [29,31]. Differently from other differential flow modulation approaches, where incompatible MS-flows are used (>20 mL/min), a fine matching of these bands within the loop during the modulation timings allows for an efficient modulation with low flows (< 7 mL/min), which enables the full transfer into the ^{2}D and the detector with no need to divert the effluent, and thus preserving the sensitivity. For such a reason, the terminology (low-)flow modulation GC×GC is used. In a previous attempt, the same modulation concept was applied to an HR-ToF, with this mainly focusing on the proof-of-principle capability of coupling flow modulation with high resolution MS, where only 3.4-2.1 mL/min (43-34% of the total flow) was directed to the detector [30]. Therefore, this contribution represents the first research were a total transfer, also called modulation unit duty cycle, is successfully exploited on a ToF mass analyzer.

In Figure 1A-C, the results from the optimized conditions are showed. At first, the adjustment of the accumulation and reinjection period was performed on alkanes standards. A solution of C_9-C_{12} alkanes was subjected to GC×GC-MS analyses under isothermal conditions (150 °C), and using a loop of 20 cm × 0.51 mm id. This is a standard procedure used to optimize, as rapidly as possible, the modulation conditions. Obviously, these alkanes elute within an acceptable elution time at 150 °C. The choice of a shorter accumulation loop (and a single accumulation/re-injection step) was also made to simplify the flow modulation optimization process.

The optimized GC×GC conditions consisted of a primary and secondary column flow of 0.4 mL/min and 7 mL/min, respectively. The final P_M was 6.6 sec, consisting of 6 sec and 0.6 sec, respectively for the accumulation and the reinjection time. During the two stages of the modulation, the pressure conditions inside the loop generate different average linear velocities of the isolated chromatography bands during the accumulation and reinjection time, respectively $\bar{u}_{acc}$ and $\bar{u}_{inj}$. These conditions of gas flow generated an intra-loop $\bar{u}_{acc}$ of 1.5 cm/sec; at the end of the accumulation time (6 sec), the analyte band should occupy a loop length of 9 cm. The intra-loop conditions during the subsequent modulation stage (0.6 sec) generated a $\bar{u}_{inj}$ of 34.4 cm/sec, which was sufficient for the complete ejection of the analyte bands from the loop. Indeed, such conditions gave adequate peak-shape and baseline drop. For C_{12} alkane, a peak width of 600 msec (4σ) was observed and the untransformed chromatogram is shown in Figure 1A.

Please insert here Figure 1

Following the optimization of the modulator gas flows and velocities, a mix containing 37 compounds (isomers included) of various chemistries was flash-vaporized into the sorbent tubes (see section 2.2 for details) and injected to adjust the desorption conditions and to evaluate the overall GC×GC separation (Figure 1B). The optimized GC×GC conditions and the apolar-midpolar column set enabled a good occupation of the 2D separation space. The chromatographic performance attained can be observed in Figure 1C, which reports an untransformed 60-sec expansion relative to the elution zone of peaks 1-4. Here, eucalyptol (peak 2) is baseline separated from benzyl alcohol (peak 3, $R_s \geq$ 1.5) and the column bleed.

3.2 Beer analysis and data processing workflow

Twenty chromatograms were obtained from the 5 sample types (n=4) and were aligned based on retention times and mass spectra, obtaining a total of 457 peaks (see section 2.3 for alignment details). After artifact removal (siloxanes, phthalates, and bleed from the columns), a refined list of 358 peaks was obtained.

