

## Article

# Profiling Croatian Whisky Using GC/MS-MS and UHPLC-QTOF

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**Abstract:** This study investigates the chemical profiling of Croatian whisky using advanced analytical techniques: Solid-phase microextraction followed by gas chromatography coupled to mass spectrometry (SPME-GC-MS) and ultra-high-performance liquid chromatography quadrupole time-of-flight (UHPLC-QTOF). To our knowledge, there are no studies on profiling Croatian whiskies to date. Therefore, the primary objective is to identify and quantify the volatile and non-volatile compounds contributing to the flavor and aroma profile of Croatian whisky. The methods applied included sample preparation, extraction, and subsequent analysis using these advanced analytical techniques. The results revealed a diverse range of compounds, including esters, alcohols, phenols, and acids, which are integral to the whisky's sensory characteristics. Furthermore, key volatile and non-volatile compounds were identified in Croatian whiskies, revealing a complex aromatic profile influenced by compounds like D-limonene, linalool, and  $\beta$ -damascenone, which contribute citrus, floral, and fruity notes. The findings also highlighted compounds associated with aging, such as esculetin and ellagic acid, which enhance flavor complexity and emphasized the role of maturation, grain type, and production methods in shaping the unique sensory characteristics of Croatian whiskies. This study concludes that SPME-GC-MS and UHPLC-QTOF are effective tools for the detailed chemical profiling of whisky, providing valuable insights for quality control and product differentiation in the whisky industry.

**Keywords:** Croatian whisky; SPME-GC-MS; UHPLC-QTOF; chemical profiling; volatile compounds; non-volatile compounds; flavor profile; aroma profile



Academic Editors: Monica Gallo and Ioannis G. Roussis

Received: 20 December 2024

Revised: 27 February 2025

Accepted: 28 February 2025

Published: 4 March 2025

**Citation:** Lončarić, A.; Castillo, A.; Celeiro, M.; Miličević, B.; Bušić, H.; Anadolac, T.; Skoko, A.-M.G.; Lores, M. Profiling Croatian Whisky Using GC/MS-MS and UHPLC-QTOF. *Appl. Sci.* **2025**, *15*, 2738. <https://doi.org/10.3390/app15052738>

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## 1. Introduction

Whisky or whiskey production relies on three main ingredients: cereal, yeast, and water. Whisky production follows a relatively homogeneous fundamental process that is in some way regulated by the EU Regulation 2019/787 [1,2]. EU Regulations define whisky or whiskey as a spirit drink produced following three main production operations:

- (i) Distilling a mash from malted cereals, with or without unmalted cereal grains that have been saccharified by malt enzymes or other natural enzymes and then fermented by the action of yeast;
- (ii) Every distillation is carried out at less than 94.8% vol., preserving flavor and aroma from the raw materials;
- (iii) Maturation of the final distillate from at least three years in wooden casks no larger than 700 L. The final distilled product, to which only water and plain caramel (for coloring) may be added, must retain the color, aroma, and flavor imparted by the production processes outlined in points (i), (ii), and (iii).

Additionally, the EU regulation requires that whisky has a minimum alcohol content of 40% and prohibits the addition of any extra alcohol. Despite its relatively standardized production framework, governed by EU Regulation 2019/787, the final whisky profile is significantly influenced by numerous variables such as cereal variety, fermentation conditions, distillation techniques, maturation time, and cask type.

These factors result in whisky with distinct flavors, shaped by numerous active flavor compounds known as congeners. The main congeners in whisky include alcohols, aldehydes, acids, esters, phenols, terpenes, tannins, and lactones, which are produced at different stages of the production process and may be more prominent depending on the specific process involved [1]. Traditional whisky-producing regions such as Scotland, Ireland, and the United States have well-documented profiles, yet the chemical composition of whiskies from emerging regions like Croatia remains underexplored. Croatian whisky production, influenced by local ingredients, environmental conditions, and modern techniques, offers a distinctive character that warrants detailed scientific investigation. Advanced analytical techniques such as gas chromatography-mass spectrometry (GC-MS/MS) and ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UHPLC-QTOF) provide robust platforms for comprehensive profiling of both volatile and non-volatile compounds. These methods enable the identification and quantification of key flavor compounds, offering insights into the biochemical pathways and production processes that define the sensory attributes of whisky. The types of grains used in the mash provide a range of aroma and flavor compounds to the new distillate, contributing to the distinct characteristics of each whisky. For example, compounds like lyoniresino and its glycosides have been identified in whisky by Kyraleou et al. [3], while syringaresinol is commonly found in grains such as barley, rye, and corn [4]. Niemi et al. [5] also detected syringaresinol and other lignans in enzymatically fractionated extracts of brewer's spent grain, which consists of grain and malt residues left after mashing [6,7]. Additionally, (E)-2-nonenal, a compound formed through the oxidation of linoleic and linolenic acids during malting, can also be introduced from wood during maturation [8], and it can be found in trace levels in barley [6,9]. (E)-2-nonenal contributes to the green notes in Scotch malt whisky [10], and it was recognized as an important compound in the aroma profile of both new make and aged Bourbon [7,11]. Aldehydes could be derived from raw materials and be produced post-harvest throughout the malting process via enzymatic transformations of lipids in the presence of oxygen. This process occurs primarily through the lipoxygenase pathway (e.g., hexanal) [6,12,13]. After malting, fermentation is a crucial step in whisky production and significantly influences its aroma profile. During fermentation, the conversion of sugar into alcohol and other compounds generates a wide spectrum of volatile compounds, including esters, fatty acids, and higher alcohols, which contribute to the complexity and depth to the final products of aroma. Esters are an important group of compounds predominantly formed by yeast during alcoholic fermentation. They play a critical role in imparting fruity (e.g., ethyl hexanoate and isoamyl acetate) and floral (e.g., 2-phenylethyl acetate) aromatics to the final product [1,14,15]. In

addition to esters, other alcohols, such as 2-methyl-1-butanol and 3-methyl-1-butanol, are generated by yeast through the decarboxylation of  $\alpha$ -keto acids, followed by the reduction of the resultant aldehydes to alcohols. Other chemical groups, including terpenes, ketones, aldehydes, sulfur-containing compounds, and furans, also play key roles in forming the complex aroma profile of newly distilled spirits [16–19]. Distillation is a crucial step in whisky production where ethanol and congeners are separated and incorporated into the distillate [20]. The distillation process further shapes the whisky's aroma profile by concentrating certain compounds and removing others. The volatile compounds with boiling points lower than ethanol are concentrated during distillation, while heavier, less volatile compounds remain in the stillage. This separation process leads to the refinement and concentration of specific aroma compounds, contributing to the distinctive scent and flavor of the final product [14,15]. Additionally, the particular distillation methods used, such as pot still and column distillation, have a significant impact on the flavor and aromatic profile of the distilled spirit [21,22]. Pot stills are commonly used in Scotch production and in some part in Irish whiskies, while column stills are more typical in American bourbon and rye whisky production. The number of distillation runs and the shape and size of the still can contribute to the final aroma and flavor profile of the whisky. Moreover, fractional separation during distillation permits the modulation of the quantity and the characteristics of volatile aromatic compounds in the distillates. Such distillation processes lead to the concentration of volatile aromatic compounds, thereby enhancing their sensory impact. Finally, the maturation process in oak casks plays a crucial role in enriching the whisky's complexity and depth of aromas. During this stage, flavors are extracted from the wood, imparting unique characteristics to the spirit [23]. Some compounds associated with longer ageing of the whisky are octadecanoic acid, esculetin, ellagic acid, flavonoids, and lyoniresinol and its derivatives: dihydroxy- or hydro-peroxy, methylated, and lyoniresinol xyloside. Understanding the interplay between these compounds and the production process is essential for characterizing the unique attributes of Croatian whisky. This comprehensive profiling helps in identifying key biomarkers for quality control and offers insights into how specific production variables impact the final sensory experience. Additionally, it provides a scientific basis for differentiating Croatian whisky in the global market, emphasizing its distinctiveness rooted in local raw materials and traditional craftsmanship. This study represents the first comprehensive analytical characterization of Croatian whisky using solid-phase microextraction gas chromatography-mass spectrometry (SPME-GC-MS) for volatile compounds and untargeted UHPLC-QTOF for non-volatile compounds. Unlike previous studies focused on whiskies from established regions, this research highlights the unique chemical composition of Croatian whisky, shaped by indigenous raw materials, local fermentation practices, and specific maturation conditions.

The novelty of this study lies in its dual analytical approach, combining SPME-GC-MS and UHPLC-QTOF, to deliver an integrated profile of Croatian whisky's volatile and non-volatile constituents. This approach not only identifies key flavor-active compounds but also uncovers previously unreported non-volatile metabolites, contributing to a deeper understanding of the factors influencing Croatian whisky's distinctive sensory characteristics. The findings have the potential to enhance quality control, support product differentiation, and promote Croatian whisky within the global spirits market.

## 2. Materials and Methods

### 2.1. Whisky Sample

Two commercial whisky samples from Croatia were selected for this study, including single malt and four grain whisky. The whiskies were analyzed using SPME-GC-MS and UHPLC-QTOF. The other six commercial whiskies, four Scotch blended and two Scotch

single malt whiskies, were only analyzed using UHPLC-QTOF to examine untargeted non-volatile compounds to determine differentiation from Croatian whiskies.

## 2.2. Reagents and Materials

Water and methanol (both MS grade) were supplied by Scharlau (Barcelona, Spain). Formic acid was purchased from Sigma Aldrich (Steinheim, Germany). Commercial 50/30  $\mu\text{m}$  divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber and a manual SPME holder were obtained from Supelco (Bellefonte, PA, USA). Prior to first use, the fiber was conditioned as recommended by the manufacturer, inserting it in the GC injector under helium flow for 30 min at 270 °C.

## 2.3. SPME (Solid-Phase Microextraction)

SPME was carried out using 3 mL of diluted sample (1:10, *v/v* in water) placed into a 20 mL glass vial and sealed with an aluminum cap furnished with PTFE-faced septa. The vial was immersed in a water bath maintained at 100 °C. After 3 min of temperature equilibration, the DVB/CAR/PDMS fiber was exposed to the headspace over the sample (headspace sampling mode, HS) for 20 min. Afterwards, the fiber was retracted into the needle of the syringe holder and immediately thermally desorbed at 260 °C in the GC injection port for 3 min, after which GC-MS analysis was carried out.

## 2.4. GC-MS Analysis

The screening of volatile compounds was performed by GC-MS employing an Agilent 7890A (GC) coupled to an Agilent 5975C inert MS detector with a triple axis detector from Agilent Technologies (Palo Alto, CA, USA). Separation was carried out on a ZB-Zebron Semivolatiles capillary column (30 m  $\times$  0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness) obtained from Phenomenex (Torrance, CA, USA). Helium (purity 99.999%) was employed as the carrier gas at a constant column flow of 1 mL min<sup>-1</sup>. The GC oven temperature was programmed from 50 °C (held 3 min) to 200 °C at 4 °C min<sup>-1</sup> (held 2 min) and, finally, to 290 °C at 20 °C min<sup>-1</sup>. The total run time was 50 min. The injector temperature was set at 260 °C and splitless mode was used (1 min, 75 mL min<sup>-1</sup>). The mass spectrometer detector (MSD) was operated in the electron ionization (EI) positive mode (+70 eV), and the temperatures of the transfer line, the quadrupole, and the ion source were set at 290 °C, 150 °C, and 230 °C, respectively. Full Scan (FS) acquisition mode was employed monitoring mass/charge (*m/z*) fragments between 30 and 800. The system was operated by Agilent MSD ChemStation E.02.00.493 software (Agilent Technologies, USA). The analytes' tentative identification was performed by comparison (match > 80%) between the obtained experimental MS spectral and those provided by the commercial spectral library database (NIST). The SPME and GC-MS analyses were carried out in duplicate to ensure accuracy and reproducibility of the results.

