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Characterization of sulfur compounds in whisky by full evaporation dynamic headspace and selectable one-dimensional/two-dimensional retention time locked gas chromatography–mass spectrometry with simultaneous element-specific detection

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ABSTRACT

A method is described for characterization of sulfur compounds in unaged and aged whisky. The method is based on full evaporation dynamic headspace (FEDHS) of 100 μL of whisky samples followed by selectable one-dimensional (^{1}D) or two-dimensional (^{2}D) retention-time-locked (RTL) gas chromatography (GC)–mass spectrometry (MS) with simultaneous element-specific detection using a sulfur chemiluminescence detector (SCD) and a nitrogen chemiluminescence detector (NCD). Sequential heart-cuts of the 16 sulfur fractions were used to identify each individual sulfur compound in the unaged whisky. Twenty sulfur compounds were positively identified by a MS library search, linear retention indices (LRI), and formula identification using MS calibration software. Additionally eight formulas were also identified for unknown sulfur compounds. Simultaneous heart-cuts of the 16 sulfur fractions were used to produce the ^{2}D RTL GC-SCD chromatograms for principal component analysis. PCA of the ^{2}D RTL GC-SCD data clearly demonstrated the difference between unaged and aged whisky, as well as two different whisky samples. Fourteen sulfur compounds could be characterized as key sulfur compounds responsible for the changes in the aging step and/or the difference between two kinds of whisky samples. The determined values of the key sulfur compounds were in the range of 0.3–210 ng mL^{-1} (RSD: 0.37–12%, $n = 3$).

1. Introduction

Sulfur compounds in alcoholic beverages such as wine, beer, and spirits are of particular interest because of their low sensory threshold and possible resulting effect on product flavor [1–5]. Moreover, the presence of several medium and high boiling sulfur compounds can improve the flavor. These sulfur compounds have hydrophilic properties and are present at ng mL^{-1} levels in complex matrices, which include several high concentration compounds (μg mL^{-1} levels) such as fusel alcohols, fatty acids, and esters. Therefore, in order to analyze sulfur compounds in those alcoholic beverages, it is essential to have powerful extraction and enrichment steps before gas chromatographic analysis. Although liquid–liquid extraction (LLE) has been the most widely used technique, LLE is tedious, time consuming, labor intensive, and requires large amounts of organic solvents. Headspace techniques, e.g. static headspace (SHS), dynamic headspace (DHS), and headspace solid phase microextraction (HS-SPME), have been frequently used in analysis of sulfur compounds in wine because they are simple, solvent-less, and (can be) fully automated [1]. However, these techniques are generally more selective for more volatile and/or hydrophobic compounds. In 2012, a full evaporation dynamic headspace (FEDHS) method, based on a classical full evaporation technique (FET) developed by Markelov and Guzowski in 1993 [6], was demonstrated for uniform enrichment of odor compounds including hydrophilic sulfur compounds (e.g. 2-acethylthiazole and 2-formylthiophene) in aqueous samples at ng mL^{-1} levels [7]. By using FEDHS of 100 μL of aqueous sample at 80 °C, a wide range of odor compounds with different polarities could be uniformly recovered (>85%) in contrast with conventional DHS and HS-SPME, while leaving most of the low volatile matrix behind [8].

Several authors reported that gas chromatography–mass spectrometry (GC–MS) in combination with simultaneous selective detection is a powerful tool for the identification of sulfur compounds [1]. Bouchilloux et al. demonstrated combinations of GC with olfactometry, flame photometry, and MS for identification of three aromatic thiols in red wine [9]. In certain cases, identification of trace sulfur compounds in complex samples by GC–MS with

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simultaneous selective detection can be challenging because co-elution interferes with mass spectral identification of individual compounds. A more effective way to improve the identification capability and separation resolution is through two-dimensional (^{2}D) GC with simultaneous mass spectrometric and selective detection. There are two established ^{2}D GC approaches: heart-cutting ^{2}D GC (GC–GC) [10,11] and comprehensive ^{2}D GC (GC × GC) [10,12]. The former approach is commonly used in target analysis of specific compounds in a sample. The latter approach is mainly used in exhaustive analysis of a sample for total profiling. Although several injections are often required for the identification of multiple target compounds, heart-cutting ^{2}D GC–MS with simultaneous selective detection has higher ability to obtain a clean mass spectrum for each target peak because of a much longer second column and proper temperature programming, resulting in higher peak capacity and sample capacity in the second dimensional separation compared to GC × GC. Heart-cutting ^{2}D GC–MS in combination with a sulfur chemiluminescence detector (SCD) and nitrogen chemiluminescence detector (NCD) was successfully applied for analysis of trace sulfur and nitrogen compounds in whisky [13–15]. Recently, a novel selectable one-dimensional (^{1}D) or ^{2}D GC–MS (^{1}D/^{2}D GC–MS) with simultaneous selective detection was demonstrated for simple and fast operation of both ^{1}D GC–MS and ^{2}D GC–MS using GC equipped with LTM-technology in combination with single quadrupole MS [8,16,17]. With this system, simultaneous mass spectrometric and selective detection can be performed for both ^{1}D GC and ^{2}D GC separations, without any instrumental set-up change. Therefore, the selection and confirmation of target compounds in ^{2}D GC analysis can be easily performed with both mass spectral and olfactometric/element-specific information.

In this study, a combined approach consisting of FEDHS, ^{1}D/^{2}D GC–MS with SCD and NCD for characterization of sulfur compounds in two kinds of unaged and aged whisky is described.

2. Experimental

2.1. Reagents and materials

4,5-Dimethyl-1,3-thiazole, ethyl 2-methyl sulfanyl acetate, ethyl 3-methyl sulfanyl propanoate, 1-(1,3-thiazol-2-yl)ethanone (2-acetyl thiazole), 3-methylthiophene-2-carbaldehyde (3-methyl-2-formylthiophene), 5-methylthiophene-2-carbaldehyde (5-methyl-2-formylthiophene), (methyltrisulfanyl)methane (dimethyl trisulfide), thiophene-2-carbaldehyde (2-formyl thiophene), 1-thiophen-2-ylethanone (2-acetyl thiophene), and 1-thiophen-3-ylethanone (3-acetyl thiophene) were kindly obtained from Dr. Katsumi Umano of Takata Koryo Co., Ltd. (Hyogo, Japan).

Two kinds of malt whisky ("A" from Speyside, Scotland and "G" from Highland, Scotland), unaged and 15 years old, were used for the analysis. Both whiskies were of the "single malt" variety, i.e. produced exclusively in the same distillery and not a mixture of malt whiskies from different distilleries. The unaged whisky was at 65% (v/v) ethanol. The aged whisky was at 40% (v/v) ethanol.

