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A novel approach to assess the quality and authenticity of Scotch Whisky based on gas chromatography coupled to high resolution mass spectrometry

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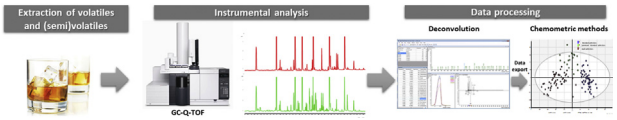
HIGHLIGHTS

- Two sample handling strategies were compared for isolation of whisky components.
- Non-target fingerprinting of Scotch Whiskies by GC-Q-TOF was applied.
- Chemometric methods were employed for the assessment of the authenticity.
- Characteristic markers were found and tentatively identified.
- Identification of fake samples based on GC-Q-TOF fingerprints is documented.

GRAPHICAL ABSTRACT



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ABSTRACT

Whisky is one of the most popular spirit drinks in the world. Unfortunately, this highly valued commodity is vulnerable to fraud. To detect fraudulent practices and document quality parameters, a number of laboratory tests based on various principles including chromatography and spectroscopy have been developed. In most cases, the analytical methods are based on targeted screening strategies. Non-targeted screening (metabolomics fingerprinting) of (semi)volatile substances was used in our study. Following the pre-concentration of these compounds, either by solid phase microextraction (SPME) or by ethyl acetate extraction, gas chromatography (GC) coupled to tandem mass spectrometry (Q-TOF mass analyser) was employed. Unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS–DA) were used for evaluation of data obtained by analysis of a unique set of 171 authentic whisky samples provided by the Scotch Whisky Research Institute. Very good separation of malt whiskies according to the type of cask in which they were matured (bourbon versus bourbon and wine) was achieved, and significant 'markers' for bourbon and wine cask maturation, such as N-(3-methylbutyl) acetamide and 5-oxooxolane-2-carboxylic acid, were identified. Subsequently, the unique sample set was used to construct a statistical model for distinguishing malt and blended whiskies. In the final phase, 20 fake samples were analysed and the data processed in the same way. Some differences could be observed in the (semi)volatile profiles of authentic and fake samples. Employing the statistical model developed by PLS-DA for this purpose, marker compounds that positively distinguish fake samples were identified.

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1. Introduction

The presence of counterfeit foods and beverages in worldwide markets is a continuous problem, with counterfeiting techniques becoming increasingly more sophisticated [1]. The worldwide popularity of Scotch Whisky encourages unscrupulous traders to fraudulently sell their non-genuine products under its protected name, unfairly trading on its global reputation [2]. Unsurprisingly, this scenario is not only a concern for consumer protection agencies, Scotch Whisky producers and legitimate traders but also inspection and custom authorities [3]. During the Operation Opson V' [4] action taken by Interpol in 2015 and early 2016, over 385 thousand litres of alcoholic beverages were seized; one of the most counterfeited commodities was Scotch Whisky. Typically, brands are counterfeited using liquid formulations based upon: (i) a cheaper version of whisky belonging to the same category as the genuine brand (e.g. a deluxe Scotch Whisky being replaced by a locally bottled one), (ii) a cheap local alcohol mixed with whisky from the genuine category, or (iii) a cheap local alcohol with added flavourings and colouring [5]. Fraudulent Scotch Whisky may also be mislabelled as to its maturation in oak casks. The maturation period and the history of the casks in which maturation occurred are important quality characteristics, particularly in the case of premium brands.

The Scotch Whisky Regulations 2009, SI 2009/2890 [6], which is a part of UK legislation, defines the following requirements as to the process of Scotch Whisky production: Scotch Whisky is *inter alia* (i) distilled at a distillery in Scotland from water and malted barley to which only whole grains of other cereals may be added, (ii) matured only in oak casks of a capacity not exceeding 700 L, (iii) matured only in Scotland, (iv) matured for a period of not less than three years and (v) not allowed to contain any additives other than plain caramel and/or water.

Under these conditions, a number of facts have to be taken into consideration when authenticating Scotch Whisky. The water, the cereals used, the potential application of peat smoke during barley malting, and the distillation process will all have an influence, to a greater or lesser extent, on the final product. The oak cask in which maturation takes place is an important contributor to overall whisky quality. During the time that the raw distillate spends in the cask, major changes occur in the chemical composition of the spirit, resulting in a product which has mellowed and become more acceptable to the palate [7]. In general, the change in the profile of flavour significant compounds during the maturation of distilled beverages, including whisky, is due to a number of ongoing processes (i) direct extraction of wood compounds, (ii) decomposition of wood macromolecules, such as lignin, and the extraction of their products into the distillate, (iii) reactions between wood components and the constituents of the raw distillate, (iv) reactions involving only wood extractives, (v) reactions involving only the distillate components and (vi) evaporation of volatile compounds [8].

Scotch Whisky authentication has been the subject of a number of papers in the scientific literature, employing a range of analytical techniques. Recently, the use of portable authentication techniques, designed to provide a rapid indication of a sample's authenticity, has received particular attention. The employment of a portable device based on UV–Vis spectroscopy was demonstrated by MacKenzie and Aylott for the authentication of Scotch Whisky brands. Counterfeits, most of which were combinations of cheap local alcohol flavoured with a smaller proportion of whisky and colouring, could be distinguished from genuine samples [9]. The same approach was used by Martins et al.; large amounts of authentic ($n = 164$) and counterfeit samples ($n = 73$) were analysed and in combination with PLS-DA resulted in a brand prediction efficiency of at least 93% [10]. Such methods rely on the construction of a large database of UV–Vis spectra from individual brands, for comparison with suspect samples, and thus tend to be best undertaken by brand owners. In addition to brand profiling, an abnormal UV–Vis spectrum may indicate the presence of non-permitted compounds in spirit samples [5,11].

Raman spectroscopy has also been explored for its potential to authenticate Scotch Whiskies, since the opportunity of analysis through the spirit drink bottle using such a technique is an attractive option for fraud detection [12,13]. Other spectroscopic techniques that have been looked at for the authentication of different spirit drink categories include mid-infrared spectroscopy [14,15] and fluorescence spectroscopy [16]. Unlike UV–Vis spectroscopy, such (potentially) portable technologies, though promising, have not developed to the state of being routinely used for spirit drink authentication.

Portable spectroscopic techniques are typically based on an untargeted chemometric analysis of the spectral range measured. Consequently, application has been to the authentication of specific Scotch Whisky brands (brand authentication), where spectral deviations are contained by an effort to maintain consistency of product. Application to the authentication of a product category such as Scotch Whisky (generic authentication) is difficult using such techniques, due its chemical diversity. However, application of portable UV–Vis spectroscopy to the quantification of carbohydrates has been demonstrated, to identify counterfeit Scotch Whisky irrespective of brand [17]. Raman and NIR spectra have also been used to measure alcohol strength (ethanol content) and therefore, potentially, demonstrate non-compliance with the category's minimum alcohol strength requirement [18].