Please insert here Figure 2

For further data analysis and to make the downstream data analysis more reliable and robust, an inclusion criterion was applied to consider only the peaks detected in at least 3 out of 4 replicates (FOO of 75%) within each group type. These are defined here as the most consistent features and they resulted in a list of 285 peaks. Figure 2 shows the overall flow of data treatment applied in the present investigation. The Venn diagram in Figure 2 (right side) depicts the qualitative distribution of the features amongst the 5 beer types. As can be observed, the majority (65) of the peaks consistently detected in the headspace are shared by all the fruit beers analyzed; interestingly, each beer is characterized by a unique set of compounds, ranging from 13 (peach) to 21 (apple), and contributing to their volatile composition and aroma. It must be said that, beside the qualitative odor of the compound, the odor threshold is the most important quantitative parameter for the aroma formation and characterization [7]. The information of these representative peaks, along with retention indices and odor characteristics, are reported in Table 1.

Unmodulated GC-MS profiles were concurrently acquired to evaluate the additional value provided by GC×GC, namely the higher sensitivity and separation power. In Table 1, *S/N* values of representative peaks and unique for each beer type (*i.e.* those features at the extremities of the ellipses in the Venn diagram, Figure 2), are reported for the unmodulated GC and GC×GC runs. A characteristic ion for each peak was selected to extrapolate the *S/N* values. For 12 compounds, some factors made difficult the extrapolation of *S/N* values in the unmodulated chromatograms. This was due to one or the combination of the following issues: 1) the lower sensitivity of the unmodulated approach, 2) the more frequent occurrence of co-elutions combined with non-selective ions for *S/N* calculation. For such peaks, unmodulated GC *S/N* values are not reported.

Signal intensities resulted higher in the GC×GC experiment due to the rapid second dimension elution conditions. On the other hand, the noise amplitude is comparable, because the same MS acquisition frequency (*i.e.* 150 Hz) was used in both unmodulated and GC×GC analysis. In terms of *S/N* values, an average ×3-fold increase is obtained in the GC×GC trace, ranging from a factor ×1.2 (1-dodecanol) to ×5.2 (*trans*-rose oxide). One of the reported peaks (*i.e.* 10-undecen-1-ol) shows almost identical *S/N*. The reason for this S/N range can be explained by a higher extent of band

broadening due to the greater retention on the secondary column, which affects negatively the signal intensities of the more polar compounds.

It must be said that for a more appropriate S/N comparison with traditional GC, a single column configuration should be used. Indeed, the unmodulated conditions are far from ideal in terms of band broadening, because of the contribution of the accumulation loop and the ^{2}D column. Another way to increase the sensitivity is the adoption of fast-GC methods with a higher column flow and/or narrow bore columns (*i.e.* ≤ 0.18 mm id).

Please insert here Table 1

The application of a FOO threshold greatly improves the data analysis and interpretation (step c, Figure 2), directing the attention to the more consistently detected peaks. The raw area (*i.e.* not normalized or log-transformed) of these features was used to evaluate the degree of correlation and repeatability within the groups. To do this, Spearman correlation and Euclidean distances were used as metrics [36]. The former is generally used to measure the strength of a correlation and the resulting correlation matrix is illustrated in Figure 3A. Here, the sample groups resulted very strongly correlated, with average Spearman values ranging from 0.85 (apple) to 0.91 (berries).

To evaluate the repeatability of the overall method in the untargeted analysis, a distance matrix was built considering the samples. The average values were extrapolated and are showed in Figure 3B. Here, a value close to 0 would indicate the near proximity of the samples, and thus their similarity in the qualitative and quantitative VOCs composition. From the table inset of Figure 3B, it can be noted that the intra-group distances on the diagonal are 3 times smaller than the inter-group distances between different sample-types. Such a distance matrix and the dendrogram overview is an indication of 1) the high repeatability of the overall method for samples of the same group, and 2) the separation of the groups based on their proximities.

Please insert here Figure 3

Once the intra-group repeatability and correlation were assessed, the next step involved the untargeted analysis of the samples using the VOCs. The 285 consistent features obtained from the neat unsupervised data analysis workflow illustrated in Figure 2 were plotted in the principal component space to visualize the variance between these sample types (Figure 4A). The data

scaling (auto-scaling) for PCA was the only data manipulation realized. Indeed, as a result of the controlled analytical conditions and the distinctive sample-types, the 285 features were not further treated (*i.e.*, transformation or normalization). However, for larger long-term studies, the addition of a water-soluble internal standard would be beneficial to monitor and correct the variations in case of instrumental drift.