2.2. Instrumentation

FEDHS was performed using a GERSTEL DHS module (GERSTEL, Mülheim an der Ruhr, Germany) that enables dynamic purging of the headspace above a sample combined with trapping of purged analytes on an adsorbent trap using a dual-needle design [18]. The trapped compounds were subsequently analyzed by thermal desorption (TD)-^{1}D/^{2}D GC-SCD/NCD/MS using a MPS2 robotic arm and a TDU thermal desorption unit placed on top of a CIS4 programmable temperature vaporizing (PTV) inlet (GERSTEL). The Agilent 7890 gas chromatograph (host GC) was equipped with a CTS2 cryo-trap system from GERSTEL, a dual LTM-GC system (Agilent), a SCD (Agilent) and a NCD (Agilent). A 5975C mass spectrometer from Agilent was used. The dual LTM-GC-SCD/NCD/MS system was configured as ^{1}D/^{2}D GC–MS with simultaneous selective detection previously described [16], which enables simple and fast operation of both ^{1}D GC–MS and ^{2}D GC–MS with simultaneous selective detection without any instrumental setup change.

The ^{1}D/^{2}D GC-SCD/NCD/MS system was equipped with dual wide format LTM-GC column modules (5 in.; 1 in. = 2.54 cm), an Agilent capillary flow technology (CFT) Deans switch, a 2-Way splitter and a 3-way splitter (with make-up gas line), which were controlled with a pressure control module (PCM). PCM has two pressure control capabilities. One is called PCM (main) and the other is called Auxiliary (AUX).

2.3. Sample preparation

One hundred micro-liters of whisky sample were transferred into an empty 10 mL screw cap headspace vial. No further sample preparation was necessary.

2.4. FEDHS and thermal desorption (TD)

For FEDHS, samples were transferred from the sample tray to the DHS module at 80 °C. Analytes in the headspace vial were immediately purged with 3 L of nitrogen gas at a flow rate of 100 mL min^{-1} and trapped at 40 °C on a TDU tube packed with Tenax TA. The TDU tube was transported to, and subsequently desorbed in the TDU. The TDU was programmed from 30 °C (held for 0.5 min) to 240 °C (held for 3 min) at 720 °C min^{-1} with 50 mL min^{-1} desorption flow. Desorbed compounds were focused at 10 °C on a Tenax TA packed liner in the PTV inlet. After desorption, the PTV inlet was programmed from 10 °C to 240 °C (held for GC run time) at 720 °C min^{-1} to inject trapped compounds onto the analytical column. The injection was performed in the split mode with a split ratio of 1–1 using the low split option (Gerstel KK, Tokyo, Japan) controlled by the pneumatic box of the TDU system.

2.5. Selectable ^{1}D/RTL ^{2}D RTL GC-SCD/NCD/MS

Separations were performed on a 30 m, 0.25 mm i.d., 1.0 μm film thickness DB-1 column (Agilent) as the first dimensional (^{1}D) column and a 30 m, 0.25 mm i.d., 0.25 μm film thickness DB-Wax column (Agilent) as the second dimensional (^{2}D) column. The column temperature for the ^{1}D DB-1 was programmed from 40 °C (held for 2 min) to 240 °C (held for 8 min) at 5 °C min^{-1}. After the retention time of 50 min, the sample matrix was back flushed. The column temperature for the ^{2}D DB-Wax was kept at 40 °C during ^{1}D GC analysis, and programmed from 40 °C at 10 °C min^{-1} to 280 °C (held for 10 min) for ^{2}D GC analysis. The host GC oven was kept at a constant temperature of 250 °C. The inlet pressure and the pressure of AUX of PCM for the 3-way splitter were 361 and 27 kPa, respectively. For the ^{2}D RTL GC-SCD/NCD/MS analysis, five individual runs, each at a different constant pressure of the PCM for the Deans switch, were initially performed with injections of the locking compound {3-methylthiophene-2-carbaldehyde (3-methyl-2-formylthiophene)}. This is followed by a 3-methyl-2-formylthiophene retention time versus the Deans switch pressure (for the ^{2}D column) regression calibration [19] to allow calculation of the exact required pressure to achieve the desired retention time of the locking compound. The pressure of the PCM was initially set at 261 kPa so that the retention time of 3-methyl-2-formylthiophene is exactly 64.80 min. A single run with injection of 3-methyl-2-formylthiophene was performed to check and relock the PCM pressure before every sequence.

For simultaneous mass spectrometric and element-specific detection, a split ratio of 1:1:1 was set to the MS, the SCD, and the NCD. A deactivated fused silica capillary with 1.0 m × 0.20 mm i.d., was used for connecting from the splitter to the SCD, and 1.2 m × 0.20 mm i.d., for connecting from the splitter to the NCD and the MS. The MS was operated in scan mode using electron ionization at 70 eV. Scan range was set from m/z 29 to 300 and a sampling rate equal to three was used, resulting in scan rate of 2.68 scan s^{-1}. For the formula identification using MassWorks software ver. 2.0.2.0 (Cerno Bioscience, CT, USA), the MS was operated in "raw scan mode" which generates a profile mass spectrum (10–20 measurements per each integer m/z value in the spectrum). The SCD burner temperature was set to 800 °C and its flow rate was 63 mL min^{-1} and 45 mL min^{-1} for air and hydrogen, respectively. The NCD burner temperature was set to 950 °C and its flow rate was 10 mL min^{-1} and 5 mL min^{-1} for oxygen and hydrogen, respectively.

2.6. Data analysis

ChemStation ver. E.02.01.1177 (Agilent) and Aroma Office ^{2}D database ver. 2.01.00 (Gerstel KK) were used for a combined search using a MS library and a linear retention indices (LRI) database. Aroma Office ^{2}D contains the most comprehensive database of odor compounds available (>73,000 entries). This software is a searchable database which contains LRI information for a wide range of odor compounds from many literature references. MassWorks software ver. 2.0.2.0 (Cerno Bioscience) was used for the formula identification. MassWorks is a novel MS calibration software which calibrates for isotope profile as well as for mass accuracy allowing highly accurate comparisons between calibrated and theoretical spectra. This calibration process has been published and detailed elsewhere [20,21]. Principal component analysis (PCA) was performed using Pirouette software ver. 4.0 (Infometrix, WA, USA).

