



Article

A New Simple Method for the Determination of Complex Wine Aroma Compounds Using GC-MS/MS—The Case of the Greek Variety “Agiorgitiko”

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Abstract: Wine exerts a fundamental influence on the global market, and its aroma remains a crucial attribute contributing to its commercial value. The market could benefit significantly if a simple and cheap method of analyzing a wine’s aromatic profile were developed. The purpose of this study is to develop such a method. A multi-analytical method for quantifying 39 volatile compounds of wine aroma was developed and validated using liquid–liquid extraction and gas chromatography/mass spectrometry/mass spectrometry (GC-MS/MS). The method was validated for its linearity, reproducibility, recovery, limit of detection, and limit of quantification and showed excellent results for almost all compounds. The method was applied to 25 commercial Protected Designation of Origin “Nemea” wines, and the results were compared and correlated with the sensory analysis results by a trained panel. The correlations among the parameters indicated that the newly developed GC-MS/MS method produces similar results to human responses.

Keywords: wine; aroma; GC-MS/MS; Nemea; Agiorgitiko; sensory



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1. Introduction

Wine has a considerable global trade value. The international trade of wine by value has exhibited a clear increasing trend over the last twenty years. Specifically, the world wine export value reached a record high in 2021, with very high annual increases. Bottled wine is the main product with a significant share of trade volumes and values (53% and 68%, respectively, in 2022). The five leading countries in wine production are Italy, France, Spain, USA, and Australia, with percentages of world wine production ranging from 4.9% to 19.3%. Greece produces between 2.0 and 3.0 mhl per year, approximately 1% of the world’s wine production [1].

Wine aroma is a key factor in its quality and commercial value. Hence, many studies have explored consumer behavior and sensory preferences for wine [2–4]. Studies have also reported gender differences in wine flavor preferences [5], providing valuable insights for marketing strategies to wineries. Moreover, wine aroma has been associated with consumers’ emotions, a relevant topic for the wine industry [6]. Wine aroma can be considered multidimensional. Various factors such as grape variety, soil type, and fungicide treatment influence it [7]. Yeasts also play a crucial role in wine aroma formation, as they produce a large number of volatile compounds from grape-derived aroma precursors [8]. Moreover, lactic bacteria strains can have a significant impact on the aromatic profile of the wine produced [9,10]. The oenological practices are also important for the formation and preservation of wine aroma, i.e., storage temperatures are critical for maintaining the

wine aroma that has been formed. The supplementation of must with nitrogen is also an important factor that affects the aroma [11]. The aroma and flavor of wine are dynamic, as they depend strongly on post-fermentation treatments such as fining, filtration, and maturation in tanks, wooden containers, or bottles [12]. Specifically, during aging in barrel containers, the fruity character and the oak aroma change. Moreover, the compounds whose concentration changes during the aging process can be classified into categories. There are compounds that increase their concentration, such as those extracted from wood or formed by precursors in a process not influenced by wood. Conversely, there are compounds that decrease or even lose their concentration due to oxidation processes or processes of sorption to the wood [13]. The choices of the type of barrel and the toasting temperatures are very important for the aromatic profile of the wine aged in wood [14]. The high-toasted barrels release higher concentrations of volatile phenols and lower concentrations of furanic compounds and phenolic aldehydes [15].

Wine fraud is a growing concern in the global commercial trade [16]. One of the main forms of wine fraud is counterfeit wine. Studies have shown that wine consumers base their purchases on the region of origin and the designation of origin (collective brand) of the product. Moreover, the reputation of the product and the individual winemaker significantly influence their willingness to buy a wine. Therefore, wine authentication is an urgent market demand. Wine authentication relies on analytical data obtained by using a combination of different techniques, along with the development of statistical models [17]. Several studies have aimed to identify the typicity of wines, which refers to the aromatic profile that characterizes wines from a specific variety and region. To this end, analytical methods using gas chromatography coupled with sensory analysis have been employed [18,19]. Volatile compounds of the aroma and their concentrations have been used as markers for typicity in various grape varieties [20,21]. The aromatic profile has also been used as evidence for distinguishing a geographical region as unique for certain varieties [22]. The increasing interest in using gas chromatography for authenticity verification is evident from its application to other alcoholic beverages, as well [23].