The use of portable techniques, such as UV–Vis spectroscopy, allow rapid authenticity evaluations to take place in the laboratory or at key points in the supply chain, for example at point of sale. However, they are currently employed as a screening tool, to reduce the number of suspect counterfeit samples that will be analysed by traditional, authoritative, laboratory reference methods. The typical laboratory authentication strategy used for Scotch Whisky is based on commonly used gas and liquid chromatography techniques (including GC-FID and LC-UV). These are used to quantify a number of common constituents of Scotch Whisky, the results of which can then be used for either generic and brand authentication. Generic authenticity analysis of complex sample categories, such as Scotch Whisky, is significantly more complicated compared to brand authenticity analysis, which is based on relatively narrow analytical fingerprints. Analytical data for generic category analysis needs to adequately represent all the types and styles of product in a respective category [3].

Attempts have been made to circumvent the complication of analytical diversity in the generic authentication of Scotch Whisky by addressing specific chemical characteristics unique to all Scotch Whiskies. Meier-Augenstein et al. used high temperature conversion-isotope ratio mass spectrometry for the ^{2}H and ^{18}O isotope analysis of eleven whisky samples from several Scotch Whisky distilleries. The potential of this approach to detect false statements relating to geographical location of production (i.e. Scotland) was reported [19]. However, this is complicated by the fact that whilst Scotch Whisky must be produced in Scotland, it may be diluted to bottling strength elsewhere in the world. The isotopic properties of the water used for bottling will affect the usefulness of this technique.

Within the compounds analysed as part of a typical analytical strategy for the authentication of Scotch Whiskies (brand or generic), the concentrations and ratios of major volatile compounds, particularly methanol, n-propanol, 2-methylpropan-1-ol and 2- and 3-methyl butanol, have been found to be important

factors in the authenticity decision-making process. The absence of these congeners, or their presence in abnormal concentrations, contributes to the decision making process [3]. Within this study, our goal was to reveal new compounds characterising authentic samples and compounds occurring in fraudulent whiskies using advanced analytical techniques.

A similar approach was adopted by Kew et al., who applied Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to the assessment of chemical diversity and complexity in Scotch Whisky. The data obtained by analysis of 85 whisky samples were interpreted by multivariate analysis, including Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). Discrimination between types of Scotch Whisky (blend or malt) was achieved, as well as discrimination based on the type of cask in which the product was matured. 'Markers' characterising the type of whisky or cask type were detected. Syringic acid, ellagic acid and gallic acid were tentatively identified as possible discriminants for single malt whisky, reflecting the longer periods of maturation malt whiskies typically undergo. These compounds, along with glucono delta-lactone were also responsible for the discrimination between samples matured in sherry and bourbon casks (representing molecules occurring in greater quantities in whiskies aged in sherry cask). Decanoic, dodecanoic, hexadecanoic, hexadec-9-enoic and tetradecanoic acids were typically higher for samples matured in bourbon casks [20].

The current case study has been conducted within the EU-funded FoodIntegrity project (www.foodintegrity.eu), the aim of which is the development of effective strategies to support food integrity – i.e. food's safety, quality, authenticity and traceability. The objective of this study was to develop a novel analytical strategy for Scotch Whisky authentication, based on a non-targeted fingerprinting approach utilising gas chromatography coupled to tandem high-resolution mass spectrometry (Q-TOF mass analyser). For isolation of GC-amenable sample components, solid phase microextraction technique (SPME) and ethyl acetate extraction were employed as possible alternatives. The acquired data set of unique fingerprints was then processed using multi-dimensional chemometric strategies represented by PCA and PLS-DA.

Contrary to other studies concerned with GC-MS analysis of whisky volatiles [21–23], identification of all the detected compounds was not the primary objective, since many of the (semi) volatile compounds occur in different whisky brands (and other spirits) at comparable levels, and thus can contribute poorly to their unequivocal classification. Instead, recording unique sample fingerprints seemed to be the more challenging, but rewarding, option. For this purpose, chromatographic separation (gas chromatography, in this particular case) with high resolution mass spectrometric detection clearly represented the technique of choice.

The developed strategy was applied to a set of genuine Scotch Whisky products containing certain known quality characteristics, as well as a set of pre-identified fraudulent whisky products. As such, it was possible to test the strategy's ability to authenticate both Scotch Whisky (when compared to known 'fakes'), or a particular characteristic of genuine Scotch Whisky, such as its maturation history. It was also possible to identify marker components associated with such distinctions that could be employed successfully in a targeted analysis.

2. Experimental

2.1. Whisky samples

191 whisky samples (15 mL each in a tightly closed glass vial) were delivered by the project partner, the Scotch Whisky Research Institute (SWRI, Riccarton, UK). The sample batch contained several subsets: (i) 71 authentic malt whiskies; (ii) 77 authentic blended Scotch Whiskies; (iii) 20 samples identified as fake'; and (iv) 23 supplemental malt whiskies specially selected as having been matured in more than one type of cask. The samples described as fake' had been previously identified as fraudulent by the analytical strategy employed in the SWRI's laboratory. Malt whiskies from respective distilleries were characterised (where known) by their maturation age and cask type, and, for some of them, information about peating was provided too. After delivery, the samples were stored in a refrigerator (4 °C). Before analysis (completed within four weeks of delivery), the samples were equilibrated to room temperature.

2.2. Chemicals and reagents

Analytical grade ethyl acetate, methanol and formic acid were purchased from Merck (Darmstadt, Germany). Water purified by a Milli-Q® Integral system supplied by Merck was used throughout the study. 2,3,4,5,6-pentadeuteriophenol (98% purity), used as a standard for retention time locking, was obtained from Sigma-Aldrich (Steinheim, Germany). Anhydrous sodium sulphate (Penta Praha, Czech Republic) was activated by heating at 600 °C for 6 h and further stored in a desiccator.

2.3. Sample preparation

Two alternative sample preparation strategies prior to GC-MS analysis were used; their optimisations are described below.

2.3.1. Solid phase microextraction

Three SPME fibres (all supplied by Supelco, Bellefonte, USA) were tested: (i) 100 μm polydimethylsiloxane (PDMS), (ii) 85 μm polyacrylate (PA) and (iii) 50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS). Prior to use, all fibres were conditioned following the manufacturer's recommendations. All the key parameters that may affect SPME performance, such as type of fibre coating, incubation time (5, 10, 20 and 30 min) and temperature (30, 40, 50, 60 and 70 °C), and extraction time (5, 10, 20 and 30 min) and temperature (30, 40, 50, 60 and 70 °C), were tested on whisky samples in 10 mL glass vials. Different whisky sample preparations were also tested: dilution with water (1:1, 1:2 and 1:3 *v/v*) and the addition of a saturated solution of NaCl. For method development and quality control (QC), a pooled sample of all individual samples was used. The optimal parameters selected for the entire study were as follows: SPME fibre – DVB/CAR/PDMS, incubation temperature – 60 °C, sample incubation time – 10 min, extraction temperature – 60 °C, extraction time 10 min. For the preparation of the whisky samples, their dilution with water (1:3 *v/v*) and saturation with NaCl to the final volume of 3 mL (0.75 mL of the sample and 2.25 mL of water) was found to be optimal.