3. Results and discussion

3.1. FEDHS recovery of sulfur compounds in ethanol–water samples

Table 1 shows FEDHS recovery at 80 °C for the test sulfur compounds in 40% ethanol–water and 65% ethanol–water at 100 ng mL^{-1}. The logarithm of the octanol–water distribution coefficient ($\log K_{ow}$) of the test sulfur compounds ranged from 0.67 {1-(1,3-thiazol-2-yl)ethanone (2-acetyl thiazole)} to 2.09 (4,5-dimethyl-1,3-thiazole). The $\log K_{ow}$ values were calculated with a SRC-KOWWIN v1.68 software package (Syracuse Research, Syracuse, NY, USA). FEDHS was performed in six replicate analyses. The recovery was calculated by comparing peak areas with those of a calibration curve prepared by automated direct liquid injection of a standard solution injected into a micro-vial in a thermal desorption liner through a septum head of the TDU (TDU liquid option, GERSTEL). Very good recoveries in the range of 92–99% and 88–98% were obtained for both ethanol–water samples. Repeatabilities were also good for both samples resulting in a relative standard deviation (RSD) of less than 3.9%. As already described elsewhere [7], FEDHS allows sample matrix independent analysis. Here, the high concentration level of ethanol is assumed to be the main matrix component during headspace sampling. FEDHS can provide high recovery of hydrophilic sulfur compounds in ethanol–water samples.

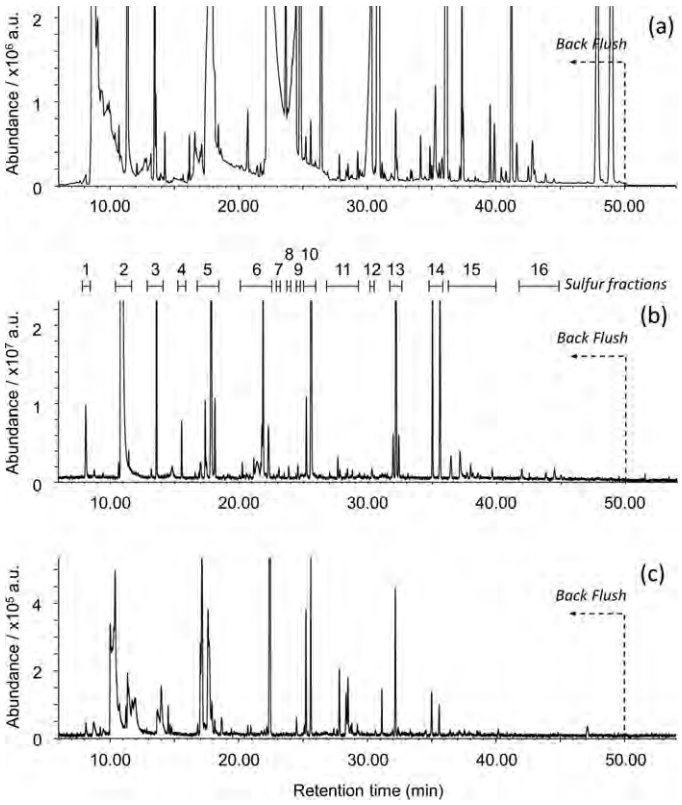

Fig. 1. ^{1}D total ion chromatogram (TIC), SCD and NCD chromatograms of the unaged whisky "A". (a) ^{1}D TIC; (b) ^{1}D SCD chromatogram; (c) ^{1}D NCD chromatogram.

3.2. Identification of sulfur compounds in single malt whisky

In order to identify sulfur compounds in the whisky samples, the unaged and aged whisky "A" and "G" were first analyzed with FEDHS-^{1}D GC-SCD/NCD/MS. From the comparison of the ^{1}D SCD chromatograms, the unaged whisky "A" showed the highest number of sulfur peaks. Fig. 1 shows the ^{1}D total ion chromatogram (TIC) (Fig. 1a), SCD and NCD chromatograms (Fig. 1b and c) of the unaged

Table 1
FEDHS recovery at 80 °C for the test sulfur compounds in 40% ethanol–water and 65% ethanol–water at 100 ng mL^{-1}.

Compound (common name)[a]	$\log K_{ow}$ [b]	40% ethanol–water		65% ethanol–water	
		Recovery (%)	RSD (%), $n=6$	Recovery (%)	RSD (%), $n=6$
1-(1,3-Thiazol-2-yl)ethanone (2-acetyl thiazole)	0.67	92	2.0	98	1.1
Ethyl 2-methyl sulfanyl acetate	0.95	95	3.0	93	2.1
Ethyl 3-methyl sulfanyl propanoate	1.44	99	3.7	94	2.7
1-Thiophen-2-yl ethanone (2-acetyl thiophene)	1.49	93	2.1	96	1.7
Thiophene-2-carbaldehyde (2-formyl thiophene)	1.53	94	1.8	98	2.0
(Methyltrisulfanyl)methane (dimethyl trisulfide)	1.87	92	2.4	88	3.9
5-Methylthiophene-2-carbaldehyde (5-methyl-2-formyl thiophene)	2.08	93	1.9	96	1.9
4,5-Dimethyl-1,3-thiazole	2.09	92	1.5	91	1.7

[a] Common name was shown in a parenthesis.
[b] $\log K_{ow}$ values were calculated with SRC-KOWWIN software (Syracuse Research, Syracuse, NY, USA).

Table 2
Identification of sulfur compounds in the unaged whisky "A" by FEDHS-^{1}D/^{2}D RTL GC-SCD/NCD/MS.