## 2.5. UHPLC-QTOF Analysis

The whisky samples were filtered through a 0.22  $\mu\text{m}$  PTFE filter. Untargeted analysis was performed by ultra-high performance liquid chromatography (Elute UHPLC 1300) coupled to a compact model quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Fremont, CA, USA). The stationary phase consisted of a Bruker Intensity Solo 2 C-18 column (100 mm  $\times$  2.1 mm, 2.0  $\mu\text{m}$ ) set at 40 °C. The mobile phase (A) and (B) consisted of 0.1% formic acid in water and methanol, respectively. The mass deviation correction is adjusted by injecting a calibrant consisting of a salt solution (NaOH 1-10-3 M) isovolumetric 2-propanol: water with 0.2% formic acid as calibrant. The gradient consisted of 0.4 min 95/5 (A/B) where the calibrant enters to then perform an increasing ramp of 70/30 (4.5 min), 63/37 (8 min), 50/50 (9 min), and 10/90 (11 min), which was held for 4 min

and returned to the initial conditions at 16 min (kept for 4 min). The total run time was 20 min. Acquisition was carried out in AutoMS/MS mode with negative polarity, using an electrospray ionization (ESI) source detecting mainly pseudomolecular  $[M-H]^-$  ions, as described by Castillo et al. [24]. Briefly, an acquisition rate of 8 Hz in 1 s cycles in a voltage range of 10–105 eV and a filtering amplitude of 20–1000  $m/z$  were used using the Compass HyStar 5.0. acquisition software (Bruker, USA). The UHPLC-QTOF analysis was carried out in duplicate to ensure accuracy and reproducibility of the results.

### 2.6. Statistical Analysis

Through MetaboScape<sup>®</sup> Version 4.0.4 (Build 19) and using the T-REX 3D algorithm, the different study groups were categorized. MetaboScape<sup>®</sup>'s built-in statistical package was used to analyze the results using an unsupervised PCA model, employing Pareto as the scaling algorithm with a 10% cross-validation mode and a minimum variance explanation of 98%. Detailed comparison between pairs of groups was carried out using a *t*-test analysis, generating a volcano plot, taking as marker compounds those bounded by a  $p < 0.05$  and Fold change limit 2. For the prediction of molecular formulae, the maximum mass deviation error of 5 mDa and mSigma 50 were set as acceptability parameters in all cases. The generated identifications were checked against spectral libraries: Mass Bank European and MassBank of North America (MoNA) and online databases: Chemical Entities of Biological Interest (ChEBI) and National Center for Biotechnology Information (NCBI).

## 3. Results and Discussion

This study's primary focus is on Croatian whisky from the growing interest in emerging whisky-producing regions and the need for scientific characterization of their products. Unlike traditional whisky-producing nations such as Scotland, Ireland, and the United States, Croatia's whisky industry is still in its developmental stages, with limited literature available on its chemical composition. Croatian whisky is influenced by locally sourced raw materials, distinctive climatic conditions, and modern distillation and maturation techniques, making it a valuable subject for analytical investigation.

A comparative aspect with Scotch whisky is incorporated to contextualize the findings. Scotch whisky serves as a benchmark due to its well-documented chemical profile and global recognition. By analyzing six Scotch whisky samples using UHPLC-QTOF, this study assesses the extent to which Croatian whisky aligns with or diverges from established Scotch whisky profiles. This comparative analysis helps elucidate the impact of raw materials, fermentation conditions, distillation methods, and maturation processes on whisky chemistry.

### 3.1. Aromatic Profile of Croatian Whisky Determined by SPME-GC-MS

The analysis of aromatic compounds in Croatian whisky using solid-phase microextraction-gas chromatography coupled to mass spectrometry (SPME-GC-MS) identified a wide variety of chemical compounds contributing to its complex flavor profile. The main advantage of SPME is that in a single step the extraction and concentration of the analytes is carried out, allowing the determination of compounds even at trace concentration levels. A total of six alcohols, twenty-one esters, two aldehydes, seven fatty acids, three terpenes, and one lactone were detected (Table 1). These compounds contribute to the fruity, floral, and spicy notes in whisky.

**Table 1.** Aromatic profile of Croatian whiskies determined by SPME-GC-MS.

| Compound             | Retention Time (min) | Kovats RI <sup>a</sup> | MS Ions (Relative Abundance, %) | Mass | Formula  | Group      | Descriptor   | Odor Type   | Samples     |            |
|----------------------|----------------------|------------------------|---------------------------------|------|--|------------|--|-------------|-------------|------------|
|                      |                      |                        |                                 |      |  |            |  |             | Single Malt | Four Grain |
| Ethanol              | 0.934                | 668                    | 31 (100), 45 (51), 46 (21)      | 46   | C <sub>2</sub> H <sub>6</sub> O                | Alcohol    | ethanolic  |             | x           | x          |
| Ethyl hexanoate      | 10.343               | 1002                   | 88 (100), 43 (85), 99 (53)      | 144  | C <sub>8</sub> H <sub>16</sub> O <sub>2</sub>  | Ester      | red apple, aniseed, cherry-like, red fruits, pear                        | Fruity      | x           | x          |
| D-Limonene           | 11.426               | 1030                   | 68 (100), 93 (59), 67 (44)      | 136  | C <sub>10</sub> H <sub>16</sub>                | Terpene    | sweet, orange, citrus  | Fruity      | x           | x          |
| Ethyl heptanoate     | 14.11                | 1100                   | 88 (100), 113 (45), 101 (33)    | 158  | C <sub>9</sub> H <sub>18</sub> O <sub>2</sub>  | Ester      | fruity pineapple, sweet, banana, berry, green, seedy                     | Fruity      | x           | x          |
| Linalool             | 14.181               | 1100                   | 71 (100), 93 (76), 55 (63)      | 154  | C <sub>10</sub> H <sub>18</sub> O              | Terpene    | flowery, citrus  | Floral      | x           | x          |
| 2-phenylethanol      | 14.609               | 1118                   | 91 (100), 92 (56), 65 (23)      | 122  | C <sub>8</sub> H <sub>10</sub> O               | Alcohol    | floral, rose, dried rose flower, rose water                              | Floral      | x           | x          |
| (E)-2-Nonenal        | 16.375               | 1147                   | 43 (100), 55 (89), 70 (86)      | 140  | C <sub>9</sub> H <sub>16</sub> O               | Aldehyde   | fried, toasted, fatty  | Fatty       | x           | x          |
| 2-nonanol            | 16.82                | 1187                   | 45 (100), 69 (17), 55 (134)     | 144  | C <sub>9</sub> H <sub>20</sub> O               | Alcohol    | waxy, green, creamy, citrus, orange, cheesy, fruity                      | Waxy        | x           | x          |
| Octanoic acid        | 17.562               | 1279                   | 60 (100), 73 (62), 43 (42)      | 144  | C <sub>8</sub> H <sub>16</sub> O <sub>2</sub>  | Fatty acid | fatty, waxy, rancid, oily, vegetable, cheesy                             | Fatty       | x           |            |
| Ethyl octanoate      | 17.749               | 1198                   | 88 (100), 101 (35), 127 (26)    | 172  | C <sub>10</sub> H <sub>20</sub> O <sub>2</sub> | Fatty acid | sweet, waxy, fruity, pineapple, with creamy, fatty, mushroom             | Waxy        | x           | x          |
| Isoamyl alcohol      | 19.941               | 697                    | 55 (100), 42 (89), 70 (71)      | 88   | C <sub>5</sub> H <sub>12</sub> O               | Alcohol    | fusel, alcoholic, pungent, ethereal, cognac, fruity, banana and molasses | Fermented   | x           | x          |
| Ethyl nonanoate      | 21.215               | 1224                   | 88 (100), 101 (36), 141 (18)    | 186  | C <sub>11</sub> H <sub>22</sub> O <sub>2</sub> | Fatty acid | waxy, fruity, apple, banana, tropical, winey                             | Waxy/fruity | x           | x          |
| cis-Whisky lactone   | 21.964               | 1245                   | 99 (100), 43 (39), 71 (26)      | 156  | C <sub>9</sub> H <sub>16</sub> O <sub>2</sub>  | Lactone    | spicy, woody, coconut, creamy, nutty                                     | Woody       | x           | x          |
| ethyl hydrocinnamate | 22.914               | 1330                   | 104 (100), 91 (56), 107 (40)    | 178  | C <sub>11</sub> H <sub>14</sub> O <sub>2</sub> | Ester      | hyacinth, rose, honey, fruity  | Floral      |             | x          |
| Butyl caprylate      | 22.944               | 1388                   | 56 (100), 57 (87), 145 (64)     | 200  | C <sub>12</sub> H <sub>24</sub> O <sub>2</sub> | Ester      | butter   | Buttery     | x           | x          |
| n-Decanoic acid      | 23.826               | 1373                   | 60 (100), 73 (89), 129 (33)     | 172  | C <sub>10</sub> H <sub>20</sub> O <sub>2</sub> | Fatty acid | soapy, waxy, fruity  | Fatty       |             | x          |
| β-Damascenone        | 23.934               | 1386                   | 69 (100), 121 (60), 190 (16)    | 190  | C <sub>13</sub> H <sub>18</sub> O              | Terpene    | sweet, fruity, rose, plum, grape, raspberry, sugar                       | Floral      | x           |            |
| Ethyl 4E-decenoate   | 24.015               | 1375                   | 88 (100), 110 (97), 69 (96)     | 198  | C <sub>12</sub> H <sub>22</sub> O <sub>2</sub> | Ester      | green, pear, pineapple, apple  | Green       |             | x          |
| Ethyl caprate        | 24.544               | 1398                   | 88 (100), 101 (41), 155 (14)    | 200  | C <sub>12</sub> H <sub>24</sub> O <sub>2</sub> | Ester      | sweet, waxy, fruity, apple   | Waxy        | x           | x          |
| Isoamyl octanoate    | 26.119               | 1433                   | 70 (100), 127 (34), 145 (12)    | 214  | C <sub>13</sub> H <sub>26</sub> O <sub>2</sub> | Ester      | sweet, fruity, waxy, green, fatty, pineapple, coconut-like               | Fruity      | x           | x          |

Table 1. Cont.