2.3.2. Ethyl–acetate extraction

3 mL of whisky sample was transferred into a 50 mL polypropylene centrifuge tube and 100 μl of 2,3,4,5,6-pentadeuteriophenol, 10 mL of 1% (*v/v*) aqueous formic acid solution and 15 mL of ethyl acetate were added. The mixture was vigorously shaken for 2 min by hand. Centrifugation (10,000 rpm, 5 min) was used for phase separation. The upper phase (ethyl acetate) was dried by filtration through 10 g of sodium sulphate. The extract was evaporated to the last drop by a gentle stream of nitrogen and the residue was re-dissolved in 0.5 mL of ethyl acetate. 1 μl of the final extract was injected into the GC-MS instrumentation.

2.4. GC-MS

An Agilent 7200b system consisting of Agilent 7890B gas chromatograph equipped with a multimode inlet, PAL RSI 85 for automated head space–solid phase microextraction (HS–SPME) and direct injection, and quadrupole–time of flight mass spectrometer (Q-TOF) (Agilent Technologies, Palo Alto, California, USA), was employed. For the instrument control and data acquisition MassHunter GC/MS Acquisition (Agilent Technologies, Palo Alto, California, USA) software (B.07.03.2129) was used. Sample components were separated on a 25 m HP–INNOWax capillary column (0.2 mm id, film thickness: 0.2 μm; Agilent Technologies, Palo Alto, California, USA).

For both sample preparation strategies, samples were injected in splitless mode (splitless period 1 min) at 230 °C and the oven temperature program was as follows: 60 °C (1 min), 20 °C/min to 240 °C (8 min).

To prevent deviations of the retention times of compounds, retention time locking was applied. For the calibration and locking of the GC method, 2,3,4,5,6-pentadeuteriophenol at a retention time of 7.247 min with flow 1.2 mL min^{-1} was used.

The mass spectrometric detector was operated in the electron ionization (EI) mode. The temperature of the ion source was 230 °C. The mass range was 50–550 m/*z* and the resolution of the mass analyser was set >12,500 (FWHM). The temperatures of the ion source and quadrupole in positive chemical ionization (PCI) were 300 °C and 150 °C, respectively.

2.5. Data analysis

MassHunter Unknowns Analysis (Version B.08.00) software was employed for raw data processing. Following spectral deconvolution, all detected signals were exported to Mass Profiler Professional (v.B.13.0) where peak alignment according to their mass (±10 ppm) and retention time (±0.1 min) together with filtration according to frequency occurrence were performed. In the next step, the data set obtained by filtration was exported to MassHunter Quantitative Analysis (v.B.08.00) software for recursive analysis. All software were from Agilent Technologies, Palo Alto, California, USA.

In the final phase, the normalised data (relative intensities of each signal obtained by division of the sum of all signals) was investigated by multivariate chemometric analysis. SIMCA (UMETRICS, Umea, Sweden) software was employed for unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). A Variable Importance for the Projection (VIP)-plot illustrating the distribution of the detected compounds involved in the statistical evaluation was used as a tool for the selection of 'markers'. Compounds positioned on the left side of the VIP-plot can be considered as 'markers' with the highest importance for the separation of samples.

2.6. Identification of marker compounds

Marker compounds were identified and verified using NIST17 library, isotopic pattern, exact mass (mass error <5 ppm), Kovats retention index and positive chemical ionization in MS and MS/MS modes. The latter technique, thanks to soft ionization, enables obtaining adducts of molecular ions of compounds which are extensively fragmented under EI conditions.

3. Results and discussion

3.1. The choice of analytical strategy

As mentioned in the Introduction, a number of counterfeiting techniques for whisky have been documented over time, and various analytical approaches have been used for their detection. In this study, we decided to base authentication on the unique 'metabolomic' fingerprints of (semi)volatile whisky components using GC-HRMS.

The benefit of employing a HR-TOF-mass analyser was demonstrated when comparing the number of peaks in the total ion chromatogram (TIC) before and after the application of the deconvolution function. Although chromatographically unresolved, the number of detected compounds increased on average by 55% (median 260) when using the SPME sample preparation technique. As illustrated in Fig. 1, even more compounds, particularly in the case of malt whisky, were found when analysing the ethyl-acetate extract (median of 493 compounds, after deconvolution). This contained a number of semivolatile compounds that have minimal presence in the sample headspace and are therefore hardly detectable when employing SPME sampling. With regards to these preliminary results, the ethyl-acetate extraction seemed to be the more suitable sample preparation technique.

Compared to earlier studies (shown in Table 1) in which the HS-SPME-GC-MS technique was used, the possibility to resolve co-eluted compounds by HRMS enabled not only faster analysis but also more comprehensive and faster characterisation of the sample, for which identification of all the individual compounds was not needed.

3.2. Classification of whisky according to the cask(s) used in maturation

As mentioned in the Introduction, a large number of factors, including the cask(s) used for maturation and its duration, may influence the whisky character in terms of overall chemical composition, and therefore sensory profile. A number of the chemical reactions that occur during maturation have been described previously [24].

To investigate the impact of the various casks used for maturation on the (semi)volatile fingerprints, 64 malt whiskies matured in oak casks previously used for bourbon maturation were analysed in this part of the study (selected from sample sets (i) and (iv) where maturation history is known). After several years of maturation in the 'bourbon cask', some of these whiskies had each been transferred into a cask that had previously been used for the maturation of wine (either sherry, port, red or white). The maturation of whiskies in the 'wine cask' lasts for a shorter time period (typically from a few months to a few years).

The PCA analysis of data generated by the two alternative sampling approaches (see Fig. 2) showed some clustering behaviour related to the oak cask(s) used for maturation during whisky production. The data obtained by analysis of ethyl acetate extract enabled better separation between samples aged in 'bourbon' and 'bourbon/wine' cask(s). Within the samples in the 'bourbon/wine' group, no significant clustering related to the type of wine was achieved at this stage.