No.	Compound (common name)[a]	log K_{ow} [b]	$^{2}t_R$ (min)[c]	^{2}D LRI			PBM[e]	Formula	Theoretical m/z	Mass error (mDa)	Spectral accuracy[f]
				Calculated	Database[d]	Deviation					
1	2-Methyl-1,3-thiazole	1.54	57.21	1246	1245 (n = 5)	1	83	C_4H_5NS	99.0143	−8.6	98.22
2	1,3-Thiazole	1.81	57.39	1260	1248 (n = 5)	12	91	C_3H_3NS	84.9986	−6.1	98.51
3	Diethyl sulfite	0.99	57.98	1301	–	–	96	$C_4H_{10}O_3S$	138.0351	−7.9	98.29
4	Ethyl methanesulfonate	−0.17	58.46	1335	–	–	96	$C_3H_8O_2S$	108.0245	−6.6	99.71
5	Ethyl 2-methyl sulfanyl acetate	0.95	60.18	1458	1452 (n = 1)	6	64	$C_5H_{10}O_2S$	134.0402	−8.8	98.63
6	Ethyl 3-methyl sulfanyl propanoate	1.44	61.75	1579	1567 (n = 3)	12	91	$C_6H_{12}O_2S$	148.0558	−5.3	99.13
7	Methyl sulfinyl methane (dimethyl sulfoxide)	−1.22	61.86	1585	1571 (n = 3)	14	94	C_2H_6OS	78.0139	−6.5	99.40
8	(Acetyl thiazole isomer)	0.67	62.42	1633	–	–	95	C_5H_5NOS	127.0092	−7.7	99.14
9	3-Methyl sulfanyl propyl acetate	1.44	62.55	1643	1626 (n = 2)	17	83	$C_6H_{12}O_2S$	148.0558	−5.1	99.26
10	1-(1,3-Thiazol-2-yl)ethanone (2-acetyl thiazole)	0.67	62.81	1664	1651 (n = 8)	13	96	C_5H_5NOS	127.0092	−8.5	99.25
11	Thiophene-3-carbaldehyde (3-formyl thiophene)	1.53	63.23	1698	1693 (n = 2)	5	91	C_5H_4OS	111.9983	−7.5	98.65
12	Thiophene-2-carbaldehyde (2-formyl thiophene)	1.53	63.42	1714	1718 (n = 1)	4	95	C_5H_4OS	111.9983	−7.2	99.19
13	3-Methyl sulfanyl propan-1-ol (Methionol)	0.44	63.64	1734	1734 (n = 15)	1	97	$C_4H_{10}OS$	106.0452	−5.9	99.63
14	Isothiocyanatobenzene (phenyl isothiocyanate)	3.33	63.87	1753	–	–	90	C_7H_5NS	135.0143	9.0	99.06
15	5-Methylthiophene-2-carbaldehyde (5-methyl-2-formyl thiophene)	2.08	64.43	1800	1785 (n = 1)	15	83	C_6H_6OS	126.0139	−7.5	99.47
16	3-Methylthiophene-2-carbaldehyde (3-methyl-2-formyl thiophene)	2.08	64.80	1834	–	–	83	C_6H_6OS	126.0139	−8.6	99.59
17	3-Ethylthiophene-2-carbaldehyde (3-ethyl-2-formyl thiophene)	2.01	65.37	1885	–	–	94	C_7H_8OS	140.0296	−6.8	99.18
18	2-Methyl-1,3-benzothiazole	2.72	66.18	1960	–	–	90	C_8H_7NS	149.0299	−9.2	99.14
19	1,3-Benzothiazole	2.17	66.41	1979	1961 (n = 10)	18	91	C_7H_5NS	135.0143	−7.1	99.86
20	3-Ethyl-2(3H)-benzothiazolethione	2.87	78.01	3025	–	–	89	$C_9H_9NS_2$	195.0176	−6.8	98.70

[a] Common name was shown in a parenthesis.
[b] log K_{ow} values were calculated with SRC-KOWWIN software (Syracuse Research, Syracuse, NY, USA).
[c] Second dimensional retention time (min).
[d] Average LRI obtained from Aroma Office ^{2}D database.
[e] Probability based matching of a Wiley library search. The similarity between the theoretical and measured patterns based on 100.
[f] A measure of the similarity between the measured isotope pattern and the theoretical pattern.

Table 3
Formula identification of unknown sulfur compounds in the unaged whisky "A" by FEDHS-^{1}D/^{2}D RTL GC-SCD/NCD/MS.

No.	Compound	^2t_R (min)[a]	^{2}D LRI	Formula[b]	Theoretical m/z	Mass error (mDa)	Spectral accuracy[c]
S1	S1	58.12	1309	$C_5H_{10}O_2S$	134.0402	8.5	99.08
S2	S2[d]	61.39	1549	$C_5H_{12}OS_2$	152.0330	8.6	99.16
S3	S3	66.22	1960	$C_{11}H_{23}NOS$	217.1500	4.5	99.47
S4	S4	66.24	1963	$C_3H_8OS_2$	124.0017	7.6	98.23
S5	S5	67.31	2061	$C_8H_{15}NOS$	173.0874	4.5	99.47
S6	S6	67.72	2099	C_9H9NOS	179.0405	6.5	99.26
S7	S7	69.22	2236	$C_{11}H_{15}NOS$	209.0874	4.0	98.96
S8	S8	69.98	2306	C_7H11NOS	157.0561	8.9	98.76

[a] Second dimensional retention time (min).
[b] Formula identification was performed with MassWorks software (Cerno Bioscience, CT, USA).
[c] A measure of the similarity between the measured isotope pattern and the theoretical pattern.
[d] S2 was identified as 1-ethoxy-2-(methyldisulfanyl)ethane (3,4-dithiapentyl ethyl ether) from Refs. [4,22].

whisky "A". Although numerous sulfur compounds were detected in the ^{1}D SCD chromatogram (Fig. 1b), these sulfur compounds were completely buried in the ^{1}D TIC (Fig. 1a). It is hard to extract a clean mass spectrum for each sulfur compound because of significant interference of co-eluting sample matrix. Therefore, we sequentially performed 16 heart-cuts with 16 sulfur fractions selected by the ^{1}D SCD chromatogram for the identification in the ^{2}D chromatograms. The transferred fractions were separated and profiled under ^{2}D RTL GC-SCD/NCD/MS. Fig. 2 demonstrates an example of a single heart-cut in the ^{1}D retention time (RT) 16.8–18.8 min (the sulfur fraction 5 in Fig. 1b) and both ^{1}D and ^{2}D TIC (Fig. 2a), SCD/NCD chromatograms (Fig. 2b and c) (separation obtained in ^{2}D is zoomed and given in Fig. 3). After heart-cutting, the heart-cut fraction was cryo-focused in the CTS2 at −100 °C during the rest of ^{1}D GC run. At the RT 50 min, ^{1}D GC was back-flushed and the CTS2 was rapidly heated to start ^{2}D RTL GC. For the identification of sulfur compounds in the ^{2}D SCD chromatogram, the corresponding peak in the ^{2}D TIC and the mass spectral data were used and a combined search using the MS library and the LRI database was performed. Besides this, the corresponding nitrogen peak in the ^{2}D NCD chromatogram was used for confirmation of the presence of nitrogen. Using ^{2}D LRI, Aroma Office ^{2}D database, and the MS library search, six sulfur compounds, e.g. ethyl 2-methyl sulfanyl acetate, 1-(1,3-thiazol-2-yl)ethanone (2-acetyl thiazole) and its isomer, thiophene-2-carbaldehyde (2-formyl thiophene), thiophene-3-carbaldehyde (3-formyl thiophene), and 3-methyl sulfanyl propan-1-ol (methionol), were positively identified in this fraction from only 100 μL of sample. Additionally, the same ^{2}D analysis but with "raw scan mode", which generates a profile mass spectrum, was done and the formula identification was performed with MassWorks software. After the calibration using MassWorks software, the mass peak shape involving isotope distribution is identical to the theoretical spectrum and the accuracy of mass