| Compound                       | Retention Time (min) | Kovats RI <sup>a</sup> | MS Ions (Relative Abundance, %) | Mass | Formula  | Group      | Descriptor  | Odor Type | Samples     |            |
|--------------------------------|----------------------|------------------------|---------------------------------|------|--|------------|---|-----------|-------------|------------|
|                                |                      |                        |                                 |      |  |            |   |           | Single Malt | Four Grain |
| n-Capric acid n-heptyl ester   | 29.164               | 1478                   | 57 (100), 173 (55), 173 (54)    | 270  | C <sub>17</sub> H <sub>34</sub> O <sub>2</sub> | Ester      | -   | -         | x           |            |
| Ethyl laurate                  | 30.583               | 1494                   | 88 (100), 101 (47), 183 (13)    | 228  | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub> | Ester      | sweet, waxy, floral, soapy, clean                                   | Waxy      | x           | x          |
| Phenethyl caproate             | 31.868               | 1606                   | 104 (100), 105 (24), 43 (19)    | 220  | C <sub>14</sub> H <sub>20</sub> O <sub>2</sub> | Ester      | sweet, honey, floral, waxy, woody, sweaty, green, banana, pineapple | Floral    |             | x          |
| Isoamyl decanoate              | 32.015               | 1615                   | 70 (100), 71 (42), 43 (41)      | 242  | C <sub>15</sub> H <sub>30</sub> O <sub>2</sub> | Ester      | sweet, apricot, banana, pear, pineapple, mushroom                   | Fruity    |             | x          |
| Myristic acid                  | 35.18                | 1748                   | 73 (100), 60 (90), 129 (51)     | 228  | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub> | Fatty acid | coconut, pineapple, citrus, peel                                    | Waxy      |             | x          |
| Ethyl myristate                | 36.054               | 1778                   | 88 (100), 101 (47), 89 (14)     | 256  | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | Ester      | sweet, violet, orris  | Floral    | x           | x          |
| Myristaldehyde                 | 36.668               | 1586                   | 57 (100), 82 (86), 55 (71)      | 212  | C <sub>14</sub> H <sub>28</sub> O              | Aldehyde   | creamy, pear, dry citrus peel                                       | Waxy      |             | x          |
| 2-[(9Z)-9-octadecenyl]-ethanol | 36.682               | 2336                   | 82 (100), 55 (97), 69 (77)      | 312  | C <sub>20</sub> H <sub>40</sub> O <sub>2</sub> | Alcohol    | -   | -         | x           |            |
| Isoamyl laurate                | 37.356               | 1829                   | 70 (100), 45 (50), 71 (47)      | 270  | C <sub>17</sub> H <sub>34</sub> O <sub>2</sub> | Ester      | fatty, creamy, yeasty, fusel  | Waxy      |             | x          |
| Ethyl (9E)-9-octadecenoate     | 37.843               | 2174                   | 55 (100), 41 (83), 43 (71)      | 310  | C <sub>20</sub> H <sub>38</sub> O <sub>2</sub> | Ester      | -   | -         | x           |            |
| Ethyl palmitate                | 37.855               | 1968                   | 88 (100), 101 (56), 43 (36)     | 284  | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | Ester      | fruity, creamy, milky with a balsamic nuance                        | Waxy      |             | x          |
| Ethyl 9-hexadecenoate          | 38.165               | 1855                   | 55 (100), 88 (81), 69 (73)      | 282  | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> | Ester      | -   | -         |             | x          |
| 1-Hexadecanol                  | 38.29                | 1870                   | 55 (100), 69 (82), 83 (76)      | 242  | C <sub>16</sub> H <sub>34</sub> O              | Alcohol    | clean, greasy, floral, oily   | Waxy      | x           | x          |
| Ethyl iso-allocholate          | 38.325               | n.f.                   | 43 (100), 55 (91), 41 (86)      | 436  | C <sub>26</sub> H <sub>44</sub> O <sub>5</sub> | Ester      | fruity, sharp, pineapple, apple, green and orange                   | Fruity    | x           |            |
| n-Hexadecanoic acid            | 40.393               | 1953                   | 43 (100), 73 (90), 60 (83)      | 256  | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | Fatty acid | creamy, candle waxy nuance  | Waxy      |             | x          |
| Ethyl 9-hexadecenoate          | 40.51                | 1975                   | 55 (100), 88 (81), 69 (73)      | 282  | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> | Ester      | -   | -         | x           | x          |
| Ethyl palmitate                | 41.086               | 1979                   | 88 (100), 101 (56), 43 (36)     | 284  | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | Ester      | fruity, creamy and milky with a balsamic nuance                     | Waxy      | x           | x          |
| Linoleic acid                  | 44.217               | 2095                   | 67 (100), 81 (87), 87 (73)      | 280  | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub> | Fatty acid | -   | -         | x           |            |
| Ethyl linoleate                | 44.475               | 2139                   | 67 (100), 81 (74), 55 (59)      | 308  | C <sub>20</sub> H <sub>36</sub> O <sub>2</sub> | Ester      | fatty fruity oily   | Fatty     | x           | x          |
| Ethyl oleate                   | 44.57                | 2171                   | 43 (100), 55 (69), 69 (67)      | 310  | C <sub>20</sub> H <sub>38</sub> O <sub>2</sub> | Ester      | fatty, oil, dairy, milky, waxy, tallow                              | Fatty     | x           | x          |

<sup>a</sup> RI—retention index; n.f.—not found.

### 3.2. Aliphatic and Aromatic Alcohols

Methanol, 2-butanol, and 1-butanol are not products of alcoholic fermentation but instead serve as marker compounds for identifying specific raw materials in distilled spirits. Due to their relatively high concentration thresholds, these compounds have a limited impact on overall flavor. Methanol levels, for example, are typically elevated in fruit spirits due to the enzymatic breakdown of pectin in fruits and grapes. In the case of 1-butanol, cherry distillates generally contain less than 3 mg/100 mL, while other fruit distillates may reach concentrations up to 100 mg/100 mL. Concentrations of 2-butanol higher than 50 mg/100 mL can indicate bacterial spoilage of raw materials or mash, while concentrations of 1-propanol higher than 500 mg/100 mL suggest spoilage of fruit mash [25–28]. Higher alcohols, which constitute the largest group of volatile flavor compounds, are produced as amino acid metabolites through the keto acid (2-oxo acid) degradation pathway. The key alcohols include 3-methylbutanol, 2-methylbutanol, 2-methyl-1-propanol, 1-propanol, and the aromatic alcohol 2-phenylethanol. These alcohols are commonly known as “fusel alcohols” due to their malty and burnt flavors, with the exception of 2-phenylethanol, which has a floral scent resembling rose. The concentrations of these aliphatic alcohols in distilled spirits can vary significantly and are largely influenced by the specific distillation processes used for separation and fractionation. Excessive concentrations of higher alcohols can lead to a strong, pungent and “fusel-like” smell and taste. On the other hand, optimal levels contribute to a pleasant fruity character [25–29]. The mixture of 2-methylbutanol and 3-methylbutanol, also called isoamyl alcohol, is the most abundant minor component of distilled spirits synthesized by yeasts. Depending on the nature of the raw material, these alcohols constitute from 40 to 70% of the total fusel alcohol content [29]. In studied whiskies, the following alcohols were found; ethanol, 2-phenylethanol, 2-nonanol, isoamyl alcohol, 2-[(9Z)-9-octadecenyloxy]-ethanol, and 1-hexadecanol. Ethanol is the primary alcohol found in all alcoholic beverages, including whisky. It is the result of the fermentation of sugars by yeast, and it is responsible for the alcoholic content in the drink [14,30]. The 2-phenylethanol is well known for its pleasant floral aroma reminiscent of roses, and it can be found in many foods and beverages, including whisky. It is produced during fermentation and can contribute to the complexity of the whisky’s aroma. On the other hand, 2-nonanol, which imparts a fatty, citrus like scent, is one of the many constituents that may appear in the complex profile of whisky in low concentrations [23,31]. Isoamyl alcohol, a higher alcohol known as “fusel alcohol”, is formed in small quantities during fermentation. It contributes to the fruity and a slightly harsh character of whisky’s flavor and aroma, often with a hint of banana [1,23]. The presence and specific role of 2-[(9Z)-9-octadecenyloxy]-ethanol in whisky is not commonly highlighted in the research, and limited information is available regarding its contribution. It is a less volatile type of alcohol with a complex nature, potentially resulting from intricate chemical reactions during fermentation or maturation. Interestingly, it was detected only in a single malt whisky sample. Finally, 1-hexadecanol (cetyl alcohol) is a fatty alcohol that is not usually associated with whisky’s flavor or aroma profile, but it is known as an emollient or thickening agent in other industries. In whisky, it may influence the mouthfeel or texture rather than the taste or smell [20,32].

#### 3.2.1. Aldehydes

Aldehydes are a group of volatile compounds that contribute to the aroma and flavor profile of whisky [14]. They are formed during the fermentation and aging process, adding distinct characteristics to the final product. Aldehydes found in whisky, like acetaldehyde, heptanal, and benzaldehyde, can contribute to fruity, nutty, and caramel-like notes in the whisky. One notable aldehyde, (E)-2-nonenal, is especially associated with the aging

process, often contributing a “cardboard-like” or “papery” note to aged whiskies. This compound is formed through the oxidation of unsaturated fatty acids during long-term oxidative processes [1,23,33]. The myristyl aldehyde, detected only in the four grain whisky, is not frequently highlighted in discussions about whisky flavors. Although it is less commonly discussed in the context of whisky flavor than shorter-chain aldehydes, its presence may enhance the overall aroma profile. In other hand, shorter-chain aldehydes like acetaldehyde contribute more significantly to whisky’s aroma due to their higher volatility and prevalence. The presence and concentration of such compounds can be influenced by various factors including fermentation process, ingredients used, type of cask for aging, and length of aging process [1,20].

### 3.2.2. Esters

This study identified several esters in whisky, including ethyl heptanoate, ethyl hydrocinnamate, butyl caprylate, ethyl 4E-decenoate, isoamyl octanoate, n-capric acid, n-heptyl ester, ethyl caprate, phenethyl caproate, among others. Esters, known for their pleasant taste characteristics, represent the largest group of flavor compounds [1,34]. The relative amount and ratios of these esters are crucial in defining the overall flavor profile, as their concentrations typically exceed sensory detection thresholds. Low-boiling esters, such as ethyl 2-methylbutanoate, and acetates, like ethyl acetate, are particularly influential on the flavor profile along with the others mentioned. Malolactic fermentation also effects the concentrations of esters, enhancing the fruitiness and aroma intensity [3,34]. Compounds such as ethyl hexanoate and similar esters are commonly present in whisky, contributing significantly to its flavor profile. These esters are typically produced during the fermentation process, where fatty acids react with ethanol, leading to a diverse range of fruity, floral, and other intricate aromas and flavors that define various styles of whisky. For instance, ethyl hexanoate imparts fruity, apple-like notes, while ethyl heptanoate may add fruity or wine-like characteristics. Additionally, compounds like ethyl hydrocinnamate could provide sweet, balsamic, spicy, or cinnamon-like aromas [10,35]. The composition and concentration of these compounds can differ significantly between whiskies due to factors like the type of grain, fermentation conditions, distillation methods, and aging processes. In Scotch-blended whisky, congeners such as ethyl hexanoate, ethyl octanoate, and ethyl dodecanoate are prominent in the volatile profile and may reveal the type of grain used as well as the whisky’s region due to their variation among different types of whiskies. Moreover, the type of cask used for maturation and the duration of aging also impact the presence and concentration of these esters. Extended aging tends to allow more complex reactions, which can increase the concentration of such esters. Notably, butyl caprylate (ethyl octanoate) provides sweet, fruity, and waxy notes, enhancing the whisky’s fruity profile [7,23,31,36]. Furthermore, ethyl 4E-decanoate and ethyl caprate (ethyl decanoate) can contribute fruity and floral aromas to aged spirits. Ethyl caprate notably has a sweet, tropical fruit character [31,37]. Higher molecular weight esters, such as ethyl laurate and ethyl palmitate, contribute waxy and fuller-bodied notes to the whisky’s profile. Fatty acid esters like ethyl oleate and ethyl linoleate influence both the mouthfeel and texture, often introducing a subtle fresh or fruity note [1,20,31,37]. Isoamyl octanoate, in higher concentrations, brings pronounced fruity, banana-like notes, while n-capric acid, n-heptyl ester, and ethyl laurate enrich the profile with a variety of fruity and floral notes. Phenethyl caproate and isoamyl decanoate also enhance the whisky’s fruity bouquet. Esters such as ethyl myristate and isoamyl laurate likely add waxy, fruity, and floral aspects to the spirit’s aroma. Ethyl (9E)-9-octadecenoate, ethyl palmitate, and ethyl 9-hexadecenoate may introduce faint fruity or floral character and can be found in trace amounts adding to the complexity of the whisky [1,38].