3.2.1. Selection of marker compounds and their identification

Following analysis by PCA, supervised chemometric analysis by PLS-DA was performed. The outcome is shown in Fig. 3A; distinct grouping according to the maturation cask(s) was achieved ($R^2Y = 0.921$, $Q^2 = 0.808$). To find the most important ions contributing to sample separation, a VIP-plot, shown in Fig. 3B, was constructed on the acquired data. Four compounds with the highest values in the VIP plot were identified and selected as candidate 'markers', see Table 2. Their identification was based not only on the match with spectra in the NIST library but also on Kovats indexes. Since the molecular ion was either low or absent in the EI spectra, a

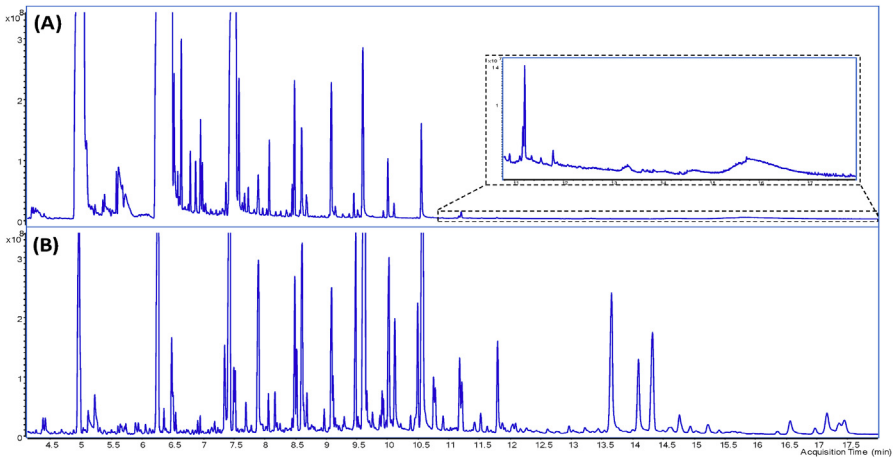

Fig. 1. GC-MS chromatograms of malt whisky volatiles isolated by SPME (A) and ethyl-acetate extraction (B) (total ion chromatogram, *m*/*z* 50–550).

Table 1
The outcome of studies focused on whisky analysis by SPME-GC-MS.

Analysed whisky	SPME fibre for sampling	Instrumentation	Number of identified/monitored compounds	Time of analysis	Reference
3 samples	CAR/PDMS	GC-sQ	86	87 min	1
_	DVB–CAR–PDMS	GC-sQ	44	70 min	2
40	PDMS	GC-IT	36	52 min	3

Note: 1 - [21], 2 - [22], 3 - [23].

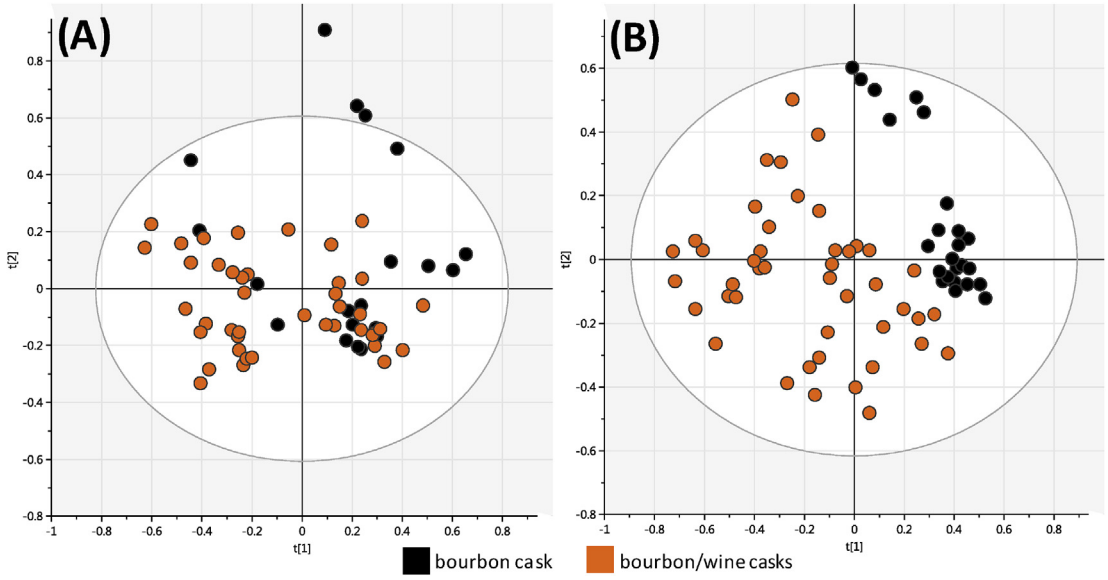

Fig. 2. PCA on samples aged in bourbon and in bourbon/wine cask(s); volatiles obtained by (A) HS-SPME and (B) ethyl–acetate extraction.

PCI analysis was also performed to confirm elemental formula and evaluate the match of isotopic pattern.

As mentioned above, all investigated samples were matured in bourbon casks and some were then transferred into casks previously used for wine maturation. The occurrence of marker compounds was clearly associated with type of maturation cask used in the latter phase of maturation. As illustrated in Fig. 4, N-(3-methylbutyl) acetamide, 5-oxooxolane-2-carboxylic acid and 4(2-hydroxyethyl)phenol were not detectable in samples matured in bourbon casks only. For ethyl 5-oxoprolinate, its relative concentration in such whiskies was significantly lower compared to the other group of samples ('bourbon/wine' samples). To the best of our knowledge, those compounds have never been reported in literature as important molecules for discrimination of different types of whisky. Interestingly, N-(3-methylbutyl) acetamide, 5-oxooxolane-2-carboxylic acid and ethyl 5-oxoprolinate were found in studies investigating wine quality [25–28], explaining their transfer from wine cask into matured whisky. 4(2-hydroxyethyl)phenol was described by Pryde et al. as a compound transferred from sherry casks to whisky [29].

The scientific literature provides explanations of the origins of the marker compounds in non-distilled beverages such as wine,