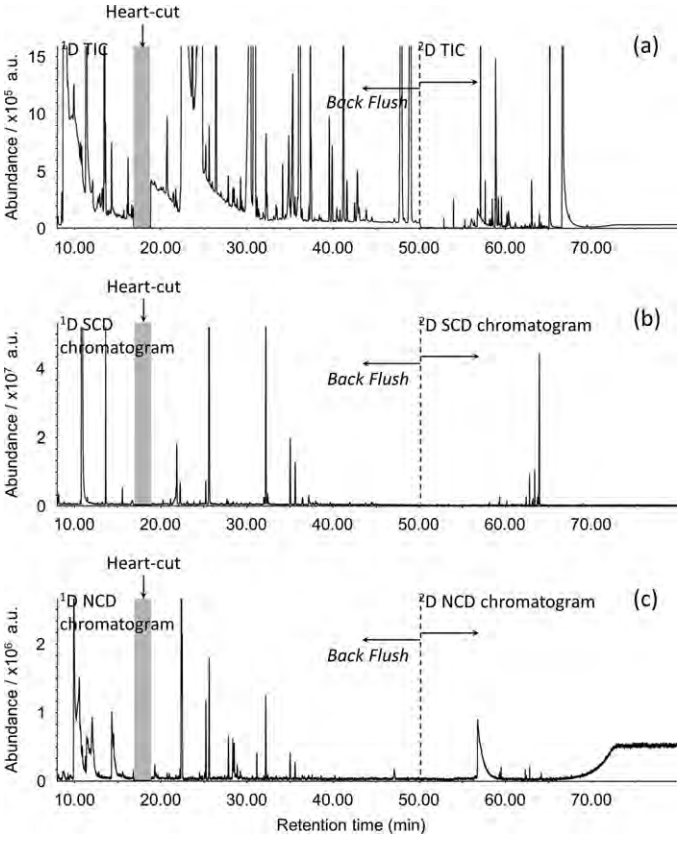

Fig. 2. An example of a single heart-cut in the ^{1}D retention time (RT) 16.8–18.8 min (the sulfur fraction 5 in Fig. 1) and both ^{1}D and ^{2}D TIC, and SCD/NCD chromatograms. (a) ^{1}D/^{2}D TIC; (b) ^{1}D/^{2}D SCD chromatogram; (c) ^{1}D/^{2}D NCD chromatogram.

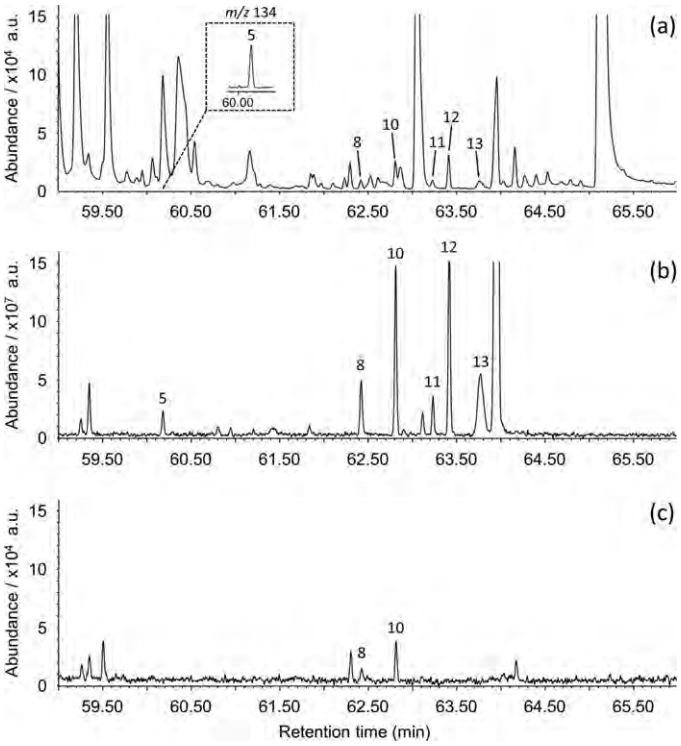

Fig. 3. ^{2}D TIC, SCD and NCD chromatograms of the sulfur fraction 5 of the unaged whisky "A". (a) ^{2}D TIC; (b) ^{2}D SCD chromatogram; (c) ^{2}D NCD chromatogram. 5, ethyl 2-methyl sulfanyl acetate; 8, acetyl thiazole isomer; 10, 2-acetyl thiazole; 11, 3-formyl thiophene; 12, 2-formyl thiophene; 13, methionol.

position is greatly improved (e.g. down to four decimal places even with the unit resolution quadrupole MS measurement). Consequently, a unique isotope distribution as well as an accurate mass could be used for the formula identification. For these six sulfur compounds, the number one candidate formula obtained by MassWorks software corresponded to the formula of the identified compound by using the MS library and/or the LRI database. The mass errors of molecular ions ranged from −5.9 to −8.8 mDa and the spectral accuracies ranged from 98.63 to 99.63 (the spectral accuracy reflects the correctness of the complete mass spectral response of an ion in the form of continuously sampled spectral error as a function of all relevant *m*/*z* values [21]). From the 16 sulfur fractions selected by the ^{1}D SCD chromatograms, 20 sulfur compounds were positively identified in the unaged whisky "A". Table 2 summarizes 20 sulfur compounds with the parameters used for the identification. Since the mass errors of less than ±10 mDa and the spectral accuracies of more than 98 were obtained for the identified 20 sulfur compounds, we used these values as criteria for the formula identification of unknown sulfur compounds. Eight additional best candidate formulas were obtained for the unknown sulfur compounds (S1–S8) in the unaged whisky "A" (Table 3). We have succeeded in identifying S2 as 1-ethoxy-2-(methyldisulfanyl)ethane (3,4-dithiapentyl ethyl ether) [4,22] and the further identification of the others are work in progress. These 28 sulfur compounds were used for multivariate analysis in the next section.