In addition, the volatile compounds of wine aroma composition have been established as an analytical tool for differentiating wines according to variety [24]. This tool can also be used to distinguish wines from different varieties and countries of origin. The data produced are considered reliable for determining the grape origin of wines [25]. The aromatic profile of wines can also serve as a reliable indicator of wines from different Protected Designations of Origin [26]. These analytical tools can be applied to white, rosé, and red wines.

Several methods for the determination of wine's aroma compounds have been developed and validated. Most of the extraction methods developed were liquid-liquid [27] or SPE-cartridges [28]. The instruments used were gas chromatography-flame ionization detection (GC-FID) [29], gas chromatography-mass spectrometry (GC-MS) [27,30], or gas chromatography-tandem mass spectrometry (GC-MS/MS) [28,31]. SPE-cartridges increase the cost of the analysis (the cost of the cartridge, laboratory labor cost). Liquid-liquid extraction methods use large volumes of reagents that also raise the cost of analysis. The use of deuterated analogs as internal standards is a complicated and costly procedure. The methods using GC-FID have problems identifying the volatile compounds. For that reason, these methods are combined with GC-MS for the identification of the aroma compounds [29]. The GC-MS methods have solved the identification difficulty, but they have relatively high limits of detection (LODs) and limits of quantification (LOQs) for several compounds.

The market could benefit more from the efforts of these studies if a simple and cheap method of analysis for the wine's aromatic profile is developed. The aim of this study was to develop and validate a new simple method for the determination of the volatile compounds of wine aroma. The method uses small volumes of reagents without any time-consuming steps and determines a significant number of volatile compounds in one single run using gas chromatography-tandem mass spectrometry (GC-MS/MS). The running time of each

chromatographic run is shorter than in other studies, increasing the laboratory's daily capacity to analyze samples.

This method was applied to 25 commercial PDO Nemea wines made from the Agiorgitiko variety.

We also developed a wine sensory analysis method in order to compare the GC-MS/MS results with the sensory analysis results of the same samples.

The novelty of this article lies in the simplicity of the analysis method, the utilization of a straightforward extraction technique, the comprehensive analysis of various compounds, and the rapid, cost-effective sample analysis procedure suitable for laboratory settings. This method is readily applicable in routine laboratory procedures, and its affordability and efficiency make it particularly attractive. Furthermore, this publication introduces a novel approach by integrating the analytical method of GC-MS/MS with human responses.

2. Materials and Methods

2.1. Wines

Twenty-five (25) commercial Nemea wines purchased by the Nemea Winemakers Association were analyzed. The price of the wines was in the range of EUR 5 to 30 per bottle. The vintages were 2015 and 2016.

2.2. Chemicals and Reagents

Water (for UHPLC, supergradient) and dichloromethane (for pesticide analysis, 99.8%) were purchased from PanReac AppliChem ITW Reagents (Barcelona, Spain). Ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, sodium sulfate Na_2SO_4 , and phenol ($\geq 99.5\%$) were purchased from Penta Chemicals Unlimited (Prague, Czech Republic). Absolute ethanol ($\geq 99.8\%$) was purchased from Honeywell (Charlotte, NC, USA) and tartaric acid (99.5%) was purchased from Sigma Aldrich (St. Louis, MO, USA).

Thirty-nine (39) reference-standard-grade pure compounds were purchased from commercial sources as follows: citronellol, decyl aldehyde, ethyl 2-methylbutyrate, ethylvanillin, 4-ethylphenol, eugenol, ethyl-caproate, ethyl-cinnamate trans, geraniol, isoamylacetate, linalool, and vanillin were purchased from Acros Organics (Geel, Belgium); ethylbutyrate, ethyl-isobutyrate, ethyl-isovalerate, hexyl-acetate, isobutyl-acetate, β -ionone, 2-phenylethyl acetate, and rose oxide were purchased from Honeywell Fluka (Charlotte NC, USA); acetovanillone, damascenone natural, 4-ethylguajacol, ethyl-caprylate, ethyl 3-hydroxybutyrate, ethyl-decanoate, ethyl-dodecanoate, guaiacol, hexanal, isoeugenol, 2-methoxy-4-vinylphenol, 2-methoxy-4-methylphenol, 3-(methylthio)propionaldehyde, 2-phenylethanol, benzyl-acetate, citral, thymol, 4-vinylphenol solution, and whiskey lactone were purchased from Sigma Aldrich (St. Louis, MO, USA). The list of the standards, Chemical Abstracts Service Number (CAS Registry Number), molecular formula, purity, and company are shown in Table A1.