### 3.2.3. Fatty Acids

The yeast cell's initiation of fatty acid biosynthesis during alcoholic fermentation begins with the formation of acetyl-coenzyme A, which combines with malonyl-coenzyme A to mainly produce saturated straight-chained fatty acids containing an even number of 4 to 18 carbon atoms. The presence of relatively low amounts of fatty acids with odd numbers of carbon atoms and unsaturated fatty acids depends on fermentation conditions. These volatile fatty acids contribute significantly to the flavor profile in fermented beverages like wine and beer, where their concentrations typically range from 100 to 250 mg per 100 mL. In distilled spirits, concentrations of free fatty acids are much lower due to esterification and separation through distillation, resulting in levels around 50 mg per 100 mL in wine distillates such as cognac [20]. Acetic acid can be generated during or after fermentation by oxidizing ethanol under aerobic conditions via the acetic acid bacteria *Acetobacter*. To avoid an undesirable vinegar-like off-flavor note, acetic acid levels in distilled spirits should remain below 100 mg per 100 mL [14].

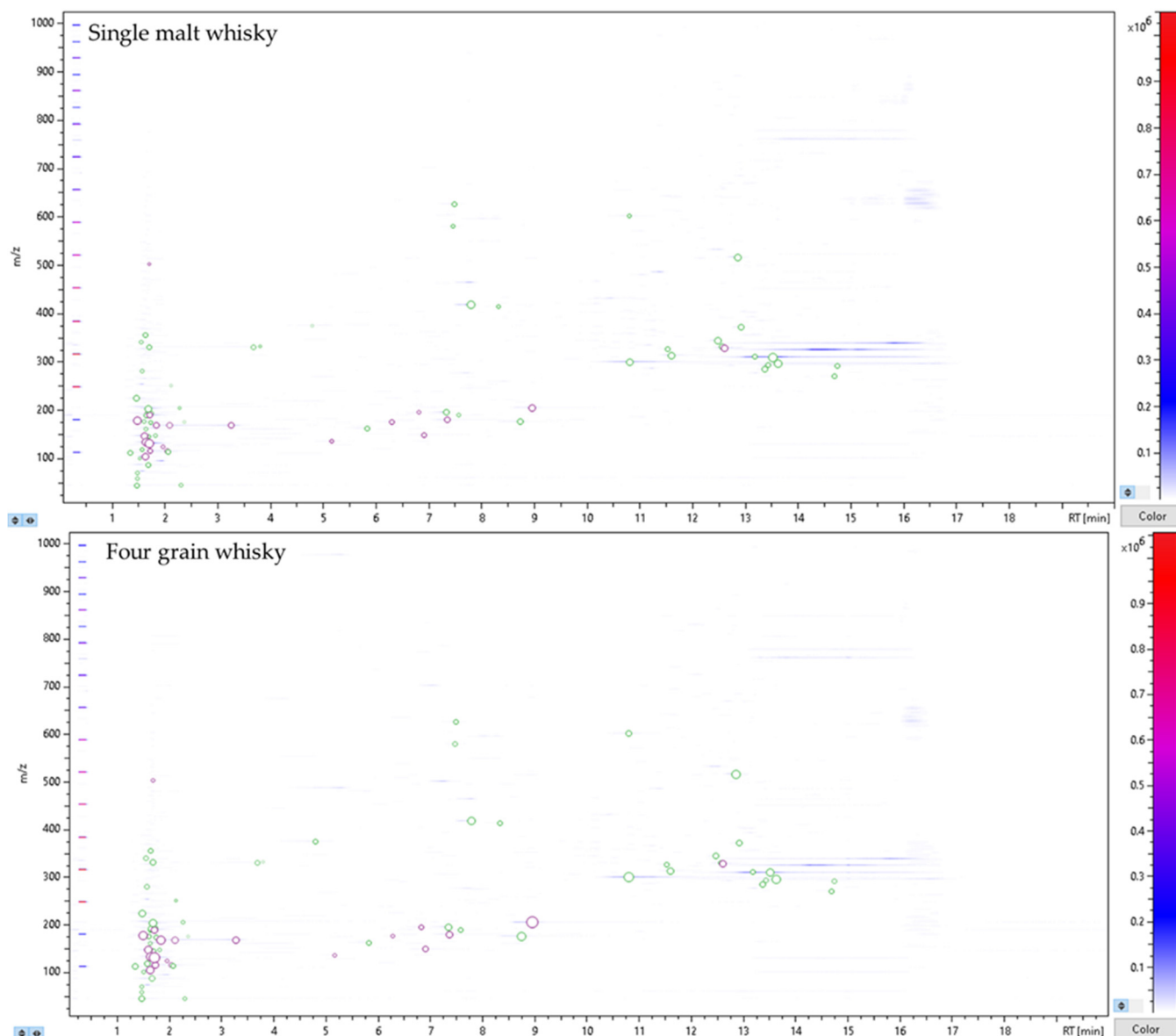
In this work, we identified several compounds such as octanoic acid (caprylic acid), ethyl octanoate, ethyl nonanoate, n-decanoic acid, myristic acid, palmitic acid (n-hexadecanoic acid), and linoleic acid that can influence whisky's flavor profile. The presence of these compounds in whisky is influenced by the choice of raw materials, fermentation techniques and maturation conditions. Octanoic acid or caprylic acid is a medium-chain fatty acid that adds a subtle waxy or fatty aroma, which in moderate levels enhances the complexity of the whisky's flavor. Ethyl octanoate works alongside these compounds to contribute fruity, sometimes slightly soapy notes to the whisky [1,23,37]. n-Decanoic acid, a long chain fatty acid, imparts creamy and fatty traits to whisky. During whisky production, it can be esterified to produce esters that impact the flavor profile. Although it may not stand out as a dominant aroma, its balanced presence contributes to the overall character of the whisky [1,31,37]. Myristic acid and n-hexadecanoic acid, both long-chain fatty acids, contribute waxy, soapy, and sometimes fatty notes in whisky. While myristic acid itself does not significantly impact whisky's aroma, it participates in forming esters that add complexity to the flavor profile. Palmitic acid, known as n-hexadecanoic acid, is a saturated fatty acid that may not have a significant direct olfactory impact but plays a role in mouthfeel and contributes to the formation of flavor-active esters [3]. Linoleic acid, a polyunsaturated fatty acid in whisky, influences both flavor and aging through oxidation reactions that occur during maturation. As a precursor to various flavor compounds, its oxidation can yield diverse sensory effects. However, high concentrations are generally undesirable due to the development of off flavors [1].

Fatty acids in whisky influence flavor directly and through the production of esters during fermentation and aging. Maturation can lead to further esterification, resulting in more pronounced specific esters in whisky. The concentration and balance of these compounds are critical, as they can significantly influence the complexity and richness of the whisky's flavor profile. While smaller quantities of fatty acids enhance flavor, higher concentrations may lead to less favorable taste or aroma attributes. The presence of cis-whisky lactone, specifically cis- $\beta$ -methyl- $\gamma$ -octalactone and trans- $\beta$ -methyl- $\gamma$ -octalactone, is reported in whiskies that are aged in wooden casks. These compounds, along with vanillin, guaiacol, eugenol, cresol, and other phenolics, migrate from the toasted wood into the distillate. These compounds are responsible for the characteristic oak wood and vanilla-like flavor that define well-aged whisky [39].

Finally, D-limonene, linalool, and  $\beta$ -damascenone are aromatic compounds that contribute to the complex flavor profile of whisky. D-limonene is a monoterpene commonly found in citrus fruit oils, contributing fresh citrus aroma notes in many foods and beverages. In whisky, D-limonene can contribute a fresh citrus note, although generally it is



This section was addressed in terms of the potential formation of specific compounds during the whisky production process, from raw material to maturation, due to the presence of non-specific and non-volatile compounds that can often arise as by-products or precursors of certain reactions. Results from the PCA model analysis, shown in Figure 2, illustrate a “topography view” presenting the intensities of the compounds plotted against their retention time and  $m/z$  values. In the figure, each circular marker represents an analyte, positioned at its primary ion’s retention time and  $m/z$ . The color-coded intensity scale (on the right) represents the highest intensity in that particular area displayed. This tool enables simple grouping of compounds with similar retention times and mass profiles, making it convenient to categorize the analyzed untargeted compounds.



**Figure 2.** Chromatography profile of the single malt and four grain whisky samples by UHPLC-QTOF.

Using UHPLC-QTOF analysis, 72 chemical compounds were detected (Table 2) in Croatian whiskies. For comparison, six other whiskies were also tested. The following text provides a description of these detected compounds and their contributions to the aroma profile of the whiskies.