3.3. Principal component analysis of sulfur compounds in whisky using ^{2}D RTL GC-SCD with 16 simultaneous heart-cuts

FEDHS of 100 μL of whisky transfer substantial amounts of non-target compounds such as esters and fatty acids to the ^{1}D column, resulting in subtle retention time shift even with the thick film column such as DB-1 with dimensions 30 m × 0.25 mm i.d. × 1.0 μm df. In this case, it is essential to have retention time alignment for all the components in all the chromatograms before multivariate analysis. In the meantime, ^{2}D GC can reduce the effect of the non-target compounds for the retention time shift. Also, the PCM and the CTS2 cryo-trap system can provide highly reproducible injection of the heart-cut fractions into the ^{2}D GC column under RTL condition. Therefore, we applied ^{2}D RTL GC-SCD with 16 simultaneous heart-cuts containing 28 sulfur compounds (listed in Tables 2 and 3) to multivariate analysis. Triplicate analyses were performed for each whisky sample. The data set which consists of 12 analyses was used for multivariate analysis to consider repeatability of analysis. Fig. 4 demonstrates an example of the 16 heart-cuts of the unaged whisky "A" and both ^{1}D and ^{2}D TIC (Fig. 4a), SCD/NCD chromatograms (Fig. 4b and c). Fig. 5 shows a comparison of the ^{2}D RTL SCD chromatograms between all whisky samples. Compared to the unaged whisky, many sulfur compounds (3, 5, 6, 8, 9, 13, 18–20, and S1–S8) dramatically decreased or were not detected in the aged whisky. Masuda and Nishimura investigated changes in sulfur compounds during whisky aging in wood casks and found that low boiling sulfur compounds {e.g. methylsulfanylmethane (dimethyl sulfide) (DMS) and (methyldisulfanyl)methane (dimethyl disulfide) (DMDS)} and some medium/low boiling sulfur compounds (e.g. 3-methyl sulfanyl propyl acetate and methionol) decrease rapidly during maturation [23]. Similar results have been reported for DMS and DMDS in Irish whiskey [24]. These sulfides have disagreeable aromas (e.g. asparagus, cabbage, and onion), but disappear rapidly during maturation. Natural evaporation is a factor in the decrease, but oak wood is also necessary for their removal. It is reported that the burnt char on the inside of the cask can act as adsorption layer to remove these disagreeable volatile sulfur compounds [25]. On the other hand, several sulfur compounds did not appreciably change (1, 15, and 17) or even increased (4, 11, and 12 for both "A" and "G", 10 for "G", and 16 for "A"). Compounds that

Table 4
Concentration of the key sulfur compounds in unaged and aged single malt whisky samples.

No.	Compound (common name)[a]	Formula	Unaged "A"		Aged "A"		Unaged "G"		Aged "G"	
			Concentration (ng mL^{-1})	RSD (%), n = 3	Concentration (ng mL^{-1})	RSD (%), n = 3	Concentration (ng mL^{-1})	RSD (%), n = 3	Concentration (ng mL^{-1})	RSD (%), n = 3
3	Diethyl sulfite	$C_4H_{10}O_3S$	100	2.2	0.52	3.4	93	3.7	0.73	7.3
4	Ethyl methanesulfonate	$C_3H_8O_2S$	28	2.0	62	1.5	50	2.1	75	0.37
9	3-Methyl sulfanyl propyl acetate	$C_6H_{12}O_2S$	35	2.5	0.32	12	20	2.6	0.35	6.1
10	1-(1,3-thiazol-2-yl)ethanone (2-acetyl thiazole)	C_5H_5NOS	13	7.6	9.1	4.4	15	12	17	3.1
11	Thiophene-3-carbaldehyde (3-formyl thiophene)	C_5H_4OS	2.8	5.9	4.5	6.0	3.4	1.7	18	4.5
12	Thiophene-2-carbaldehyde (2-formyl thiophene)	C_5H_4OS	28	3.6	21	3.1	22	2.6	28	0.59
13	3-Methyl sulfanyl propan-1-ol (methionol)	$C_4H_{10}OS$	110	3.5	nd	–	32	5.1	nd	–
16	3-Methylthiophene-2-carbaldehyde (3-methyl-2-formyl thiophene)	C_6H_6OS	9.6	4.6	17	1.1	15	2.2	13	3.2
18 + S4	2-Methyl-1,3-benzothiazole + S4	$C_8H_7NS/C_3H_8OS_2$	26	4.1	nd	–	31	1.4	nd	–
19	1,3-Benzothiazole	C_7H_5NS	210	2.3	1.2	9.5	170	2.5	1.0	4.5
S1	S1	$C_5H_{10}O_2S$	11	3.5	nd	–	21	5.1	nd	–
S2	S2[b]	$C_5H_{12}OS_2$	6.4	4.3	0.41	1.3	9.5	2.6	0.30	10
S5	S5	$C_8H_{15}NOS$	110	6.0	nd	–	100	2.7	nd	–

[a] Common name was shown in a parenthesis.
[b] S2 was identified as 1-ethoxy-2-(methyldisulfanyl)ethane (3,4-dithiapentyl ethyl ether) from Refs. [4,22].

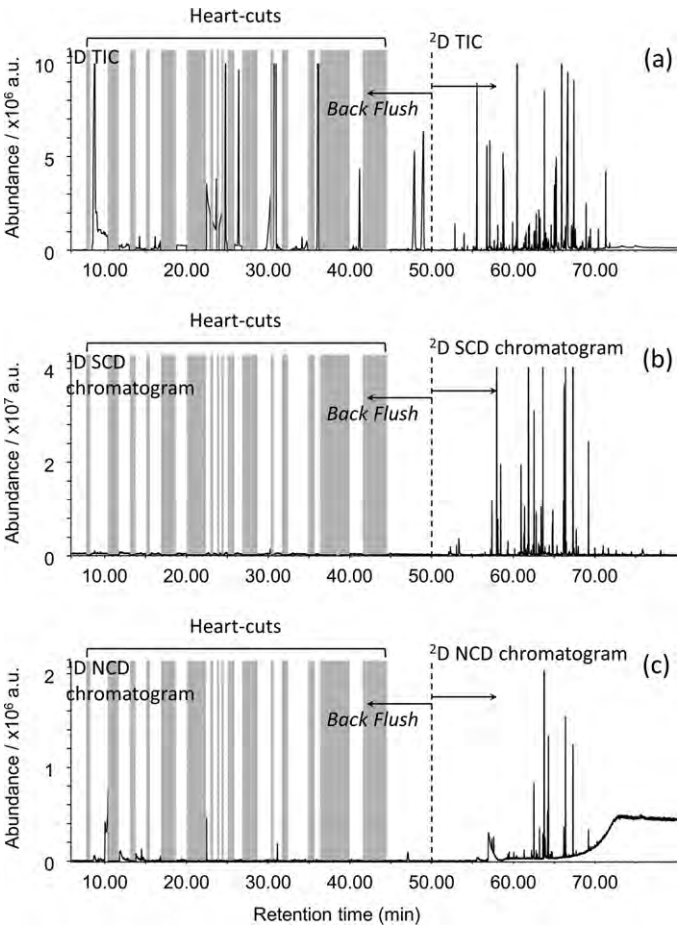

Fig. 4. An example of simultaneous 16 heart-cuts in the ^{1}D GC and both ^{1}D and ^{2}D TIC, SCD/NCD chromatograms. (a) ^{1}D/^{2}D TIC; (b) ^{1}D/^{2}D SCD chromatogram; (c) ^{1}D/^{2}D NCD chromatogram.

increase during aging, such as thiophenes, originate from the wood as Maillard reaction products. They have a roasted spicy and/or fruity aroma which can contribute positively to the whisky flavor [26].