2.3. Isolation of Volatiles

Volatile extraction was performed using the following procedure: Quantities of 3 mL of wine, 7 mL of water, 4.5 g of ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$, 1.5 mL of dichloromethane (CH_2Cl_2) , and 10 μL of phenol (10 mg/L) as an internal standard were added to a glass test tube, then the tube was vortexed for 60 s and centrifuged for 4 min at 4000 rpm. The upper phase was discarded, and 0.5 mL of the organic phase was transferred to a vial containing 0.4 g of sodium sulfate (Na_2SO_4) , then transferred to a new vial. A volume of 1 μL of the final solution was injected into the GC-MS/MS instruments.

2.4. Calibration Curve

The following procedure was used to create a calibration curve by using standard solutions (procedural standard method): Firstly, a model wine was prepared by dissolving tartaric acid (5.0 g/L) in an aqueous solution of ethanol (12.5% *v/v*) and adjusting the pH to 3.5 with NaOH. Then, five standard solutions were obtained by spiking different

volumes of the model wine with known concentrations of a mixture of volatile compounds. The final concentrations for each compound ranged from 10 to 2000 µg/L, except for 2-phenyl-ethanol, which ranged from 1 to 200 mg/L.

The standard solutions were extracted and analyzed by means of GC-MS/MS according to the method described above.

The equation for quantitation is

$$C_{\text{sample}} = C_{\text{final extract}}$$

without any condensation or dilution factor.

The equation of the calibration curve is

$$y = a + bx$$

where y represents the concentration of the substance and x represents the area of the substance in the chromatogram. The calibration curve statistics (R^2) for each substance are shown in Table 1.

Table 1. Data of repeatability and recovery for two concentration levels (10 and 500 µg/L); statistical data of reproducibility at the concentration level of 500 µg/L; LOQ and LOD; and statistical data of linearity (R^2) for each compound.