**Table 2.** Untargeted compound determined by UHPLC-QTOF analysis.

| Name                  | Formula   | Retention Time (min) | Mass [Da] | Theoretical $m/z$ [M-H] <sup>-</sup> | $m/z$ [M-H] <sup>-</sup> | mSigma | $ \Delta m/z $ [ppm] | Single Malt * | Four Grain * | <i>p</i> -Value |
|-----------------------|---|----------------------|-----------|--------------------------------------|--------------------------|--------|----------------------|---------------|--------------|-----------------|
| Fumarate              | C <sub>4</sub> H <sub>2</sub> O <sub>4</sub>    | 1.34                 | 113.991   | 113.0034                             | 112.98372                | 4.32   | 1.2480               | 20,272        | 20,676       | 0.637087        |
| Glucuheptonic acid    | C <sub>7</sub> H <sub>14</sub> O <sub>8</sub>   | 1.47                 | 226.06792 | 225.0661                             | 225.06065                | 11.14  | 0.9093               | 30,947        | 31,204       | 0.689358        |
| Acetic acid           | C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>    | 1.47                 | 60.01983  | 59.0133                              | 59.01255                 | 3.40   | 1.3024               | 7024          | 4543         | 0.001908        |
| D-Glucose             | C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>   | 1.48                 | 180.06193 | 179.0561                             | 179.05465                | 8.50   | 1.4372               | 88,945        | 67,113       | 0.000027        |
| Methylglyoxal         | C <sub>3</sub> H <sub>4</sub> O <sub>2</sub>    | 1.48                 | 72.01979  | 71.0133                              | 71.01252                 | 2.93   | 1.3373               | 6212          | 4798         | 0.004075        |
| Sorbitol              | C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>   | 1.49                 | 182.0764  | 181.0718                             | 181.06912                | 6.84   | 2.6369               | 6028          | 4756         | 0.002312        |
| Acetoacetic acid      | C <sub>4</sub> H <sub>6</sub> O <sub>3</sub>    | 1.51                 | 102.02967 | 101.0197                             | 101.02240                | 4.61   | 2.0224               | 3407          | 3006         | 0.176588        |
| Isomaltulose          | C <sub>12</sub> H <sub>22</sub> O <sub>11</sub> | 1.55                 | 342.11625 | 341.1085                             | 341.10898                | 2.68   | 0.0737               | 10,000        | 9417         | 0.28752         |
| D-Erythrose           | C <sub>4</sub> H <sub>8</sub> O <sub>4</sub>    | 1.57                 | 120.0405  | 119.0244                             | 119.03322                | 2.99   | 1.7562               | 14,238        | 18,400       | 0.004991        |
| Gluconolactone        | C <sub>6</sub> H <sub>10</sub> O <sub>6</sub>   | 1.59                 | 178.04663 | 177.0405                             | 177.03935                | 9.26   | 1.1080               | 5304          | 8936         | 0.039759        |
| Arabinose             | C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>   | 1.6                  | 150.05132 | 149.0453                             | 149.04404                | 6.72   | 1.4875               | 62,384        | 63,880       | 0.473846        |
| D-Glyceric Acid       | C <sub>3</sub> H <sub>6</sub> O <sub>4</sub>    | 1.62                 | 106.02472 | 105.0197                             | 105.01744                | 5.01   | 1.8799               | 78,915        | 62,460       | 0.003902        |
| Lactobionic acid      | C <sub>12</sub> H <sub>22</sub> O <sub>12</sub> | 1.62                 | 358.11121 | 357.1085                             | 357.10394                | 19.34  | 0.0566               | 18,164        | 13,585       | 0.002712        |
| Quinic acid           | C <sub>7</sub> H <sub>12</sub> O <sub>6</sub>   | 1.63                 | 192.06111 | 191.0561                             | 191.05384                | 8.74   | 2.2747               | 23,890        | 11,211       | 0.000819        |
| 3-Deoxyglucosone      | C <sub>6</sub> H <sub>10</sub> O <sub>5</sub>   | 1.63                 | 162.05143 | 161.0453                             | 161.04416                | 8.62   | 1.3913               | 6592          | 5858         | 0.339614        |
| Threonic acid         | C <sub>4</sub> H <sub>8</sub> O <sub>5</sub>    | 1.64                 | 136.03557 | 135.0197                             | 135.02829                | 2.33   | 1.6028               | 89,278        | 66,720       | 0.000679        |
| Gluconic Acid isomers | C <sub>6</sub> H <sub>12</sub> O <sub>7</sub>   | 1.65                 | 196.0571  | 195.0510                             | 195.04983                | 3.61   | 1.2052               | 14,154        | 12,974       | 0.189499        |
| D-Glucuronic Acid     | C <sub>6</sub> H <sub>10</sub> O <sub>7</sub>   | 1.65                 | 194.04129 | 193.0354                             | 193.03402                | 9.47   | 1.3586               | 21,166        | 19,380       | 0.096908        |
| Glyceraldehyde        | C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>    | 1.66                 | 90.02967  | 89.0244                              | 89.02240                 | 3.89   | 2.0216               | 17,883        | 23,721       | 0.001826        |
| Homocitric acid       | C <sub>7</sub> H <sub>10</sub> O <sub>7</sub>   | 1.68                 | 206.04152 | 205.0304                             | 205.03424                | 10.19  | 1.1315               | 73,705        | 48,897       | 0.000016        |
| Citramalic acid       | C <sub>5</sub> H <sub>8</sub> O <sub>5</sub>    | 1.68                 | 148.03575 | 147.0453                             | 147.02847                | 6.76   | 1.4271               | 9758          | 11,668       | 0.015119        |
| Glutaric acid         | C <sub>5</sub> H <sub>8</sub> O <sub>4</sub>    | 1.69                 | 132.04077 | 131.0197                             | 131.03349                | 5.84   | 1.4922               | 8542          | 11,138       | 0.000908        |
| Raffinose             | C <sub>18</sub> H <sub>32</sub> O <sub>16</sub> | 1.69                 | 504.1689  | 503.1407                             | 503.16162                | 22.68  | 0.1158               | 3146          | 2230         | 0.001154        |
| Glucogallin isomers   | C <sub>13</sub> H <sub>16</sub> O <sub>10</sub> | 1.69                 | 332.07491 | 331.0661                             | 331.06763                | 31.23  | 0.6328               | 20,885        | 19,268       | 0.07493         |
| Citric acid           | C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>    | 1.7                  | 192.02577 | 191.0197                             | 191.01849                | 9.53   | 1.2330               | 55,642        | 34,872       | 0.000182        |
| Malic acid            | C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>    | 1.7                  | 134.01987 | 133.0133                             | 133.01271                | 6.51   | 1.5332               | 161,207       | 128,961      | 0.000074        |
| Succinic acid isomers | C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>    | 1.71                 | 118.02478 | 117.0197                             | 117.01750                | 5.45   | 1.8254               | 27,247        | 32,954       | 0.001699        |
| Ascorbic acid         | C <sub>6</sub> H <sub>8</sub> O <sub>6</sub>    | 1.73                 | 176.03071 | 175.0244                             | 175.02344                | 8.57   | 1.3765               | 11,854        | 13,190       | 0.022258        |
| Gluconic Acid isomers | C <sub>6</sub> H <sub>12</sub> O <sub>7</sub>   | 1.74                 | 196.05695 | 195.0510                             | 195.04967                | 2.92   | 1.4249               | 7491          | 6964         | 0.182564        |
| Tartaric Acid         | C <sub>4</sub> H <sub>6</sub> O <sub>6</sub>    | 1.82                 | 150.01497 | 149.0090                             | 149.00770                | 0.54   | 1.5987               | 4857          | 5928         | 0.010689        |
| Gallic acid isomers   | C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>    | 1.83                 | 170.01977 | 169.0143                             | 169.01249                | 8.27   | 1.7693               | 42,596        | 58,844       | 0.00604         |
| Succinic acid isomers | C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>    | 2.03                 | 118.02474 | 117.0197                             | 117.01746                | 6.20   | 1.8841               | 16,860        | 17,418       | 0.584818        |

Table 2. Cont.

| Name  | Formula   | Retention Time (min) | Mass [Da]  | Theoretical $m/z$ [M-H]- | $m/z$ [M-H]- | mSigma | $ \Delta m/z $ [ppm] | Single Malt * | Four Grain * | $p$ -Value |
|---|---|----------------------|------------|--------------------------|--------------|--------|----------------------|---------------|--------------|------------|
| Maleic Acid                                   | C <sub>4</sub> H <sub>4</sub> O <sub>4</sub>    | 2.05                 | 116.00911  | 115.0034                 | 115.00183    | 5.21   | 1.8503               | 21,726        | 16,996       | 0.001163   |
| Gallic acid isomers                           | C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>    | 2.09                 | 170.0201   | 169.0143                 | 169.01282    | 7.68   | 1.5150               | 42,306        | 43,989       | 0.309906   |
| Galactosylglycerol                            | C <sub>9</sub> H <sub>18</sub> O <sub>8</sub>   | 2.11                 | 254.09952  | 253.0824                 | 253.09224    | 7.89   | 0.6184               | 1120          | 1041         | 0.116016   |
| 4-O-Methylglucuronic acid                     | C <sub>7</sub> H <sub>12</sub> O <sub>7</sub>   | 2.26                 | 208.05744  | 207.0304                 | 207.05017    | 11.58  | 0.8611               | 2293          | 927          | 0.161952   |
| Gallic acid isomers                           | C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>    | 3.26                 | 170.0202   | 169.0143                 | 169.01292    | 2.57   | 1.3128               | 48,108        | 44,930       | 0.085098   |
| Glucogallin isomers                           | C <sub>13</sub> H <sub>16</sub> O <sub>10</sub> | 3.67                 | 332.07472  | 331.0661                 | 331.06745    | 18.71  | 0.3770               | 14,178        | 8324         | 0.000001   |
| 2,3,4-Tri-O-acetyl-D-glucuronide methyl ester | C <sub>13</sub> H <sub>18</sub> O <sub>10</sub> | 3.79                 | 334.09023  | 333.0873                 | 333.08295    | 8.38   | 0.1675               | 281           | 280          | 0.972415   |
| Protocatechuic aldehyde                       | C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>    | 5.16                 | 138.03046  | 137.0197                 | 137.02318    | 7.53   | 1.2337               | 10,733        | 5956         | -          |
| Unkown 2                                      |   | 5.27                 | 978.09907  | —                        | 488.04220    | -      | -                    | 1316          | 36,852       | 0.000026   |
| Coumarinic acid                               | C <sub>9</sub> H <sub>8</sub> O <sub>3</sub>    | 5.81                 | 164.04612  | 163.0143                 | 163.03884    | 6.86   | 1.2309               | 19,322        | 13,198       | 0.00003    |
| Esculetin                                     | C <sub>9</sub> H <sub>6</sub> O <sub>4</sub>    | 6.28                 | 178.02546  | 177.0143                 | 177.01818    | 7.44   | 0.9818               | 19,605        | 7701         | 0.000002   |
| Syringic acid isomers                         | C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>   | 6.81                 | 198.05168  | 197.0453                 | 197.04440    | 5.01   | 1.8253               | 11,047        | 13,571       | 0.000047   |
| Vanillin                                      | C <sub>8</sub> H <sub>8</sub> O <sub>3</sub>    | 6.9                  | 152.04594  | 151.0399                 | 151.03867    | 5.56   | 1.4780               | 21,412        | 21,458       | 0.954648   |
| Unkown 1                                      |   | 7.25                 | 1006.12918 | —                        | 502.05731    | -      | -                    | 1425          | 55,800       | 0.000009   |
| Ethyl gallate                                 | C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>   | 7.33                 | 198.05178  | 197.0453                 | 197.04450    | 2.21   | 0.9016               | 46,371        | 46,417       | 0.969921   |
| Syringaldehyde                                | C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>   | 7.36                 | 182.05665  | 181.0509                 | 181.04937    | 2.23   | 1.2676               | 36,230        | 38,190       | 0.199999   |
| -Lyoniresinol 9-glucoside                     | C <sub>28</sub> H <sub>38</sub> O <sub>13</sub> | 7.46                 | 582.23193  | 581.1291                 | 581.22465    | 0.82   | 0.0949               | 11,722        | 7479         | 0.000249   |
| Lyoniresinol 9-glucoside + HCOOH              | C <sub>28</sub> H <sub>38</sub> O <sub>13</sub> | 7.47                 | 628.23725  | 627.1407                 | 627.22997    | 14.63  | 0.0332               | 23,604        | 14,683       | 0.000082   |
| Scopoletin                                    | C <sub>10</sub> H <sub>8</sub> O <sub>4</sub>   | 7.57                 | 192.04096  | 191.0340                 | 191.03368    | 11.77  | 1.3036               | 9119          | 14,154       | 0.00001    |
| -Lyoniresinol                                 | C <sub>22</sub> H <sub>28</sub> O <sub>8</sub>  | 7.79                 | 420.17891  | 419.1458                 | 419.17163    | 2.66   | 0.3192               | 103,323       | 63,634       | -          |
| 1-Acetoxy-pinorexinol                         | C <sub>22</sub> H <sub>24</sub> O <sub>8</sub>  | 8.32                 | 416.1466   | 415.1136                 | 415.13933    | 14.09  | 0.4193               | 8787          | 10,668       | 0.004843   |
| Coniferyl aldehyde                            | C <sub>10</sub> H <sub>10</sub> O <sub>3</sub>  | 8.74                 | 178.06147  | 177.0404                 | 177.05419    | 2.27   | 1.5286               | 44,281        | 71,629       | 0.000012   |
| Sinapoyl aldehyde                             | C <sub>11</sub> H <sub>12</sub> O <sub>4</sub>  | 8.95                 | 208.0717   | 207.0453                 | 207.06443    | 8.72   | 1.6514               | 69,392        | 162,567      | -          |
| Ellagic acid dimer                            | C <sub>28</sub> H <sub>12</sub> O <sub>16</sub> | 10.8                 | 604.01276  | 603.0136                 | 603.00548    | 23.78  | 1.1132               | 13,787        | 19,986       | 0.000549   |
| Ellagic Acid                                  | C <sub>14</sub> H <sub>6</sub> O <sub>8</sub>   | 10.81                | 302.00641  | 300.9988                 | 300.99910    | 5.26   | 0.0339               | 88,367        | 96,324       | 0.056159   |
| Salvigenin                                    | C <sub>18</sub> H <sub>16</sub> O <sub>6</sub>  | 11.51                | 328.09415  | 327.0776                 | 327.08680    | 18.08  | 2.1256               | 14,576        | 12,910       | 0.013551   |
| 3-O-methyl Ellagic acid                       | C <sub>15</sub> H <sub>8</sub> O <sub>8</sub>   | 11.58                | 316.02133  | 315.0136                 | 315.01400    | 10.21  | 0.5990               | 56,277        | 34,222       | 0.000003   |