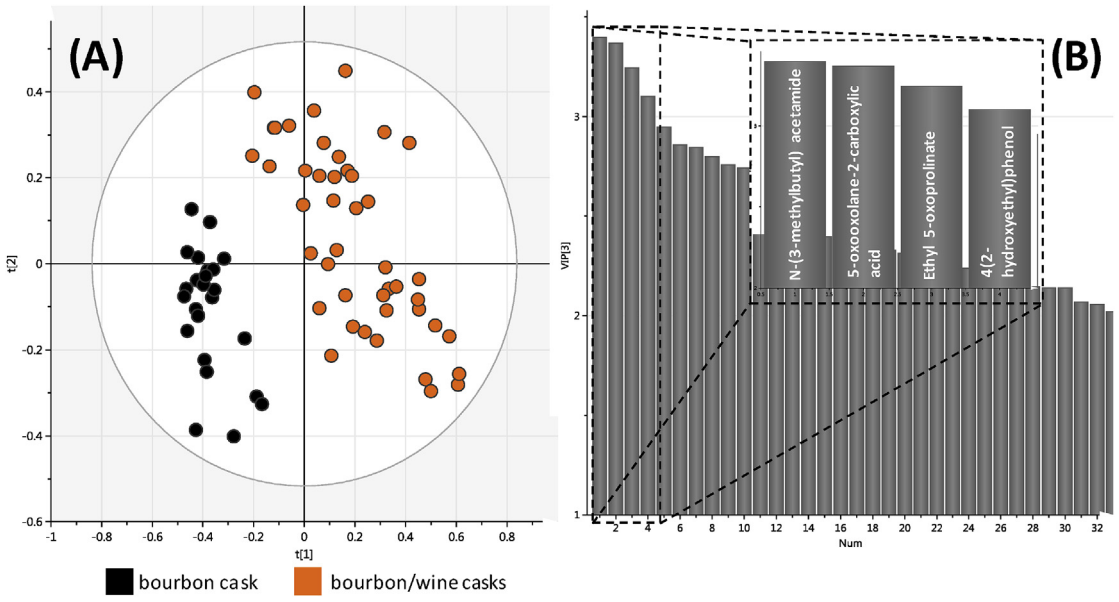

Fig. 3. (A) PLS-DA model for classification of maturation cask(s) of malt whisky samples and (B) VIP-plot with the selected markers'.

Table 2
The most significant markers' characterising whisky samples aged in bourbon/wine casks.

retention time (min)	electron ionization			positive chemical ionization			tentative identification by NIST	Kovats index	
	m/z	elemental formula	mass error (Δppm)	base peak *m/z*	elemental formula	mass error (Δppm)		exp.	NIST
7.4	129.1145*	$C_7H_{15}NO$	2.4	130.1228	$C_7H_{15}NO+H^+$	−1.2	N-(3-methylbutyl) acetamide	1862	1866
9.4	85.0284**	$C_4H_5O_2$	0.1	131.0341	$C_5H_6O_4+H^+$	−1.7	5-oxooxolane-2-carboxylic acid	2253	NA
11.2	84.0443**	C_4H_6NO	1.1	158.0811	$C_7H_{11}NO_3+H^+$	0.4	ethyl 5-oxoprolinate	2626	NA
14.8	138.0676*	$C_8H_{10}O_2$	−0.5	139.075	$C_8H_{11}O_2+H^+$	2.6	4(2-hydroxyethyl)phenol	3017	3008

Note: *molecular *m/z*, **base peak.

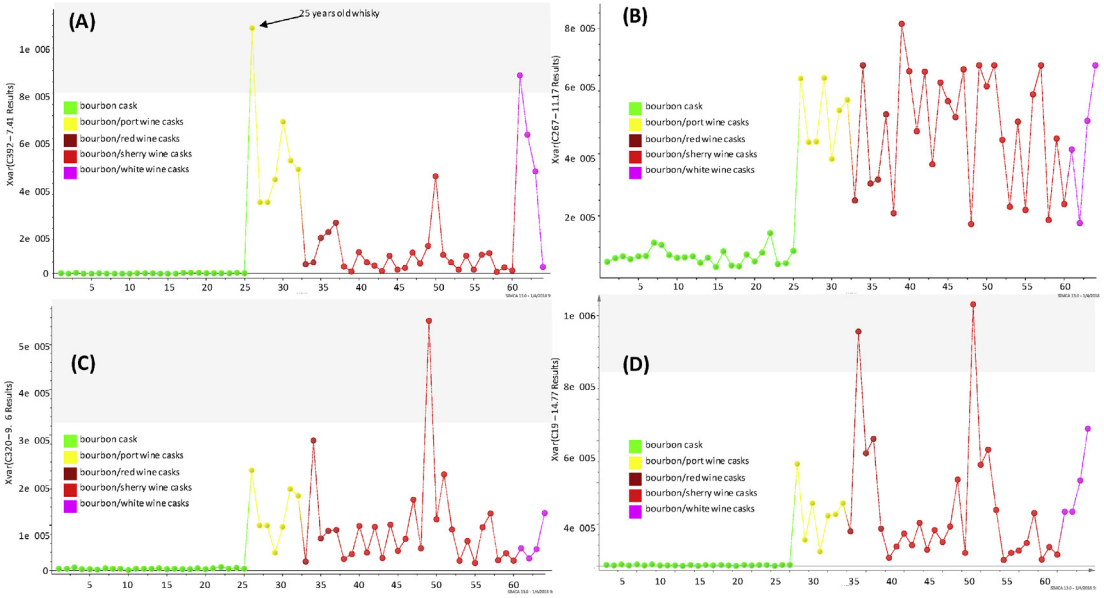

Fig. 4. Variable plots of the significant markers (**A** – N-(3-methylbutyl)acetamide; **B** – Ethyl 5-oxoprolinate; **C** – 5-oxooxolane-2-carboxylic acid; **D** – 4(2-hydroxyethyl)phenol) in malt whisky samples.

specifically 4(2-hydroxyethyl)phenol, which is formed during yeast fermentation from tyrosine [29]; many studies document its antioxidant properties [30–32]. Ethyl 5-oxoprolinate, the relative concentration of which was consistently higher in all types of whisky aged in wine casks, was identified as an important component of wine matured in oak barrels, especially pinot noir [28]. A representative compound for whisky matured in casks previously used for maturation of port wine, would be N-(3-methylbutyl)acetamide.

The application of the selected 'markers' for separation of whisky samples aged in bourbon/port wine, bourbon/sherry wine casks and the samples aged only in bourbon casks is demonstrated in Fig. 5. In this way, laboratory analysis can be simplified, based on the targeted screening of only four compounds. It is worth noting that whiskies matured in casks other than just bourbon were more scattered, clearly due to the diversities in port wine and sherry composition, and that one significant outlier (indicated in Fig. 5 by an arrow) was aged in bourbon/port wine casks fairly longer than other samples, for at least 25 years, which might be the reason for a more intensive transfer of N-(3-methylbutyl)acetamide into the whisky.

Fig. 6 documents the superiority of the TOF-HRMS detector over quadrupole or ion trap mass analysers used in other studies for whisky (semi)volatiles identification [21,23]. This can be demonstrated using the example of 4(2-hydroxyethyl)phenol that was co-eluted with another sample component. In TIC mode, only one symmetric peak was recorded; the difference in retention times was as low as 0.6 s. Thanks to the deconvolution function, peak separation was enabled and pure spectra of both compounds were obtained.

3.3. Distinguishing blended whiskies from malt whiskies

Blended Scotch Whiskies represent the largest product category by volume of sales for the Scotch Whisky industry, approximately 80% in recent years. On the other hand, single malt Scotch Whiskies are also an important product category, with many premium products on the market. Malt whiskies are produced solely from malted barley, whilst blended whisky and grain whiskies can include other cereal sources, such as wheat or maize. A single malt, or single grain, is the product of a single distillery, whereas a blended malt, or blended grain, is the product of two or more distilleries. A blended Scotch Whisky is a blend of at least one single malt and one single grain whisky (The Scotch Whisky Regulations 2009).