In the PCA of Figure 4A, the first two components explained 42.5% of the variance and a clear clustering is observed for all the sample groups. The HCA provides an intuitive visualization of the features amongst the samples. This is plotted in Figure 4B, where a complete differentiation of the 5 beer types is observed. Because of the satisfactory discrimination between the beers and the high amount of information usable with this untargeted approach, no additional data analysis steps (*e.g.*, feature selection and reduction) were necessary.

Please insert here Figure 4

The overall methodology herein reported, intended as the combination of the sampling, analytical measurements, and untargeted data processing workflows, makes possible the extrapolation of a high amount of unbiased information from the aroma, which can be used for beer differentiation and for odor notes extrapolation (Figure 4).

However, it represents one possible usage of the information provided. Indeed, a profound knowledge of the beer-making stages together with a deeper scrutiny of the compounds list would certainly help, for example, to identify markers to tailor and fix specific industrial processes.

Concluding remarks

The proposed analytical method, including the clear and straightforward data analysis workflow, was demonstrated to be a powerful and repeatable approach for aroma analysis, providing the information for the VOCs characterization of fruit beers. The use of purge-and-trap for sample preparation resulted particularly suitable for VOC extraction in beer and it represents a valid alternative to static headspace techniques (*i.e.,* SPME). The P&T device herein used can be easily implemented at the production site for *in situ* sampling.

The routine application of the entire methodology to the analysis of commercial products could be an effective tool for the monitoring of technical processes that influence fruit beer making. Furthermore, this method can easily be extended and tailored to other liquid samples and problematics as well, either in food, biological or environmental applications.

From a technical point of view, this is the first published use of P&T sampling, thermal desorption and GC×GC-MS in combination. The high sensitivity and the green nature of P&T sampling make this extraction technique an ideal up-front tool for a flow modulated GC×GC-MS system. In this regard, the concept of a differential flow modulator with total transfer at MS-compatible flows is demonstrated on a ToF MS, confirming the effectiveness of the approach, both in terms of selectivity and sensitivity, and with this research representing its initial use. In terms of sensitivity, the *S/N* gain of this (low-)flow modulation approach resulted in the range of 1.2-5.2 fold higher compared to the unmodulated GC method.

It is anticipated that future studies will exploit the present combination in biological and environmental applications.

Conflict of interest

All authors report no potential conflicts of interest.