A principal component analysis (PCA) was applied to all samples to obtain a simplified view of the relationship between unaged and aged whisky, as well as two different whisky samples. The three principal components (PC) account for 99.98% of the total variance of the data (PC1: 97.4%, PC2: 1.6%, and PC3: 0.98%, respectively). Fig. 6a shows a PCA score plot using PC1 and PC2. PC1 clearly differentiates between the unaged and aged whisky samples. From the corresponding loading plot in Fig. 6b, the sulfur compounds can be characterized according to the strength of the contribution to each PC (the compounds which have the PC factor of more than ±0.1 are labeled with a bold letter). Diethyl sulfite (3), 1,3-benzothiazole (19), and S5 show higher contribution to the positive PC1, while ethyl methanesulfonate (4) highly contributes to the negative PC1. Also, methionol (13) and 2-methyl-1,3-benzothiazole (18) + S4 (these two compounds were not separated in the ^{2}D RTL GC-SCD with the simultaneous 16 heart-cuts) show moderate contribution to the positive PC1, while 2-formyl thiophene (12) and 3-methyl-2-formyl thiophene (16) moderately contribute to the negative PC1. In general, the positive PC1 is correlated with the decrease of sulfur compounds during the aging, while the negative PC1 is correlated with the increase of sulfur compounds. PC 2 clearly differentiates between the unaged whisky "A" and "G", however, the aged whisky samples are not appreciably differentiated. From the loading plot, 2-methyl-1,3-benzothiazole (18) + S4 and S5 show higher contribution to the positive PC2, while methionol (13) highly contributes to the negative PC2. Also, diethyl sulfite (3), ethyl methanesulfonate (4), 3-methyl-2-formyl thiophene (16), S1, and S2 (3,4-dithiapentyl ethyl ether) show moderate contribution to the positive PC2, while 3-methyl sulfanyl propyl acetate (9) moderately contributes to the negative PC2. In general, the positive PC2 is correlated with higher response of sulfur compounds in the unaged whisky "G", while the negative PC2 is correlated with higher response of sulfur compounds in the unaged whisky "A".

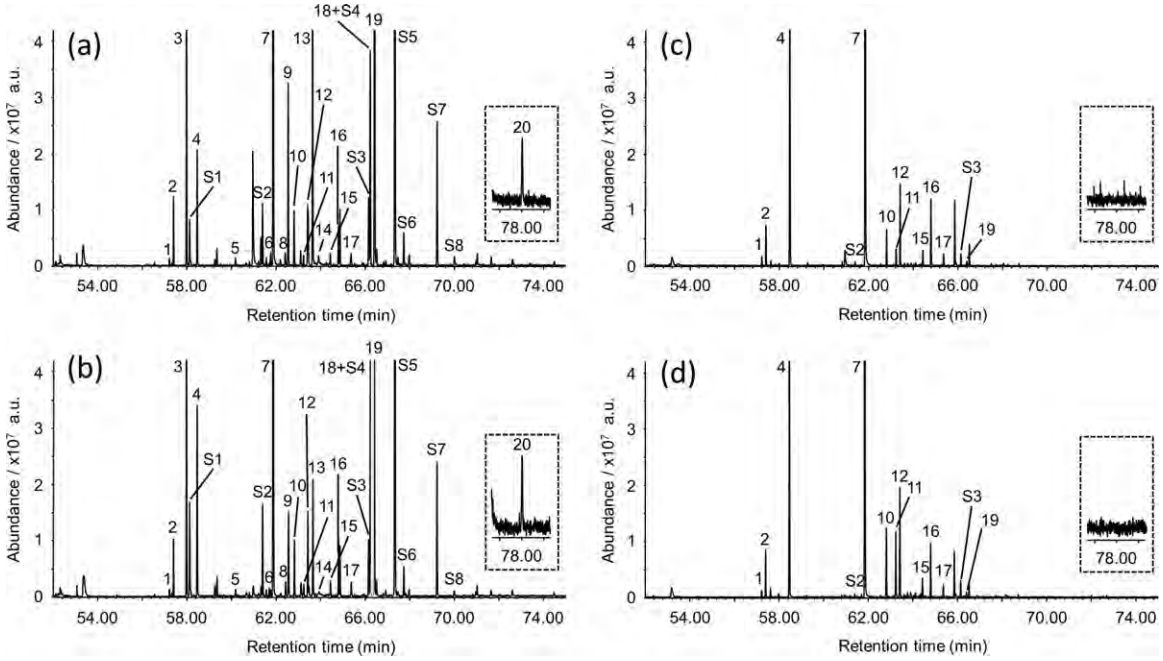

Fig. 5. A comparison of the ^{2}D RTL SCD chromatograms between all whisky samples. (a) Unaged whisky "A"; (b) unaged whisky "G"; (c) aged whisky "A"; (d) aged whisky "G". The marked peaks represent positively identified sulfur compounds (see Table 2, 1–20) and unknown sulfur compounds which have candidate formulas (see Table 3, S1–S8).