| Concentration LEVEL | 10 µg/L | | | 500 µg/L | | | 500 µg/L | | | | |
|------------------------------|-------------|------|-------|-------------|------|-------|---|--|------------|------------|----------------|
| Compound | % Re-covery | s | RSD % | % Re-covery | s | RSD % | Uncertainty of Combined Re-productibility | Uncertainty in Level of Confidence 95% (k = 2) | LOQ (µg/L) | LOD (µg/L) | R ² |
| 2-methoxy-4-methyl phenol | 87.8 | 10.0 | 11.3 | 109.4 | 14.4 | 13.2 | 7.9 | 15.8 | 0.05 | 0.015 | 0.9954 |
| 2-methoxy-4-vinylphenol | 108.0 | 13.1 | 12.1 | 97.2 | 15.3 | 15.8 | 9.5 | 19 | 0.05 | 0.015 | 0.9992 |
| 2-phenyl-ethanol | 92.3 | 13.4 | 14.5 | 81.2 | 5.2 | 6.4 | 17 | 34 | 1 | 0.3 | 0.9993 |
| 2-phenyl-ethyl-acetate | 88.8 | 20.0 | 22.5 | 119.7 | 5.1 | 4.2 | 16.3 | 32.6 | 0.05 | 0.015 | 0.9948 |
| 3-methylthio-propionaldehyde | 109.3 | 11.8 | 10.8 | 110.7 | 10.3 | 9.3 | 8.7 | 17.4 | 0.5 | 0.15 | 0.9996 |
| 4-vinyl-phenol | 94.9 | 15.3 | 16.1 | 92.3 | 7.4 | 8.0 | 8.4 | 16.8 | 5 | 1.5 | 0.9986 |
| Acetovanillone | 90.9 | 8.6 | 9.5 | 106.7 | 7.5 | 7.0 | 9.5 | 19 | 0.05 | 0.015 | 0.9995 |
| β-Ionone | 111.2 | 5.1 | 5.5 | 182.6 | 29.6 | 16.2 | 16.6 | 33.2 | 0.1 | 0.03 | 0.9885 |
| Benzyl-acetate | 94.3 | 13.0 | 13.8 | 111.2 | 9.7 | 8.7 | 6.2 | 12.4 | 0.1 | 0.03 | 0.9997 |
| Citral | 92.8 | 13.3 | 14.3 | 94.1 | 8.2 | 8.7 | 36.2 | 72.4 | 5 | 1.5 | 0.9937 |
| Citronellol | 95.3 | 12.6 | 13.2 | 102.7 | 10.3 | 10.0 | 7.4 | 14.8 | 1 | 0.3 | 0.9912 |
| Damascenone | 96.8 | 17.4 | 18.0 | 112.7 | 11.1 | 9.8 | 5 | 10 | 5 | 1.5 | 0.9963 |
| Decyl-aldehyde | 92.0 | 18.7 | 20.3 | 96.3 | 8.6 | 9.0 | 12.7 | 25.4 | 1 | 0.3 | 0.9974 |
| Ethyl-2-methyl butyrate | 112.7 | 13.6 | 12.1 | 114.8 | 9.0 | 7.9 | 7.5 | 15 | 0.05 | 0.015 | 0.9975 |
| Ethyl-3-hydroxybutyrate | 115.9 | 5.0 | 4.3 | 110.8 | 9.0 | 8.2 | 5.3 | 10.6 | 0.1 | 0.03 | 0.9979 |
| Ethyl -butyrate | 113.4 | 5.2 | 4.6 | 111.3 | 7.7 | 6.9 | 6.3 | 12.6 | 0.5 | 0.15 | 0.9962 |
| Ethyl -caproate | 109.4 | 4.7 | 4.3 | 107.3 | 9.7 | 9.0 | 5.3 | 10.6 | 0.05 | 0.015 | 0.9939 |
| Ethyl-caprylate | 109.4 | 5.9 | 5.4 | 110.2 | 4.2 | 3.8 | 7.2 | 14.4 | 0.1 | 0.03 | 0.9972 |
| Ethyl-cinnamate | 89.2 | 10.2 | 11.5 | 106.3 | 13.1 | 12.4 | 6.7 | 13.4 | 0.1 | 0.03 | 0.9969 |
| Ethyl-decanoate | 111.3 | 7.1 | 6.4 | 115.9 | 4.1 | 3.5 | 12.9 | 25.8 | 0.05 | 0.015 | 0.9889 |
| Ethyl-dodecanoate | 88.6 | 15.8 | 17.8 | 110.9 | 7.3 | 6.6 | 15.1 | 30.2 | 0.05 | 0.015 | 0.9968 |
| Ethyl-guaiacol | 89.7 | 10.2 | 11.4 | 104.7 | 7.2 | 6.9 | 14.1 | 28.2 | 0.5 | 0.15 | 0.9978 |
| Ethyl-isobutyrate | 114.0 | 9.3 | 8.2 | 115.7 | 5.7 | 5.0 | 14.2 | 28.4 | 1 | 0.3 | 0.9972 |
| Ethyl-isovalerate | 102.1 | 13.0 | 12.8 | 91.3 | 6.7 | 7.3 | 6.7 | 13.4 | 1 | 0.3 | 0.9895 |
| Ethyl-phenol | 87.6 | 15.6 | 17.8 | 90.7 | 11.6 | 12.8 | 6 | 12 | 0.1 | 0.03 | 0.997 |
| Ethyl-vanillin | 101.3 | 12.8 | 12.7 | 112.4 | 9.7 | 8.7 | 8.3 | 16.6 | 0.1 | 0.03 | 0.9991 |
| Eugenol | 93.2 | 10.0 | 10.7 | 100.8 | 8.7 | 8.6 | 7.6 | 15.2 | 0.5 | 0.15 | 0.9999 |
| Geraniol | 99.7 | 13.7 | 13.8 | 103.0 | 4.6 | 4.5 | 10.8 | 21.6 | 1 | 0.3 | 0.9936 |
| Guaiacol | 98.9 | 10.3 | 10.4 | 111.8 | 4.2 | 3.8 | 7.2 | 14.4 | 0.05 | 0.015 | 0.998 |
| Hexanal | 94.2 | 18.4 | 19.5 | 112.1 | 8.7 | 7.8 | 9.3 | 18.6 | 1 | 0.3 | 0.9979 |
| Hexyl-acetate | 93.5 | 7.9 | 8.5 | 105.8 | 7.7 | 7.3 | 4.8 | 9.6 | 1 | 0.3 | 0.9995 |
| Isoamyl-acetate | 103.2 | 19.5 | 18.8 | 103.7 | 12.7 | 12.3 | 9.7 | 19.4 | 1 | 0.3 | 0.9998 |
| Isobutyl-acetate | 112.5 | 9.5 | 8.5 | 105.8 | 8.1 | 7.7 | 11.4 | 22.8 | 1 | 0.3 | 0.9997 |