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Figure legends

Figure 1. Raw chromatograms and contour plot resulting from the (low-)flow modulated GC×GC-ToF MS optimization. A) Untransformed chromatogram expansion of C_9 alkane; B) contour plot of the 37 stds mix (including isomers). C) Raw chromatogram expansion of the rectangle in B; marked with * the highest modulated peaks. All the chromatograms are visualized using the total ion current (TIC). For peak identification, refer to Supplementary Table S1.
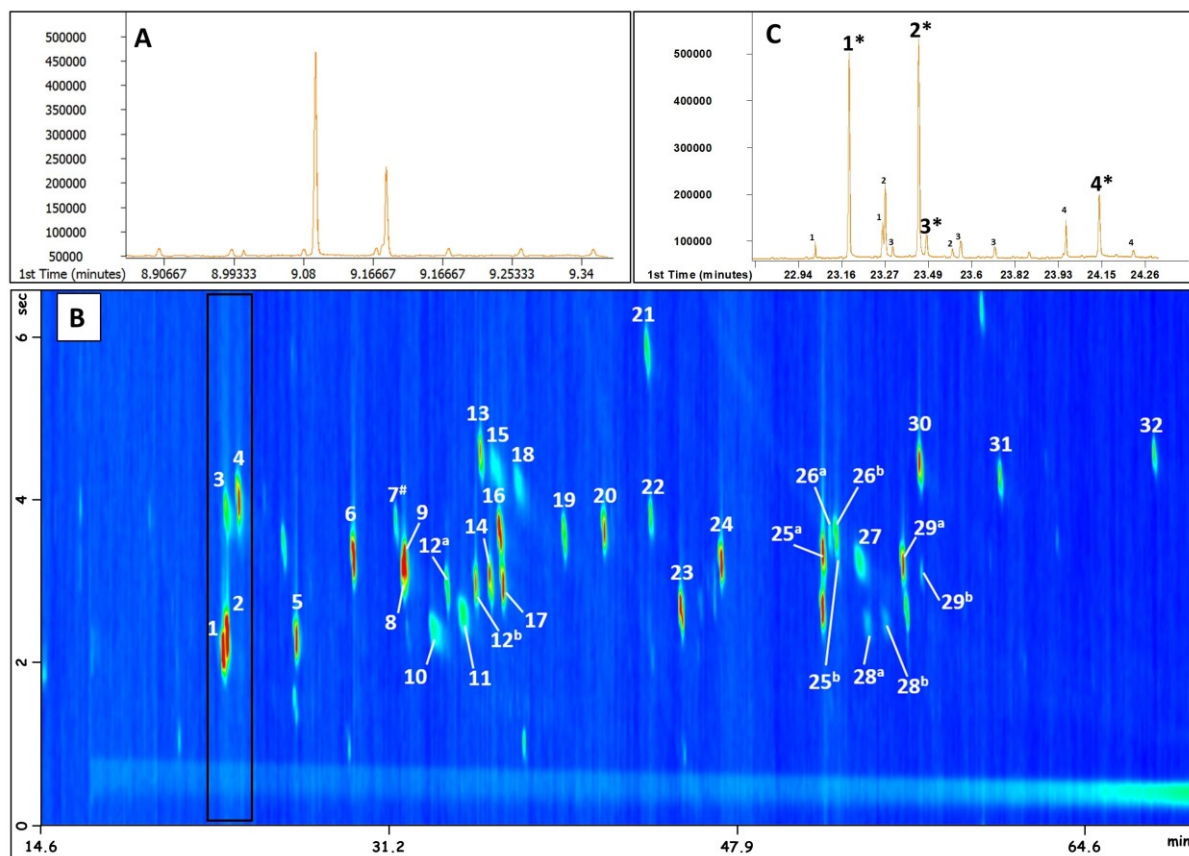
Figure 2. Scheme illustrating the flow of data treatment for the beer analysis. The numbers in the circles relate to the number of analytes detected. The Venn diagram shows the qualitative distribution of the compounds amongst the different beer types. Step a) Chromatograms alignment; Step b) artifact removal using scripts; Step c) adoption of an inclusion criterion to retain the more consistent features (presence in at least 3/4 replicates within each group type).
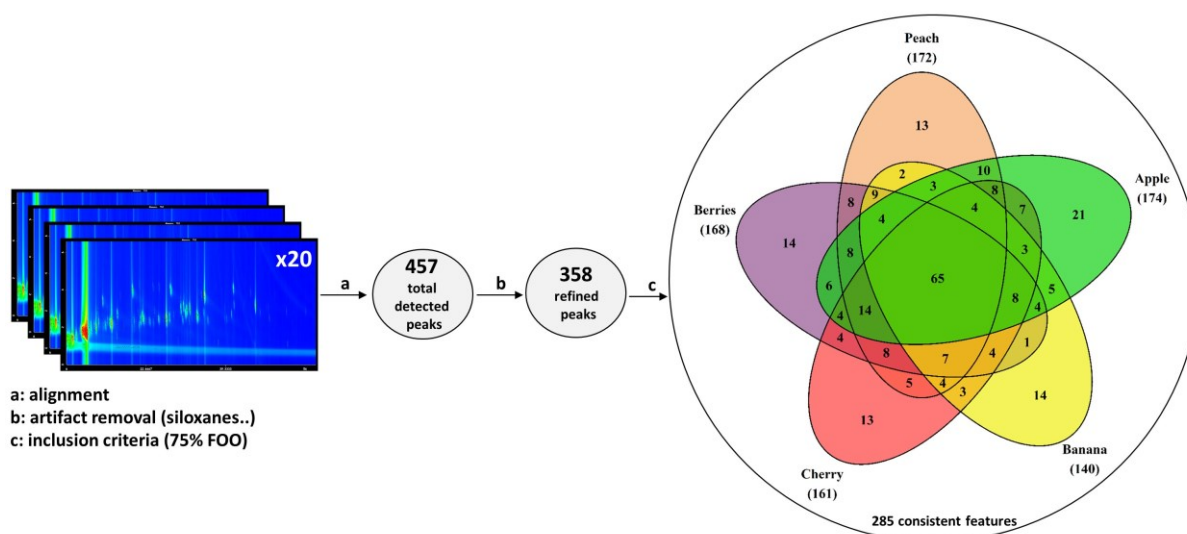
Figure 3. *Correlation matrix (A) and dendrogram from hierarchical clustering (B), using the 285*