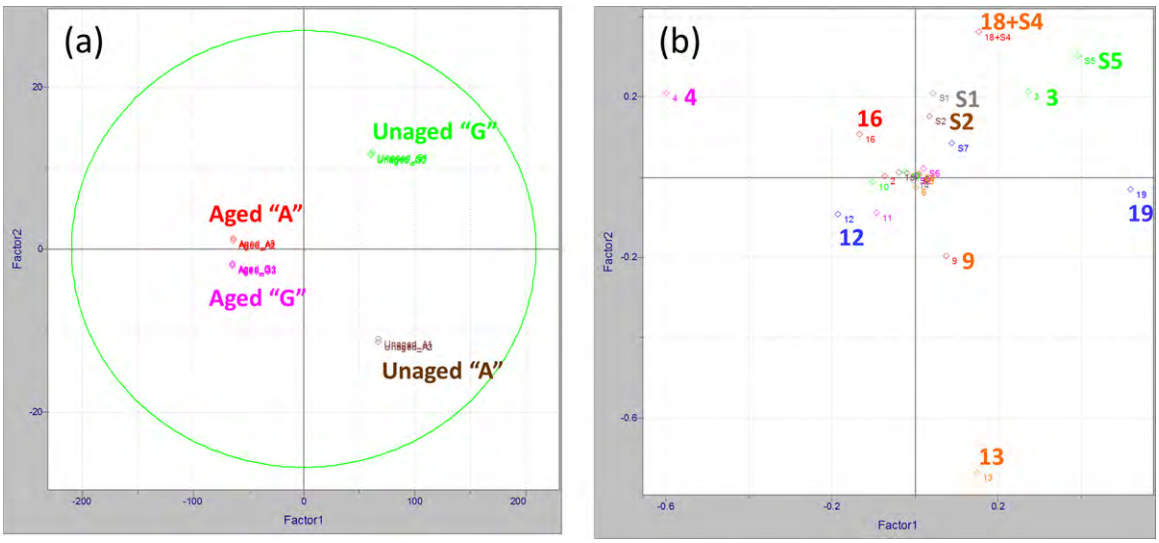

Fig. 6. PCA score plot using PC1 and PC2 (a) and the corresponding loading plot (b). The compounds which have the PC factor of more than ±0.1 are labeled with a bold letter.

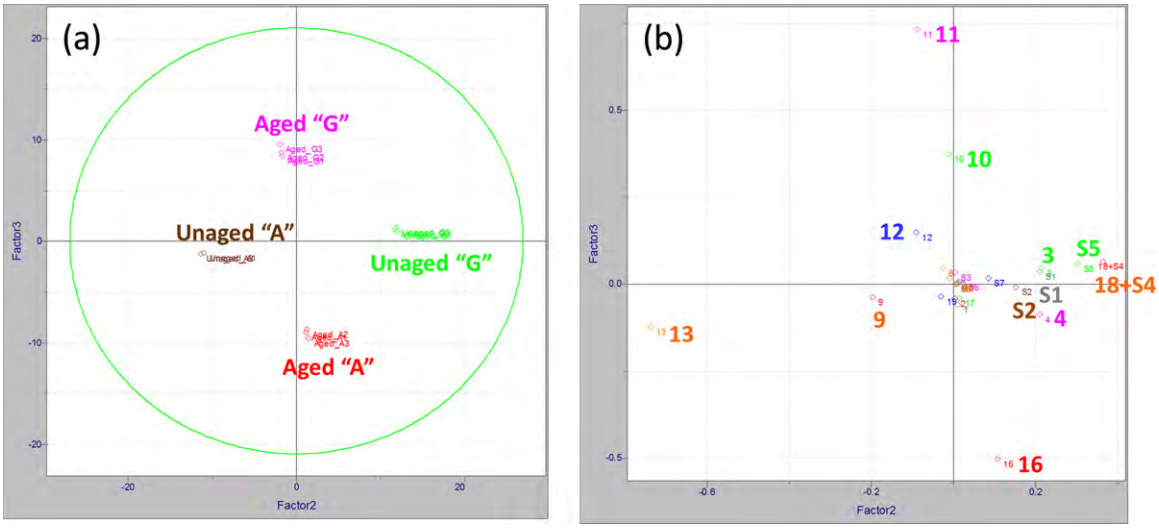

Fig. 7. PCA score plot using PC2 and PC3 (a) and the corresponding loading plot (b). The compounds which have the PC factor of more than ±0.1 are labeled with a bold letter.

Although PC1 does not differentiate between the aged whisky samples and PC2 shows very small difference, PC3 clearly differentiates those samples. Also, the score plot using PC2 and PC3 can clearly differentiates all whisky samples (Fig. 7a). From the corresponding loading plot in Fig. 7b, 2-acetyl thiazole (10), 3-formyl thiophene (11), and 2-formyl thiophene (12) show higher contribution to the positive PC3, while 3-methyl-2-formyl thiophene (16) highly contribute to the negative PC3. The positive PC3 is correlated with higher response of sulfur compounds in the aged whisky "G", while the negative PC3 is correlated with higher response of sulfur compound in the aged whisky "A", if those sulfur compounds are still detected in the aged samples.

Finally, quantification of fourteen sulfur compounds (3, 4, 9, 10, 11, 12. 13, 16, 18 + S4, 19, S1, S2, and S3), which could be characterized as key sulfur compounds with the PC factor of more than ±0.1 in the loading plot, was performed using a linear and equimolar response of the ^{2}D RTL GC-SCD to sulfur compounds [27]. 1-Thiophen-3-ylethanone (3-acetyl thiophene) (log K_{ow}: 1.49), which was not present in the samples, was chosen as a standard and spiked into the sample between 1 and 200 ng mL^{-1} (7.9 and 1600 nmol mL^{-1}). The recovery of 3-acetyl thiophene in the unaged whiskey "A" spiked at 100 ng mL^{-1} was 101% (RSD: 2.0%, n = 5). Concentrations of the key sulfur compounds in the unaged and aged whisky samples were in the range of 0.3–210 ng mL^{-1} (RSD: 0.37–12%, n = 3). Table 4 summarizes the determined values of the key sulfur compounds.

Using the FEDHS-^{1}D/^{2}D RTL GC-SCD/NCD/MS system, once the key sulfur compounds are identified in ^{2}D GC mode (and subsequent PCA), then it may be possible to routinely monitor and quantify them in ^{1}D GC mode with the selective detection such as SCD. This would be a very user-friendly option, especially because no instrumental configuration changes need to be made.

4. Conclusion

The combination of FEDHS, ^{1}D/^{2}D RTL GC-SCD/NCD/MS, the combined search using mass spectra and LRI, formula identification, and PCA, offers a very effective synergy for identifying key sulfur compounds in the unaged and aged whisky. Twenty sulfur compounds were positively identified in the unaged whisky by sequential heart-cuts of the 16 sulfur fractions. Also, 8 formulas could be obtained for unknown sulfur compounds.

^{2}D RTL GC-SCD data obtained by simultaneous heart-cuts of the 16 sulfur fractions clearly demonstrated the changes of sulfur

compounds during the aging step. PCA of the ^{2}D RTL GC-SCD data proved to be a remarkable tool for distinguishing between unaged and aged whisky, as well as two different whisky samples. Fourteen sulfur compounds could be characterized as key sulfur compounds and determined at sub-ng mL^{-1} to ng mL^{-1} levels.

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