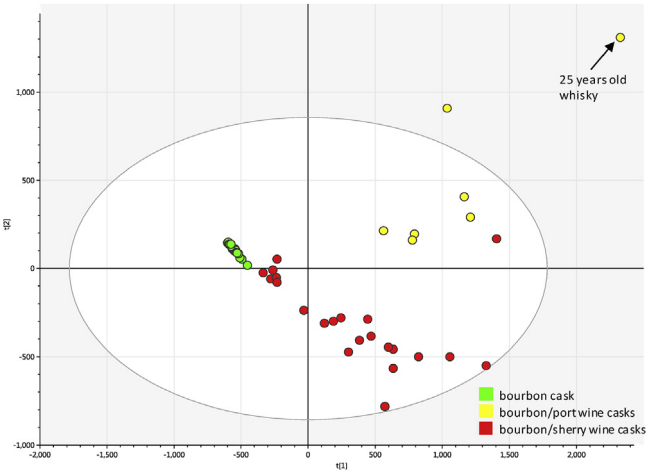

Fig. 5. PCA based on four selected markers, according to cask used for whisky maturation (bourbon/port wine, bourbon/sherry wine and bourbon cask(s)).

Fig. 7 presents the results of the PCA analysis of data, generated by the fingerprinting strategy described above, for the 148 genuine malt and blended Scotch Whiskies. Some clustering of malt and blended whiskies can be seen, with malt whiskies of Islay origin (one of five regions protected by law for whisky production, with distilleries typically producing peated whisky) forming the outlying cluster. Phenolic compounds, such as phenol and 4-ethyl-phenol, contributed to the separation of the peated whiskies from the other malt whiskies. As a group, these are responsible for the 'smoky/medicinal' flavour of this category of malt whiskies (its intensity depends on peating level).

It is worth noting that whilst malt whiskies typically command higher prices than blended whiskies, there is also some differentiation in perceived quality of blended whiskies as well. Blended Scotch Whisky marketed with some form of 'premium' quality designation will tend to have more malt whisky content and be matured for longer in the cask. Fig. 8 demonstrates very good separation between blended whisky samples, malts and the 'premium' blended whisky samples. The youngest malt whisky (8 years old) is the closest to the cluster of blended whiskies. On the other hand, the oldest 'premium' blended whiskies (21 years old) are more or less in the centre of the cluster of malt whiskies. Such observations suggest the horizontal principal component is heavily influenced by maturation related compounds. In the cluster of 'premium' blended whiskies are three samples of a malt whisky that originated from one distillery, their positions reflecting some aspect of their shared production process.

Additionally, PLS-DA (see Fig. S1) was subsequently constructed to identify 'markers' for malt samples. Cross validation was performed using both types of the extracts to evaluate the performance of the PLS-DA model. In this case, the data set was randomly split 5-times into a calibration (training) set (4/5 of samples) with the remaining samples (1/5) being used as a test set. Using this internal validation, high recognition (99%) as well as prediction (94%) abilities were achieved.

The six most significant 'markers', shown in Table 3, that were responsible for the distinction between malt and blended whisky were found and identified by the same statistical procedure described above for cask type. Fig. 9A and B compare representation of 4-hydroxy-3-methoxybenzaldehyde (vanillin) and 1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one (β-damascenone) in the sample set of tested whiskies. It should be noted, that the processed data has been normalised by percent of total area and the occurrence of compounds is expressed as relative concentration. Interestingly, vanillin was found as a marker compound for blended whiskies, although its average absolute signals in blended whisky are lower compared to malt whiskies. The reason is that its contribution to the total integral of compounds in blended whisky is higher compared to malt whisky. Malt whiskies have higher concentrations of volatiles due to the different distillation process used compared to the grain whisky component of blends, and are typically aged for longer, resulting in a richer profile of maturation related compounds.

Higher relative concentrations of ethyl 4-hydroxy-3-methoxybenzoate (ethyl vanillate) and 1-propanone-1-(4-hydroxy-3-methoxyphenyl) (propivanillone) were found to be characteristic compounds for malt and 'premium' blended whisky. On the other hand, in blended whiskies the relative concentration of vanillin and phenylmethanol were higher.

A typical 'marker' for malt whisky was β-damascenone, i.e. a compound considered as a key odour component in many alcoholic beverages (wine, beer, whisky) [21,33].

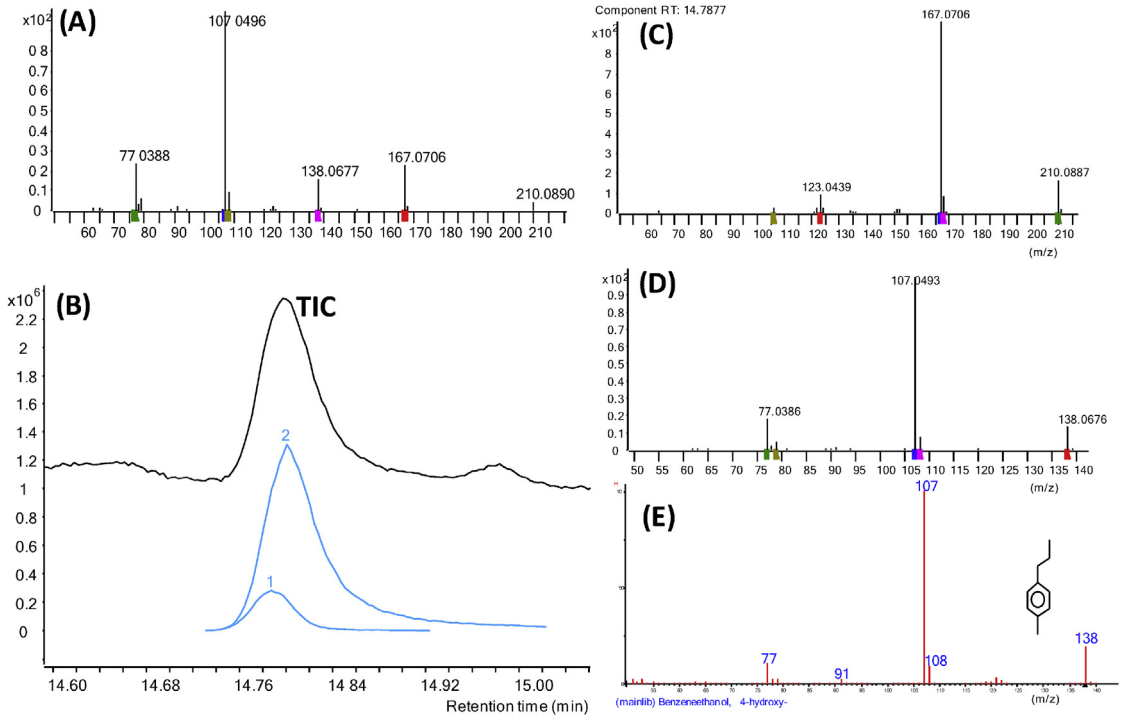

Fig. 6. Application of deconvolution function on peak recorded by GC-TOF-HRMS during analysis of whisky at retention time 14.78 min (A) in TIC (B) and the mass spectra after deconvolution: C - 'unimportant compound'; D -'marker'- 4(2-hydroxyethyl)phenol; E – NIST spectrum of 4(2-hydroxyethyl)phenol.