Table 2. Cont.

| Name  | Formula  | Retention Time (min) | Mass [Da] | Theoretical $m/z$ [M-H] <sup>-</sup> | $m/z$ [M-H] <sup>-</sup> | mSigma | $ \Delta m/z $ [ppm] | Single Malt * | Four Grain * | <i>p</i> -Value |
|---|--|----------------------|-----------|--------------------------------------|--------------------------|--------|----------------------|---------------|--------------|-----------------|
| 9,10-Dihydroxyoctadecanedioic acid                | C <sub>18</sub> H <sub>34</sub> O <sub>6</sub> | 12.47                | 346.23501 | 345.2286                             | 345.22770                | 8.84   | 0.7230               | 62,592        | 21,891       | 0.000001        |
| Phloionolic acid                                  | C <sub>18</sub> H <sub>36</sub> O <sub>5</sub> | 12.55                | 332.25517 | 331.2160                             | 331.24790                | 6.37   | 1.1021               | 35,414        | 14,756       | -               |
| <i>_Z_-</i> 5,8,11-trihydroxyoctadec-9-enoic acid | C <sub>18</sub> H <sub>34</sub> O <sub>5</sub> | 12.59                | 330.23985 | 329.2234                             | 329.23250                | 16.92  | 0.7698               | 51,717        | 31,121       | -               |
| Perulactone                                       | C <sub>30</sub> H <sub>46</sub> O <sub>7</sub> | 12.84                | 518.32346 | 517.0826                             | 517.31610                | 5.03   | 0.8606               | 63,212        | 61,474       | 0.489593        |
| Octadecanedioic acid                              | C <sub>18</sub> H <sub>34</sub> O <sub>4</sub> | 13.16                | 314.24394 | 313.2339                             | 313.23660                | 15.99  | 1.5553               | 22,013        | 12,244       | 0.000004        |
| Hexadecanedioic acid                              | C <sub>16</sub> H <sub>30</sub> O <sub>4</sub> | 13.36                | 286.21301 | 285.2026                             | 285.20570                | 3.61   | 1.3977               | 39,767        | 22,202       | 0.000001        |
| 9-Hydroxy-10,12-octadecadienoic acid              | C <sub>18</sub> H <sub>32</sub> O <sub>3</sub> | 13.42                | 296.23307 | 295.2272                             | 295.22570                | 21.95  | 2.0755               | 24,004        | 7891         | 0.000004        |
| 9-Octadecenedioic acid, <i>_9Z_-</i>              | C <sub>18</sub> H <sub>32</sub> O <sub>4</sub> | 13.5                 | 312.22608 | 311.2160                             | 311.21880                | 8.45   | 3.1642               | 153,689       | 54,914       | 0.000025        |
| Ricinoleic acid                                   | C <sub>18</sub> H <sub>34</sub> O <sub>3</sub> | 13.63                | 298.24965 | 297.2428                             | 297.24230                | 5.51   | 1.1424               | 119,254       | 68,432       | 0.000005        |
| 2-Hydroxyhexadecanoic acid                        | C <sub>16</sub> H <sub>32</sub> O <sub>3</sub> | 14.67                | 272.23387 | 271.2329                             | 271.22660                | 2.38   | 1.2719               | 16,334        | 16,856       | 0.717006        |
| Gingerol  | C <sub>17</sub> H <sub>26</sub> O <sub>4</sub> | 14.72                | 294.18503 | 293.1764                             | 293.17770                | 21.45  | 2.9489               | 16,251        | 9036         | 0.025321        |

\* Expressed as the peak area.

### 3.3.1. Sugars in Croatian Whisky

In Croatian whiskies, several sugars were detected, including D-glucose, isomaltulose, raffinose, sorbitol, and arabinose. These sugars originate from multiple sources, such as raw materials, produced during enzymatic degradation of starches and cell walls as well as from the oak cask used in maturation and its possible previous contents, especially sweet wine. D-glucose is an essential component of the grains in the mash. During fermentation by yeast in the whisky-making process, it produces ethanol and other by-products. Any presence of residual glucose in the final product can impact the sweetness and body of the whisky [31,37]. Isomaltulose, a disaccharide similar to sucrose, may be derived from a breakdown of sugars in the grains. Its presence in whisky could contribute to a different type of sweetness because it is metabolized more slowly by humans compared to sucrose [1,37]. Raffinose, a trisaccharide of galactose, glucose, and fructose, is often found in small amounts after the breakdown of complex plant materials during mashing. It has the potential to influence viscosity and mouthfeel in the body due to its sugar component [20,31]. Sorbitol, a sugar alcohol commonly found in fruits, can contribute mild sweetness and influence the mouthfeel of the whisky. Arabinose, a pentose sugar, is found in the raw materials or through the breakdown of hemicellulose from grains. It contributes to the sweetness and complexity of whisky by adding a sweet note, and it is likely derived from the breakdown of polysaccharides from grains or wooden casks used to age the spirits [37]. The presence of pentose sugars is also important for the production of key congeners through the Maillard reactions [41].

### 3.3.2. Maillard Reactions and Congeners

In whisky production, some of the sugars can be involved in the Maillard reaction (MR), a complex series of reactions between reducing sugars and amino groups accelerated by thermal energy. This reaction produces N-substituted glycosylamine, which then transforms further to contribute flavor compounds (congeners) [42–44]. Detected congeners in Croatian whiskies include methylglyoxal, D-erythrose, glyceraldehyde, and 3-deoxyglucosone. Methylglyoxal is a reactive compound that forms in small amounts through the MR and caramelization processes. It adds to the whisky's complexity with its pungent aroma contributing to both color and flavor. D-erythrose, a tetrose sugar, has the potential to take part in MR during the heating stages of whisky production. D-erythrose interactions and breakdown products could potentially add to the overall array of flavors. It may be found in whisky as a result of decomposition of larger carbohydrates during fermentation or aging, though its flavor influence remains minor due to potentially low concentrations [1]. Glyceraldehyde is an intermediate in sugar metabolism, contributing to color and flavor through MR. As a simple sugar, it could also contribute sweetness, and it is an intermediate in several metabolic pathways [31,34,37]. 3-Deoxyglucosone, a product of the MR, can occur during whisky production and contributes a desirable browned food flavor that also occurs during the cooking or kilning phase of whisky making or aging processes in barrels [31,45].

### 3.3.3. Organic Acids in Whisky

Ten organic acids were identified in tested whiskies: fumaric acid, glucoheptonic acid, acetic acid, D-glyceric acid, lactobionic acid, threonic acid, gluconic acid, homocitric acid, citramalic, and glutaric acid. Fumaric acid is an intermediate in the Krebs cycle. As a product of fermentation, fumarate can contribute to the acidity and therefore flavor profile of whisky [31,37]. Glucoheptonic acid, sugar acid, may form from the oxidation of sugars during fermentation and aging processes in whisky production, influencing its sweetness and mouthfeel characteristics [1,20]. Acetic acid is a by-product of yeast

fermentation that contributes the tart flavor profile of whisky. Acetic acid also can act as a precursor to form secondary metabolites, such as higher alcohols, esters, fatty acids, and other lipids when it reacts with acetyl-CoA. For example, acetic acid can serve as a precursor to ethyl acetate, which contributes sweet and fruity aromas. Elevated levels of ethyl acetate could be attributed to the initially produced acetic acid (by *Lactobacillus*) being subsequently metabolized by yeast or formed through esterification during one of the distillation stages. Usually, acetate esters provide fruity notes in new-make spirit [1,14,38]. These findings are consistent with research by Ensor et al. [45], which demonstrated that higher concentrations of acetaldehyde and ethyl acetate are achieved when a distillery fermentation is co-inoculated with *Lactobacillus*. Acetoacetic acid, a  $\beta$ -keto acid, may be present in whisky as a result of yeast metabolism during fermentation. It can contribute certain acidic notes to the whisky's profile and at higher concentrations it could lead to off-flavors [31]. D-glyceric acid is an intermediate in carbohydrate metabolism, and it can be found in whisky as a metabolic product from yeast during fermentation. Moreover, it can contribute to the acidity and overall flavor profile of the whisky. Another sugar-based acid detected in whisky is lactobionic acid, a congener derived from lactose oxidation, which can enhance the sweetness and mouthfeel of the whisky [1,23,31]. Threonic acid, an oxidation product of ascorbic acid, may also impact the flavor profile. Gluconic acid influences the acidity/taste, and it is formed through glucose oxidation. D-glucuronic acid might influence flavor due to its presence in maturation processes/sources such as grains or fermentation metabolites [3,14,46]. Homocitric acid is associated with the citric acid cycle. It is found in specific fermentation processes, likely as a result of yeast metabolism during whisky production, and may impact whisky's acidity profile [1,37]. Citramalic acid and glutaric acid are both dicarboxylic acids, and their presence may influence flavor and mouthfeel characteristics. Citramalic acid forms during metabolic processes, likely arising during fermentation, providing subtle flavor nuances. Glutaric acid, another dicarboxylic acid, could be produced during the fermentation or derived from the grain raw materials [15].