consistent features. The correlation was calculated using Spearman's coefficient and the average

values for each group are reported in table inset. The dendrogram was obtained using the Euclidean

distance metric on the samples and the average values for each combination of sample groups are

reported in the table inset.
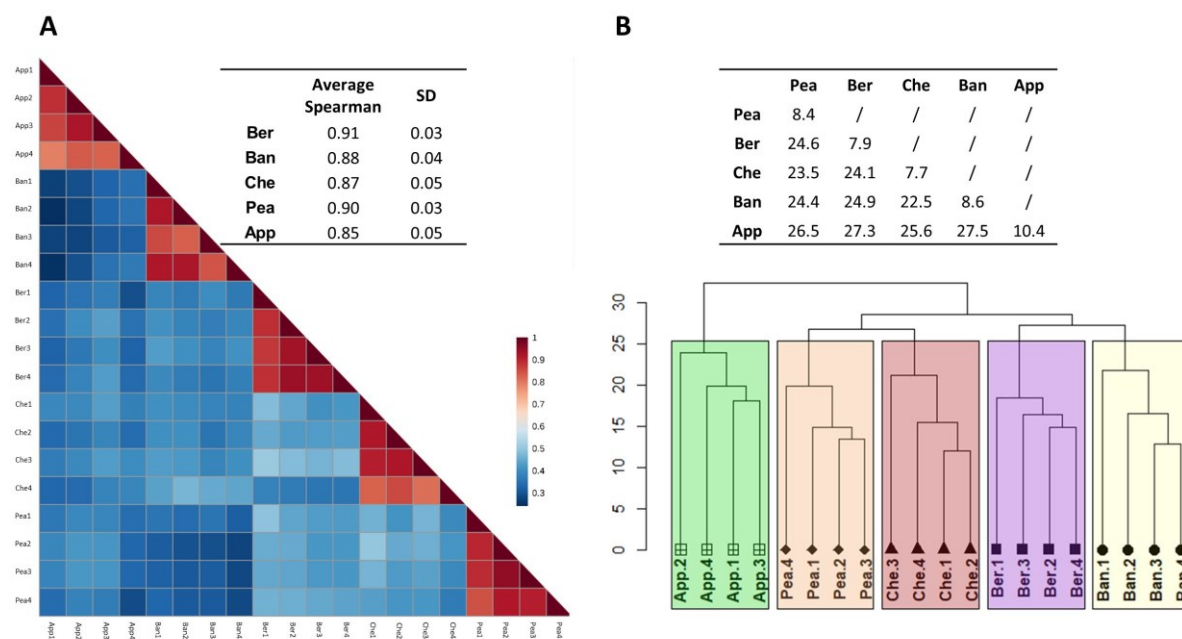

Figure 4. *Principal component analysis (A) and heatmap generated using hierarchical clustering*