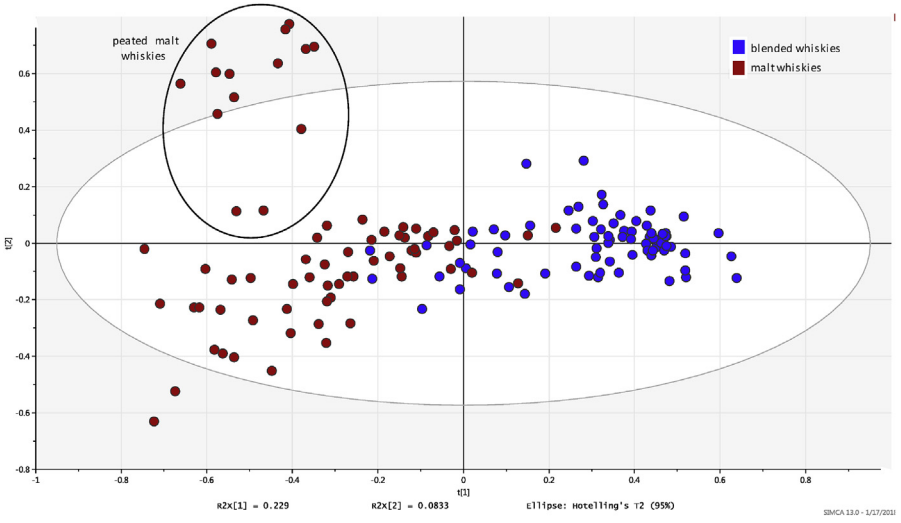

Fig. 7. PCA score plot of malt (peated and non-peated) and blended whiskies.

3.4. Analysis of fake samples

In this experiment, 20 counterfeit samples were analysed to reveal the differences between fake and genuine samples, as well as to detect and identify important 'markers' for genuine Scotch Whiskies. As shown in Fig. 10, a very good separation between genuine and fake samples using PCA was achieved. The three compounds contributing most to the separation of fake and genuine whisky were 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde), ethyl dodecanoate and 1-(3-acetylphenyl)ethanone, see Table 4. Syringaldehyde and 1-(3-acetylphenyl)ethanone were either absent or present at only low concentrations in the fake whiskies. The concentration of ethyl dodecanoate was at a comparable level in some fake samples but in the others, the concentrations were significantly lower. For detailed illustration of the occurrence of the 'markers' characterising the genuine samples using variable plots, see Fig. S2.

Syringealdehyde is derived from oak wood lignins, and its concentration increases during maturation in cask [34,35]. Hence, unsurprisingly, the higher concentration was found in malt and 'premium' blended whiskies, which tend to be matured for longer periods. Ethyl dodecanoate was described in three studies [20,21,29] as an important component of whisky aroma. The concentration of this compound in 14 fake samples was similar in concentration to genuine samples. However, in the rest of fake samples, the ethyl dodecanoate levels were significantly lower.

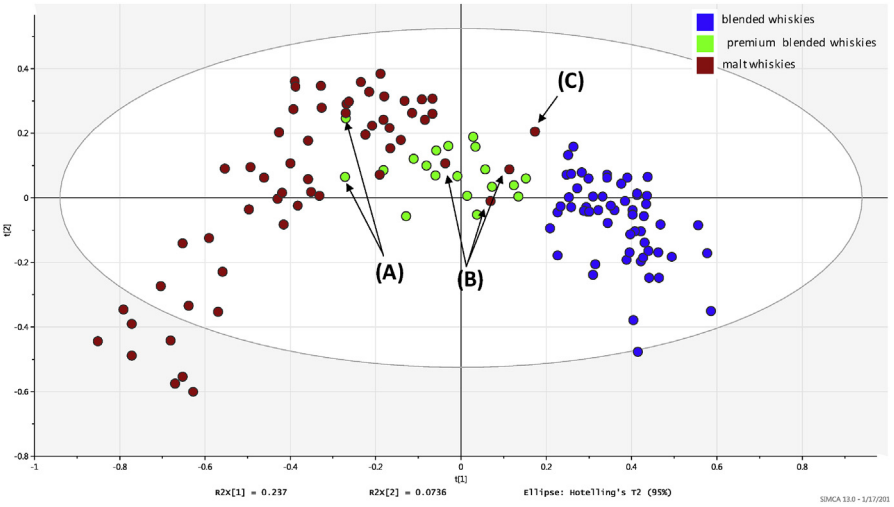

Fig. 8. Clustering within malt, 'premium' blended and blended whiskies (PCA). A – 21 years old samples. B – Samples originated from the same distillery. C – the youngest malt whisky.

Table 3
Significant marker compounds responsible for the distinction between malt, 'premium' blended and blended whiskies.

retention time (min)	electron ionization			positive chemical ionization			tentative identification by NIST	Kovats index	
	m/z	elemental formula	mass error (Δppm)	base peak *m/z*	elemental formula	mass error (Δppm)		exp.	NIST
7.3	190.1353*	$C_{13}H_{18}O$	−0,44	191.1425	$C_{13}H_{18}O+H^+$	2.9	1-(2,6,6-trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	1836	1835
7.6	108.0566**	C_7H_8O	3.4	109.0645	$C_7H_8O+H^+$	1.7	phenylmethanol	1885	1895
9.6	191.1427**	$C_{13}H_{19}O$	1.8	207.1739	$C_{14}H_{22}O+H^+$	2.1	2,4-ditert-butylphenol	2306	2312
11.0	151.0385**	$C_8H_7O_3$	3.1	153.0544	$C_8H_8O_3+H^+$	1.5	4-hydroxy-3-methoxybenzaldehyde	2590	2597
11.3	151.0386**	$C_8H_7O_3$	2.4	197.0805	$C_{10}H_{12}O_4+H^+$	1.7	ethyl 4-hydroxy-3-methoxybenzoate	2649	2654
11.8	151.0389**	$C_8H_7O_3$	2.1	181.0856	$C_{10}H_{12}O_3+H^+$	1.8	1-propanone-1-4-hydroxy-3-methoxyphenyl	2723	2719

Note: *molecular ion, **base peak.