#### 3.3.4. Influence of Microflora on Whisky Flavor

In whisky production, there is no boiling stage during wort production, resulting in 'non-sterile' conditions for fermentation. This allows bacteria and other microflora to develop throughout fermentation from the local environment or raw materials [47–49]. These microorganisms, along with the *Saccharomyces* yeast strain used, significantly influence the flavor profile of the fermented wort, known as wash, once fermentation is complete. Comprehensive studies on bacterial communities in distillery settings reveal that species of *Lactobacillus* are dominant, likely due to its ability to survive at high alcohol concentrations, low pH, and anaerobic conditions [50]. Although these bacterial cultures are well studied, their specific impact on the flavor characteristic of the new-make spirit remains poorly understood. Certain metabolic pathways of *Lactobacillus* can form succinic acid from lactic or citric acid and contribute to the decarboxylation of cinnamic acids. Bacterial production of these metabolites can have a significant impact on the flavor and aroma profile of distilled washes [51,52]. In analyzed samples, several acids which can be connected with production of *Lactobacillus* metabolism were detected, including citric acid, malic acid, succinic acid, ascorbic acid, gluconic acid, tartaric acid, and maleic acid. Citric acid, for example, a natural organic acid found abundantly in citrus fruits, contributes to the freshness and acidity of whisky. It contributes to the overall flavor profile and adds a depth to its character [1,23]. Malic acid, commonly found in apples and other fruits, can contribute to the complexity of the flavor with a subtly sour taste. It originates from raw materials such as malted barley and can add a touch of tartness to the whisky, although

it may partially break down during fermentation. It also serves as a distinguishing factor for pattern recognition between brandies and whiskies [1,23,31]. Succinic acid plays an important role in shaping the flavor profile of fermented beverages, adding to their overall mouthfeel an umami taste. It is commonly found in whisky as a common organic acid formed during fermentation [1,14,23,53]. Ascorbic acid, or vitamin C, serves as an essential antioxidant in whisky, influencing flavor through the intricate interplay of its degradation products [1,33]. Gluconic acid is produced from the oxidation of glucose, contributing to a mild acidity and potentially enhancing sweetness. Its impact on flavor is not as substantial as other acids. Furthermore, its presence may result from the breakdown of glucose during fermentation or from oxidation processes during maturation. Tartaric acid, one of the primary acids in wine, can also be found in whisky, contributing to its acidic taste profile [1,31]. Maleic acid is a less common acidic compound that may be present in whisky, potentially influencing its tartness and flavor complexity. It is not typically present in whisky, but it is more commonly associated with wines, especially those that have undergone malolactic fermentation [37]. Gluconolactone, a by-product of oxidative processes, may also be found in whisky. As a compound resulting from the oxidation of glucose, gluconolactone brings a gentle sweetness and can influence the acidity of whisky through hydrolysis into gluconic acid [54].

### 3.3.5. Maturation and Flavor Development

After distillation, the new-make spirit is colorless and neutral, but it contains many congeners that have either been transferred from the malt and/or created or concentrated during fermentation or distillation, as previously discussed. Maturation involves the transition of the new spirit into a more mature, mellow, and smoother product, usually achieved by diluting the spirit first in water and then transferring it to wooden casks for a minimum period of three years but often much longer. This maturation stage significantly enhances the complexity of the sensory characteristics of the whisky. In the studied samples, several congeners were detected. Glucogallin, the ester of gallic acid and glucose, which may contribute to astringency and could possess antioxidant properties. It is believed to be derived from oak wood aging in barrels such as those used for whisky production [38]. Quinic acid is a compound commonly found in various plants including the oak from whisky barrels, and it contributes to the bitterness and astringency of whisky. As a cyclic polyol, it can contribute to perceived acidity and astringency while acting as an antioxidant [37,55]. Furthermore, threonic acid is a metabolite produced during the oxidation process of vitamin C. Its occurrence in whisky could potentially result from chemical reactions that occur during aging. Gallic acid, commonly derived from the breakdown of tannins, is a phenolic acid that influences the color, astringency, and antioxidant properties of whisky [1,15]. Vanillin plays a significant role in the aroma of whisky, adding sweet and creamy hints reminiscent of vanilla. Its presence and levels are vital at the initial filling of the barrel and undergo changes during maturation. Extracted from lignin in oak wood as whisky matures, it gradually permeates into the spirit over time [31]. Syringic acid and syringaldehyde, also derived from the breakdown of lignin in oak wood, contribute spicy, sweet, and smoky notes, enriching the whisky's complexity.

### 3.3.6. Lignans and Phenolic Compounds

The impact of lignans and phenolic compounds on whisky flavor is less extensively documented compared to the well-known compounds listed above, yet they play a significant role as part of the complex mix of oak-derived compounds. Lignans such as lyoniresinol and related glucosides are derived from oak wood, potentially adding astringent, bitter, or sometimes spicy notes to whisky. However, their sensory impact is not as

extensively studied in comparison to key compounds like vanillin and syringaldehyde. While lignans are not typically the primary focus in whisky flavor research, they still contribute to the complexity of whisky's flavor profile. The specific sensory attributes of lyoniresinol derivatives in whisky have not been as extensively studied as those of vanillin [37]. 1-Acetoxy-pinoreinol, also found in oak, is associated with woody or resinous flavor, potentially contributing to the bitter or astringent aspects of whisky. Although these lignans are not typically a focus in whisky flavor research, they can add to the complexity of the whisky's profile. Scopoletin, a coumarin found in the wood of certain plants including oak, may be present in whisky in trace amounts, depending on the raw materials and aging conditions. It could potentially contribute earthy or medicinal notes to the flavor profile. Coniferyl aldehyde and sinapoyl aldehyde are both phenolic aldehydes derived from lignin degradation. They could influence spicy, smoky, or woody characteristics of whisky. Their contribution would be influenced by the exact processes, such as cask preparation and aging conditions. Gallagic acid and ellagic acid both contribute significantly to the complexity of whisky, often imparting a dry, puckering quality due to their astringency. Ellagic acid, in particular, is known for its role in the perception of oakiness in whisky. It is released during the aging process and has a high antioxidant capacity, which can influence the color stability and aging potential of whisky. American oak, with its higher *cis*-to-*trans* whisky lactone ratio (8:1) and greater concentrations of ellagic and gallic acids, imparts sweeter, coconut-like flavors compared to the more tannic and subtle European oak. Therefore, it is suggested that spirits maturing in American oak can have creamier, more coconut-like aromas [54,56,57]. Gallagic acid, derived from ellagitannin hydrolysis, is not a primary flavor compound but might be a part of tannin composition affecting the astringency and mouthfeel of whisky. The presence of salvigenin in whisky is less documented, but like other phenolic compounds, it may contribute to the overall astringency or might provide subtle herbal nuances to the flavor. 3-O-methyl ellagic acid is likely to contribute to the bitter and astringent aspects of the whisky flavor profile. Both salvigenin and 3-O-methyl ellagic acid are more specific derivatives of ellagic acid, related to the oak wood tannins. Moreover, they can contribute to the overall depth of flavor through their bittering and astringency properties, but their precise contributions to whisky's flavor profile are not as well characterized as ellagic acid. Perulactone, sometimes present in oak-aged spirits, can impart a spicy, cinnamon-like flavor.

Over time, these components are extracted into the whisky, resulting in older whiskies having more intense and complex flavors compared to younger ones. The type of oak used for the barrels, as well as the degree of barrel charring or toasting, and the previous contents of the cask (such as sherry or bourbon), can all impact the levels of these compounds and subsequently influence the flavor of the whisky. Factors such as wood type, origin, cask size, and charring level will also influence the release and concentration of chemicals in whisky. Environmental conditions, including temperature, humidity, and storage location also play a crucial role in further reactions. Research has shown that toasting and charring significantly affect compound concentrations like vanilla. Additionally, it has been noted that lignin-derived phenol compounds play a crucial role in shaping whisky's flavor profile by emphasizing wood quality along with treatment during the aging process [58]. However, the perception of these compounds can vary depending on their concentration and the presence of other congeners that influence the overall flavor and aroma profile of whisky. These factors are significant in contributing flavors such as vanilla, sweet, spicy, smoky, and woody tones. In addition, lyoniresinol along with similar lignans contribute nuanced flavors to whisky especially in terms of woody and spicy notes [1,3,37]. Comparatively, there is limited research directly focusing on the specific impact of some of these compounds on whisky flavor. Most references to their presence and impact often come from the broader

research on wood extractives, their contribution to the maturation process, and the sensory properties of oak-aged beverages. For example, wood extractives like ellagic acid not only influence the taste but also contribute to the color and mouthfeel of the whisky. These compounds are also associated with the antioxidant properties that are part of the ageing process in oak barrels. The precise influence of these compounds on flavor will depend on their concentration, which is influenced by factors such as the type of oak used, the char level of the cask, the duration of aging, and the initial composition of the spirit [58].

### 3.3.7. Fatty Acids and Related Compounds

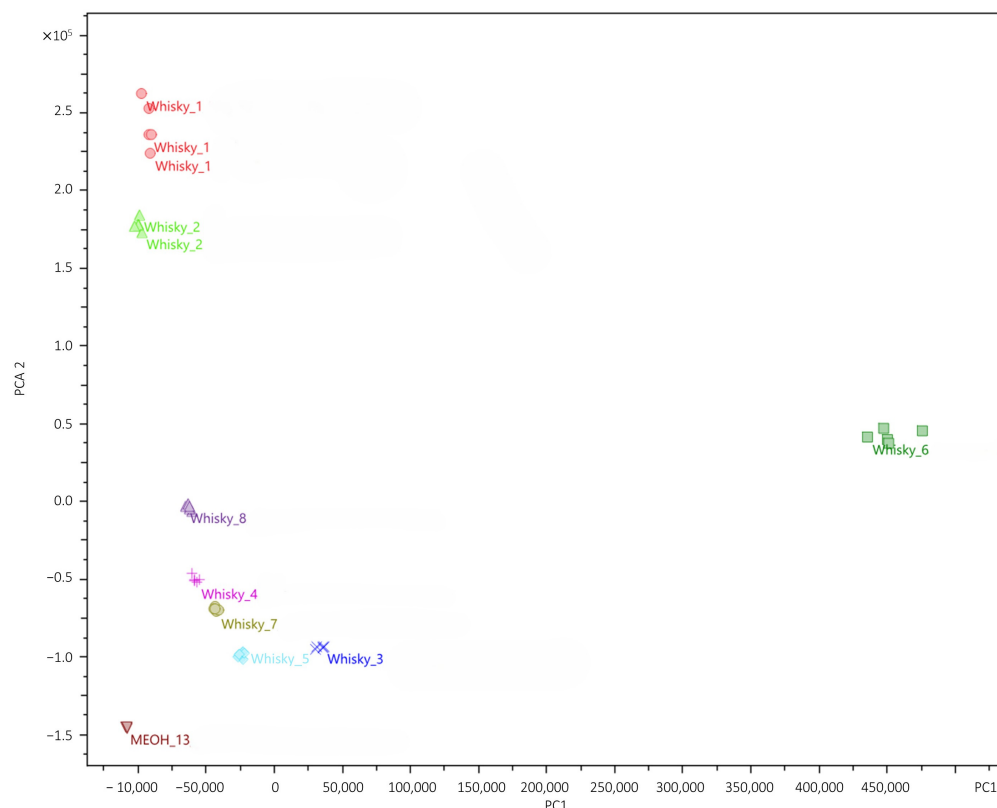
The following compounds are less common in discussions of whisky flavor and may not be major contributors to its typical profile. For example, 9,10-Dihydroxyoctadecanedioic acid, potentially related to fatty acid oxidation products, lacks specific documentation on any flavor contribution. Phloionolic acid, may be related to phenolic compounds in general and might influence flavor through astringency and complexity, particularly from the oak aging process. Similarly, Z-5,8,11-trihydroxyoctadec-9-enoic acid could be related to fatty acid oxidation products or degradation of lipid-derived materials in the aging process. Long-chain dicarboxylic acids, like octadecanedioic acid, could be related to the breakdown of fatty acids from the oak wood or from the distillation process. It is not typically cited as contributing to flavor in whisky. Hexadecanedioic acid and 9-octadecenedioic acid, both dicarboxylic acids, could potentially originate from the fatty acid metabolism within the wood of the barrel or from the distillate itself. They might add a subtle acidic or buttery profile to the whisky, contributing to the tactile sensation or mouthfeel [37]. Hydroxy acids like 9-hydroxy-10,12-octadecadienoic acid (HODE) and 2-hydroxyhexadecanoic acid may contribute to the flavor profile of the whisky through slight acidity and potentially creamy or slightly greasy notes [37]. They are often associated with the oxidative degradation of linoleic acid and might be linked with aging processes in whisky. However, HODEs' direct contribution to flavor is not well-documented. Ricinoleic acid, a fatty acid found in castor oil, is known for its unique viscosity and solubility, which could influence the mouthfeel of whisky. However, scientific literature rarely mentions its presence or flavor contribution in whisky. The monounsaturated dicarboxylic acid 9-octadecenedioic acid (9Z-) could result from the oxidation of oleic acid. It is not commonly cited in whisky flavor discussions but may contribute to the texture or mouthfeel of the whisky. Gingerol, known for its warming and spicy characteristics found in ginger, could impart such notes in whisky. Although ginger itself is not typically used in whisky production, spicy notes akin to gingerol can originate from the wood or during fermentation and distillation [37]. However, this would be quite unusual unless ginger were specifically added during production, either intentionally or it could be present in whisky due to several potential sources, such as the raw ingredients used in distillation, chemical reactions during fermentation and aging, or extraction from the wood of the casks during maturation. The comparison with other research findings is thus challenging, as these compounds themselves are not standard markers looked for in whisky research and may not be significant to the typical sensory profile as compared to other better-studied congeners such as phenolic compounds derived from wood, esters formed during fermentation, or alcohols that contribute to the body and character of whisky [1].