Table 1. Cont.

| Concentration LEVEL | 10 µg/L | | | 500 µg/L | | | 500 µg/L | | | LOQ (µg/L) | LOD (µg/L) | R ² |
|-------------------------------|-------------|------|-------|-------------|------|-------|--|--|-----|------------|------------|----------------|
| Compound | % Re-covery | s | RSD % | % Re-covery | s | RSD % | Uncertainty of Combined Re-producibility | Uncertainty in Level of Confidence 95% (k = 2) | | | | |
| Isoeugenol | 84.6 | 7.2 | 8.6 | 102.9 | 6.8 | 6.6 | 9.2 | 18.4 | 1 | 0.3 | 0.9991 | |
| Linalool | 93.4 | 13.1 | 14.0 | 111.5 | 8.2 | 7.3 | 4.2 | 8.4 | 1 | 0.3 | 0.9988 | |
| Rose oxide | 93.8 | 17.0 | 18.2 | 108.6 | 8.4 | 7.7 | 4.2 | 8.4 | 1 | 0.3 | 0.9827 | |
| Thymol | 86.9 | 17.0 | 19.5 | 110.9 | 10.3 | 9.3 | 5.3 | 10.6 | 0.1 | 0.03 | 0.9968 | |
| Vanillin | 94.6 | 18.0 | 19.0 | 102.8 | 13.1 | 12.8 | 10.4 | 20.8 | 0.1 | 0.03 | 0.9997 | |
| <i>trans</i> -Whiskey lactone | 95.2 | 15.6 | 16.4 | 108.1 | 5.2 | 4.8 | 14.2 | 28.4 | 0.5 | 0.15 | 0.9988 | |
| <i>cis</i> -Whiskey lactone | 98.8 | 19.4 | 19.7 | 102.8 | 12.1 | 11.8 | 13.1 | 26.2 | 0.5 | 0.15 | 0.9915 | |

2.5. GC-MS/MS Settings

A Quantum XLS Gas Chromatograph (Thermo Scientific Inc., Waltham, MA, USA) coupled to a Triple Quad Mass Spectrometer (Thermo Scientific Inc., Waltham, MA, USA) was used for GC-MS analysis. A TR-Pesticide II column (30 m × 0.25 mm ID, 0.25 µm film thickness, Thermo Scientific) with helium as the carrier gas (1.0 mL/min) and the following temperature program was employed: an initial temperature of 40 °C was held for 5 min, increased by 7 °C/min to 170 °C, then increased by 40 °C/min to 290 °C and held for 3 min. The injection inlet temperature was 210 °C and the injection volume was 2 µL in the splitless mode.

The MS/MS settings were as follows: experiment type SRM (selective reaction monitoring), collision gas pressure 1.5 mTorr, Q1 and Q3 peak width 0.70, cycle time 0.500 (s), solvent delay of 7 min, emission current 50 µA, source temperature 200 °C, transfer line temperature 250 °C.

A selective reaction monitoring (SRM) scan mode was employed for the quantification of the volatile compounds. The detailed MRM (multiple reaction monitoring) parameters are given in Table 2.

Table 2. Mass spectral transitions (Mr), collision energies (CEs) selected, and the retention time for analysis of the volatile compounds. The “quantitation” pair of fragments (with higher intensity) is indicated in bold for each compound.