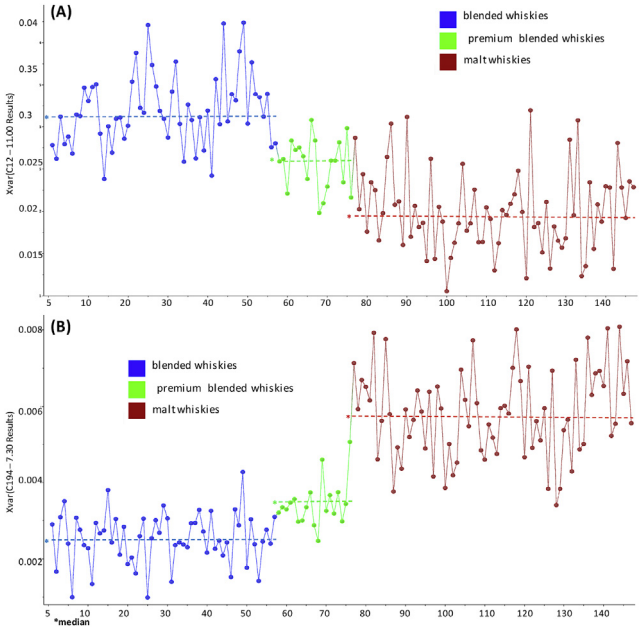

Fig. 9. Variable plots of the two significant 'markers' (**A** – vanillin, **B** – β-damascenone); in malt, 'premium' blended and blended whisky samples.

Counterfeit samples can be of variable origin and composition, so they could not be characterised as one group with identical or comparable attributes. It is impossible to find a 'marker' occurring only in counterfeit samples. However, under such conditions, non-targeted screening followed by chemometric analysis can be a powerful instrument to reveal deviations from typical fingerprints. In this way, eight compounds (summarised in Table 5) were found and subsequently identified in several fake samples at high concentration. All of these substances were obviously being used as flavouring agents. For instance, ethyl vanillin was detected in four fake samples. Whilst benzaldehyde and vanillin were found in authentic whiskies, and can be related to migration from the oak wood, especially from casks after sherry wine production [36], in two samples abnormal concentrations of these compounds were detected. Whether based on chemical knowledge or simply sensory characters, these flavours have been deliberately added to imitate the properties of whisky.

4. Conclusions

Within this study, the novel analytical strategy based on a non-targeted screening of sample components by GC-HRMS followed by multidimensional chemometric processing of generated data was employed for the assessment of the quality and authenticity of various Scotch Whiskies. The unique sample set of authentic

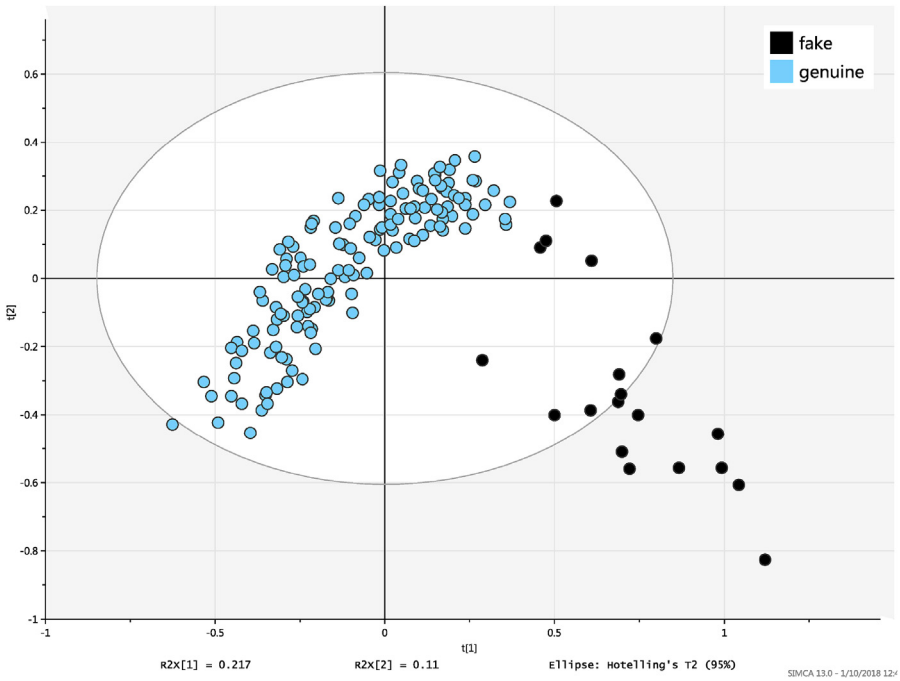

Fig. 10. PCA score plot of genuine (malt, premium' blended and blended) and fake whiskies.

Table 4
Markers characterising genuine whiskies.

retention time (min)	electron ionization			positive chemical ionization			tentative identification by NIST	Kovats index	
	base peak *m/z*	elemental formula	mass error (Δppm)	base peak *m/z*	elemental formula	mass error (Δppm)		exp.	NIST
7.3	228.2085	$C_{14}H_{28}O_2$	−0.5	229.2145	$C_{14}H_{28}O_2$	2.7	ethyl dodecanoate	1846	1841
9.9	147.0439	$C_9H_7O_2$	1.1	163.0756	$C_{10}H_{10}O_2$	−1.5	1-(3-acetylphenyl)ethanone	2360	2333
14.0	182.0571	$C_9H_{10}O_4$	1.4	183.0637	$C_9H_{10}O_4$	3.2	4-hydroxy-3,5-dimethoxybenzaldehyde	2953	2934

Note: *molecular *m/z*, **base peak.

Table 5
Flavouring agents detected in fake samples.

Compound	Positive samples
3-ethoxy-4-hydroxybenzaldehyde	4
5-butyloxolan-2-one	2
ethyl heptanoate	2
1,3-benzodioxole-5-carbaldehyde	2
2,6-dimethoxyphenol	1
1-phenylethyl acetate	1
4-hydroxy-3-methoxybenzaldehyde	1
benzaldehyde	2

whiskies, together with those identified as counterfeits, were provided by the Scotch Whisky Research Institute. The main research outcomes can be summarised as follows:

- Of two different sample handling strategies, SPME and ethyl acetate extraction, the latter one was preferred because not only volatile, but also a number of semi-volatile compounds could be detected for sample characterisation.
- The PLS-DA classification model constructed on the data obtained by analysis of ethyl acetate extracts distinguished whiskies according to the type of cask in which they were matured (bourbon versus bourbon and wine). The characteristic 'markers' occurring at elevated levels for samples aged in the bourbon and wine casks were: N-(3-methylbutyl) acetamide, 5-oxooxolane-2-carboxylic acid, ethyl-5-oxoprolinate and 4(2-hydroxyethyl)phenol.
- In the same way, ethyl vanillate, propiovanillone and β-damascenone for malt, and 2,4-ditert-butylphenol, vanillin and phenylmethanol for blended whiskies, were tentatively identified as characteristic markers.
- Fake whiskies could be identified based on a difference in obtained GC-HRMS fingerprints. A number of synthetic flavourings such as ethyl vanillin were identified.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.09.017.

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