analysis (B) of the 5 fruit beer types. The ellipse in A represents the 95% confidence interval. Cluster
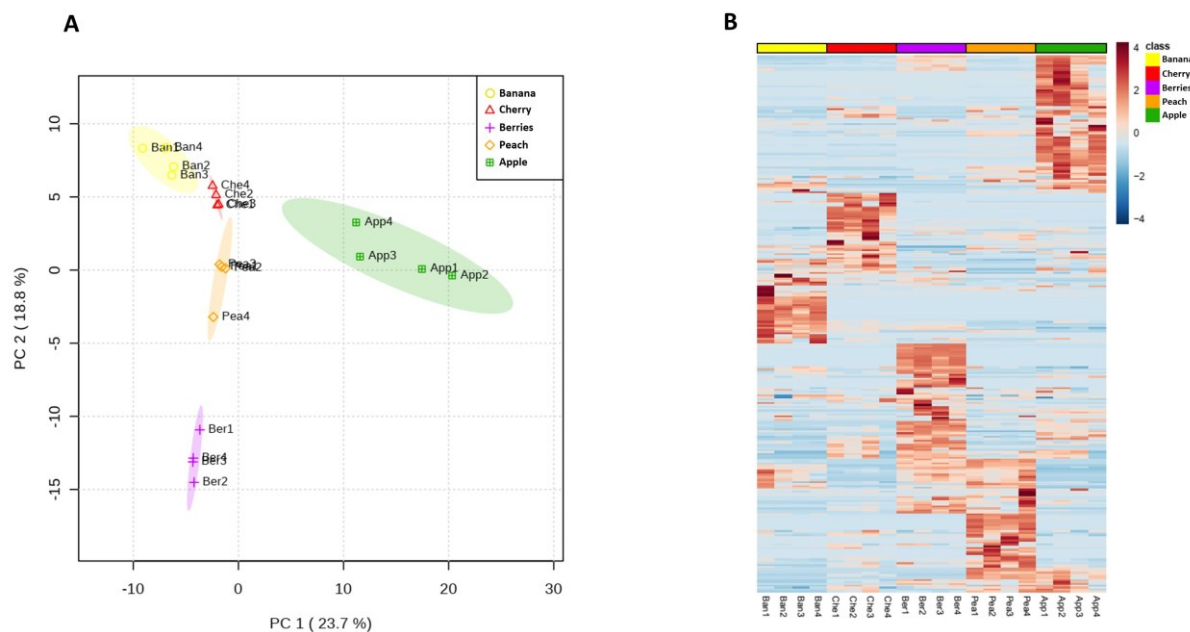
algorithm for HCA: Ward.

Table 1. *Representative analytes from the uniquely detected peaks in each beer type. The* IUPAC name, *MS library similarity, experimental and reference RI, S/N, m/z, and odor notes are also reported.* For some compounds, the common name is also reported in parenthesis.

Tentative ID	Unique	MS similarity	exp. RI GC×GC	ref. RI#	Δ RI	m/z	S/N GC×GC	S/N GC	S/N gain	odor note*
(*E*)-hex-2-enal	App	805	849	843	6	83	525	NI	-	Green, fatty
3-methylbutanoic acid (Isovaleric acid)	App	790	869	862	7	60	1365	385	3.5	Floral, fatty, sweet, acidic
2-methylbutyl acetate	Ban	921	881	877	4	72	1779	1337	1.3	Fresh, banana, citrus
3-methyl-3-butenyl acetate	Ban	837	889	879	10	68	1360	589	2.3	Banana, fruity
Butyl butanoate	App	933	999	993	6	71	2955	1605	1.8	Fatty, flowery, fruity, rotten apple, sweet
Phenylmethanol (Benzyl alcohol)	Cher	925	1040	1038	2	79	169	NI	-	Alcohol, aromatic, fruity, floral
4-methyl-2-(2-methylprop-1-enyl)oxane (*Cis*-rose oxide)	Ber	896	1116	1112	4	69	8548	1682	5.1	Bitter, camphor, floral, fresh, green lemon, strawberry