| Name | Mr | Q1 | Q3 | CE | tR |
|-------------------------------------|---------------|---------------|---------------|-----------|--------------|
| 2-methoxy-4-methyl phenol | 138.16 | 123.18 | 94.88 | 5 | 19.39 |
| 2-methoxy-4-methyl phenol | 138.16 | 123.18 | 67.08 | 5 | 19.39 |
| 2-methoxy-4-vinyl phenol | 150.17 | 135.24 | 77.1 | 15 | 21.94 |
| 2-methoxy-4-vinyl phenol | 150.17 | 135.24 | 107.03 | 15 | 21.94 |
| 2-Phenethyl-acetate | 164.2 | 104.28 | 78.04 | 15 | 20.74 |
| 2-Phenethyl-acetate | 164.2 | 104.28 | 103.02 | 15 | 20.74 |
| 2-Phenyl-ethanol | 122.16 | 91.12 | 65.06 | 15 | 17.67 |
| 2-Phenyl-ethanol | 122.16 | 91.12 | 63.3 | 15 | 17.67 |
| 3-Methylthio propionaldehyde | 104.17 | 76.08 | 61.12 | 5 | 12.32 |
| 3-Methylthio propionaldehyde | 104.17 | 76.08 | 48.06 | 5 | 12.32 |
| 4-vinyl phenol | 120.15 | 91.2 | 65.06 | 15 | 19.9 |
| 4-vinyl phenol | 120.15 | 91.2 | 50.67 | 15 | 19.9 |
| Acetovanillone | 166 | 150.95 | 107.92 | 15 | 21.7 |
| Acetovanillone | 166 | 150.95 | 122.97 | 15 | 21.7 |
| Acetovanillone | 166 | 150.95 | 76.99 | 15 | 21.7 |
| β-ionone | 192.3 | 177.53 | 162.18 | 15 | 24.8 |

Table 2. Cont.

| Name | Mr | Q1 | Q3 | CE | tR |
|--------------------------------|---------------|---------------|---------------|-----------|--------------|
| β -ionone | 192.3 | 177.53 | 147.16 | 15 | 24.8 |
| Benzyl-acetate | 150.17 | 108.18 | 107.09 | 5 | 18.76 |
| Benzyl-acetate | 150.17 | 108.18 | 79.13 | 5 | 18.76 |
| Citral | 152.23 | 94.21 | 79.18 | 10 | 21 |
| Citral | 152.23 | 94.21 | 77.14 | 10 | 21 |
| Citronellol | 156.07 | 82.25 | 67.12 | 5 | 20.12 |
| Citronellol | 156.07 | 8225 | 65.09 | 5 | 20.12 |
| Damascenone | 190.28 | 121.35 | 105.32 | 5 | 23.32 |
| Damascenone | 190.28 | 121.35 | 118.86 | 5 | 23.32 |
| Decyl-aldehyde | 156.27 | 82.36 | 67.06 | 5 | 19.7 |
| Decyl-aldehyde | 156.27 | 82.36 | 65.14 | 5 | 19.7 |
| Ethyl-2-methyl butyrate | 130.19 | 102.25 | 73.6 | 5 | 10.62 |
| Ethyl-2-methyl butyrate | 130.19 | 102.25 | 74.34 | 5 | 10.62 |
| Ethyl-3-hydroxybutyrate | 13216 | 88.13 | 60.09 | 5 | 13.08 |
| Ethyl-3-hydroxybutyrate | 132.16 | 88.13 | 60.76 | 5 | 13.08 |
| Ethyl-butyrate | 116.16 | 88.13 | 73.05 | 5 | 9.1 |
| Ethyl-butyrate | 116.16 | 88.13 | 61.14 | 5 | 9.1 |
| Ethyl-Caproate | 144.21 | 87.94 | 60.05 | 5 | 11.66 |
| Ethyl-Caproate | 144.21 | 87.94 | 61.02 | 5 | 11.66 |
| Ethyl-caprylate | 172.3 | 87.94 | 59.98 | 5 | 19.45 |
| Ethyl-caprylate | 172.3 | 87.94 | 61.04 | 5 | 19.45 |
| Ethyl-cinnamate | 176.2 | 130.98 | 102.95 | 5 | 21.36 |
| Ethyl-cinnamate | 176.2 | 130.98 | 76.99 | 5 | 21.36 |
| Ethyl-cinnamate | 176.2 | 130.98 | 90.95 | 5 | 21.36 |
| Ethyl-decanoate | 200.3 | 87.94 | 59.97 | 5 | 23.42 |
| Ethyl-decanoate | 200.3 | 87.94 | 61.05 | 5 | 23.42 |
| Ethyl-dodecanoate | 228.4 | 87.94 | 60.01 | 5 | 25.77 |
| Ethyl-dodecanoate | 228.4 | 87.94 | 60.98 | 5 | 25.77 |
| Ethyl-guaiacol | 152.19 | 137.3 | 122.04 | 5 | 21.19 |
| Ethyl-guaiacol | 152.19 | 137.3 | 94.01 | 5 | 21.19 |
| Ethyl-isobutyrate | 116.16 | 116.16 | 73.08 | 5 | 7.8 |
| Ethyl-isobutyrate | 116.16 | 116.16 | 88.24 | 5 | 7.8 |
| Ethyl-isovalerate | 130.18 | 88.18 | 60.02 | 5 | 10.74 |
| Ethyl-isovalerate | 130.18 | 88.18 | 55.01 | 5 | 10.74 |
| Ethyl-phenol | 122.17 | 106.95 | 77 | 15 | 15.56 |
| Ethyl-phenol | 12217 | 106.95 | 78.99 | 15 | 15.56 |
| Ethyl-vanillin | 166.2 | 136.91 | 108.93 | 5 | 21.15 |
| Ethyl-vanillin | 166.2 | 136.91 | 80.97 | 5 | 21.15 |
| Eugenol | 164.2 | 164.01 | 148.98 | 5 | 19.24 |
| Eugenol | 164.2 | 164.01 | 130.97 | 5 | 19.24 |
| Eugenol | 164.2 | 164.01 | 120.96 | 5 | 19.24 |
| Geraniol | 154.25 | 93.22 | 72.25 | 5 | 20.63 |
| Geraniol | 154.25 | 93.22 | 91.07 | 5 | 20.63 |
| Guaiacol | 124.1 | 108.91 | 80.97 | 5 | 13.7 |
| Guaiacol | 124.1 | 108.91 | 53.03 | 5 | 13.7 |
| Hexanal | 100.16 | 56.07 | 41.15 | 5 | 9.08 |
| Hexanal | 100.16 | 56.07 | 39.29 | 5 | 9.08 |
| Hexyl-acetate | 144.2 | 83.94 | 54.93 | 5 | 15.2 |
| Hexyl-acetate | 144.2 | 83.94 | 56.15 | 5 | 15.2 |
| Isoamyl-acetate | 130.19 | 70.02 | 55.05 | 5 | 8.61 |
| Isoamyl-acetate | 130.19 | 70.02 | 53.06 | 5 | 8.61 |
| Isobutyl-acetate | 116.16 | 73.15 | 43.15 | 5 | 8.27 |
| Isobutyl-acetate | 116.16 | 56.09 | 41.24 | 5 | 8.27 |
| Isoeugenol | 164.2 | 164 | 148.98 | 5 | 21.03 |
| Isoeugenol | 164.2 | 164 | 130.94 | 5 | 21.03 |
| Isoeugenol | 164.2 | 164 | 120.95 | 5 | 21.03 |
| Linalool | 154.25 | 93.23 | 77.05 | 5 | 17.32 |
| Linalool | 154.25 | 93.23 | 91.03 | 5 | 17.32 |
| Rose oxide | 154.25 | 69.11 | 41.23 | 5 | 18 |
| Rose oxide | 154.25 | 69.11 | 65.29 | 5 | 18 |