Figures S1 and S2 (Supplementary Materials) highlight the presence of two unknown compounds with molecular masses of  $m/z$  1006.12918 and 978.09907, respectively. These findings suggest unique chemical entities present in certain whisky samples. The high molecular masses indicate these compounds could be large polyphenolic structures or other complex organic molecules possibly derived from the maturation process or specific ingredients used in whisky production. Further identification and characterization of these

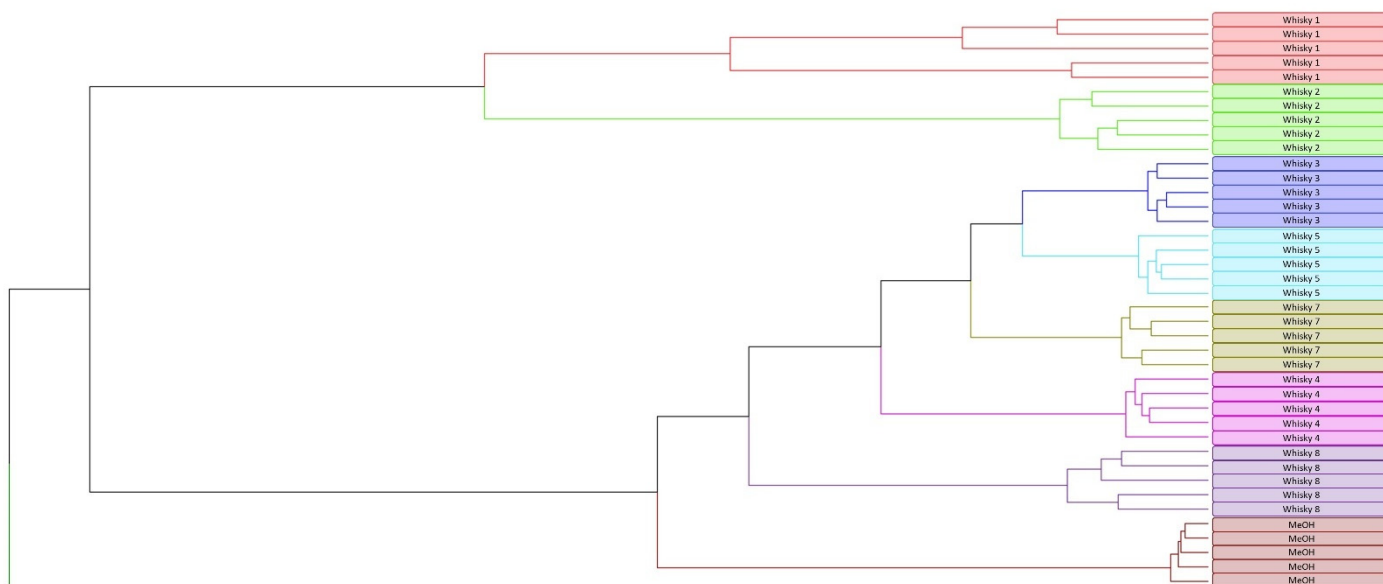
unknowns could provide insights into the distinct flavor profiles or aging processes that differentiate the whiskies. The PCA plot in Figure 3 offers a statistical evaluation of the whisky samples based on their chemical composition. By reducing the dimensionality of the dataset, PCA reveals groupings and separations among the whiskies, illustrating variability in their chemical profiles. Clusters observed in the plot may indicate distinct whisky types, such as single malt versus blended whiskies, or differences arising from production techniques, maturation periods, or geographical origins. The presence of outliers suggests certain samples have unique chemical signatures that could correspond to experimental variations or deliberate differences in their formulations. The dendrogram (Figure 4), constructed from a similarity matrix, groups whiskies based on their compositional similarities. Close clustering between certain samples implies a shared production method, source, or aging process, whereas samples located in distant branches highlight their distinctiveness. Such clustering is particularly useful for identifying counterfeit whiskies or assessing the consistency of products from the same distillery. As seen clearly in the figure, Croatian whisky (whisky 1 and 2) forms a distinct group, setting it apart from the other analyzed whiskies. Furthermore, in the Supplementary Materials (Figure S3), the data analysis methods clearly illustrate the distinction between Croatian whisky and Scotch whisky based on untargeted QToF analysis of non-volatile compounds. Principal Component Analysis (PCA) provides an unsupervised overview, revealing clear clustering patterns where Croatian and Scotch whisky occupy distinct regions of the score plot. The Partial Least Squares Discriminant Analysis (PLS-DA) refines these distinctions by maximizing variance between the groups, confirming the separation initially observed in PCA. Sparse PLS-DA (sPLS-DA) further identifies key non-volatile compounds contributing most significantly to the differentiation, narrowing down the specific markers of each whisky type. A Scores Heat Map visually emphasizes these findings, with rows corresponding to samples and columns to key compounds. The clustering within the heatmap highlights unique compound profiles for Croatian and Scotch whiskies, underscoring their biochemical differences. Lastly, the dendrogram derived from hierarchical clustering corroborates these results, organizing the samples into two distinct branches that align with their respective geographic and production origins. Together, these analytical approaches robustly demonstrate that Croatian whisky is compositionally distinct from Scotch whisky, providing a comprehensive molecular-level insight into their unique chemical signatures and regional characteristics.

Key differences between Croatian and Scotch whisky were observed, particularly in the presence and concentrations of certain esters, phenolic compounds, and aging markers. Croatian whisky exhibited a higher prevalence of D-limonene and linalool, contributing to citrus and floral notes, whereas Scotch whisky samples demonstrated higher concentrations of traditional oak-derived compounds such as vanillin and whisky lactones. Additionally, Croatian whisky displayed unique signatures of indigenous grain metabolites, further reinforcing the significance of regional influences on whisky chemistry.

By limiting the scope of this study to Croatian whisky, a comprehensive and in-depth analysis was achievable, providing foundational data for further research and industry development. Future studies may expand the comparative aspect by including additional whisky-producing regions, exploring how geographical and production variations influence whisky's chemical and sensory profiles.



**Figure 3.** Principal Component Analysis (PCA) plot of different whisky samples analyzed using UHPLC-QTOF. Whisky 1: Croatian single malt whisky; 2: Croatian four grain whisky; 3: blended Scotch whisky; 4: single malt Scotch whisky; 5: blended Scotch whisky; 6: single malt Scotch whisky; 7: blended Scotch whisky; 8: blended Scotch whisky; MEOH: methanol.



**Figure 4.** Dendrogram of clustering based on the similarity matrix of analyzed whiskies. Red: Croatian single malt whisky, light green: Croatian four grain whisky, dark blue: blended Scotch whisky, pink: single malt Scotch whisky, light blue: blended Scotch whisky, dark green: single malt Scotch whisky, olive: blended Scotch whisky, purple: blended Scotch whisky, brown: methanol.

#### 4. Conclusions

This study successfully identified and analyzed both volatile and non-volatile compounds in Croatian whiskies using SPME-GC-MS and UHPLC-QTOF methods, respectively.

Key aroma compounds identified include D-limonene, linalool, and  $\beta$ -damascenone, which contribute citrus, floral, and fruity notes, respectively. The aromatic profile of Croatian whiskies revealed a range of compounds that significantly influence their sensory characteristics. Furthermore, several compounds were associated with the longer aging of whisky: esculetin, ellagic acid, lyoniresinol 9-glucoside, lyoniresinol, and (E)-2-nonenal. These compounds contribute to the complexity and depth of the whisky's flavor, with esculetin and ellagic acid offering potent antioxidant properties, while lyoniresinol and its glucosides are linked to lignans found in grains and oak wood. The compounds identified from the single malt and four grain whiskies indicate a diverse and rich aromatic profile. Ethyl hexanoate and ethyl heptanoate contribute fruity notes reminiscent of red apple and pineapple. D-limonene provides a sweet orange citrus aroma, while linalool adds floral and spicy notes reminiscent of lavender and bergamot. The presence of (E)-2-nonenal introduces fried, toasted, and fatty aromas, which are essential for the characteristic profiles of aged whiskies. These findings highlight the intricate interplay of various chemical compounds that define the unique flavor and aroma of Croatian whiskies. The maturation process, grain type, fermentation, and distillation methods all contribute significantly to these profiles, showcasing the complexity and craftsmanship involved in whisky production.

Finally, the aromatic and non-volatile compounds identified in Croatian whiskies not only differentiate them from other whiskies but also underline the potential for further exploration in the field of whisky chemistry and flavor science. This study provides a foundation for future research aimed at understanding and enhancing the sensory qualities of whisky through detailed chemical analysis.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app15052738/s1>, Figure S1. Unknown compound 1 with molecular mass 1006.12918  $m/z$ ; Figure S2. Unknown compound 2 with molecular mass 978.09907  $m/z$ ; Figure S3. Multivariate Analyses and Clustering of Croatian and Scotch Whiskies Based on Untargeted QToF Analysis of Non-Volatile Compounds.

**Author Contributions:** Conceptualization, A.L. and H.B.; methodology, A.C. and M.C.; formal analysis, A.L.; resources, T.A. and H.B.; writing—original draft preparation, A.L. and A.-M.G.S.; writing—review and editing, M.L. and B.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available upon request from the corresponding author.

**Conflicts of Interest:** Hrvoje Bušić and Tomo Anadolac are employee of the company Spirit in the bottle. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

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