4-methyl-2-(2-methylprop-1-enyl)oxane *(Trans*-rose oxide)	Ber	856	1134	1127	7	69	756	147	5.2	Floral, rose, grass
(*E*)-non-3-en-2-one *(Trans*-3-nonen-2-one)	Pea	848	1147	1143	4	55	149	NI	-	Fruity, nutty, powdery
5-methyl-2-propan-2-ylcyclohexan-1-one *(Iso*-menthone)	Ber	935	1175	1168	7	69	5890	1348	4.4	Fresh, minty
5-methyl-2-propan-2-ylcyclohexan-1-ol (Menthol)	Ber	938	1184	1171	13	71	2777	679	4.1	Minty, fresh, grass, woody
2-(4-methylphenyl)propan-2-ol *(Para*-cymen-8-ol)	App	863	1197	1186	11	43	174	NI	-	Floral, musty, sweet, citrus
5-methyl-2-propan-2-ylidenecyclohexan-1-one (Pulegone)	Pea	891	1256	1237	19	67	121	NI	-	Mint, balsamic, pungent
2-methyl-5-prop-1-en-2-ylcyclohex-2-en-1-one (Carvone)	Pea	916	1260	1243	17	82	640	160	4.0	Mint, herbal
4-methoxybenzaldehyde *(Para*-anisaldehyde)	Che	889	1265	1258	7	135	106	NI	-	Anise, cucumber, sweet, floral, hay
(E)-3-phenylprop-2-enal *(Trans*-cinnamaldehyde)	Che	940	1283	1274	9	131	216	NI	-	Cinnamon, honey, sweet
Methyl (Z)-3-phenylprop-2-enoate (Methyl *cis*-cinnamate)	Ber	908	1313	1304	9	131	125	NI	-	Floral, sweet, strawberry
Benzyl butanoate	Ban	935	1356	1345	11	91	2542	1139	2.2	Cantaloupe, fresh, sweet, pineapple
3,7-dimethyloct-6-enyl acetate (Citronellyl acetate)	Ber	853	1356	1350	6	81	219	NI	-	Berry, citrus, fresh, rose, herbal
2-methoxy-4-prop-2-enylphenol (Eugenol)	Ban	936	1370	1366	4	164	599	240	2.5	Floral, green, herbal, sweet, vanilla, wood

Undec-10-en-1-ol	Ban	940	1372	1361	11	55	13172	13557	1.0	Fresh, floral, waxy, clean, citrus
Methyl (E)-3-phenylprop-2-enoate (Methyl *trans*-cinnamate)	Ber	925	1394	1381	13	131	4997	1097	4.6	Floral, sweet, strawberry
(E)-1-(2,6,6-trimethylcyclohexen-1-yl)but-2-en-1-one (*β*-damascone)	App	876	1401	1388	13	69	478	317	1.5	Apple, flower, fruity, herbs, sweet
(E)-4-(2,6,6-trimethylcyclohex-2-en-1-yl)but-3-en-2-one (*α*-ionone)	Pea	845	1440	1426	14	121	160	NI	-	Floral, sweet fruit, woody
2-phenylethyl butanoate	Pea	899	1455	1447	8	104	142	NI	-	Fruity
Dodecan-1-ol	Ban	901	1491	1480	11	55	497	401	1.2	Fatty, fruity, flowery, sweet, waxy
2-phenylethyl (E)-2-methylbut-2-enoate (Phenethyl tiglate)	Ber	917	1599	1590	9	104	241	NI	-	Floral, caramel, sweet, green, balsamic, herbal

NI: not identified

[#], [*]: reference RI and odor notes were obtained from AromaOffice®