Table 2. Cont.

| Name | Mr | Q1 | Q3 | CE | tR |
|------------------------|---------------|---------------|---------------|-----------|--------------|
| Thymol | 150.22 | 135.34 | 91.03 | 15 | 21.45 |
| Thymol | 150.22 | 135.34 | 115.06 | 15 | 21.45 |
| Vanillin | 120.15 | 151.97 | 150.97 | 10 | 20.25 |
| Vanillin | 120.15 | 151.97 | 122.92 | 10 | 20.25 |
| Whiskey lactone | 156.22 | 99.03 | 71.06 | 5 | 18 |
| Whiskey lactone | 156.22 | 99.03 | 41.18 | 5 | 18 |
| Whiskey lactone | 156.22 | 114.16 | 71.06 | 5 | 18 |
| Phenol | 94.11 | 94.10 | 66.10 | 10 | 13.4 |

2.6. MS Parameters

Each volatile compound was injected into the gas chromatograph at a concentration of 10 mg/L, and the MS/MS detector was set at MS mode for full scan analysis. The m/z range for each compound ranged from 50 to its molecular weight plus 10. The chromatographic peaks were checked using the NIST Library to match the right peak with the compound. This procedure determined the fragment with the highest intensity (parent ion), the exact m/z value (with accuracy at 0.01 level), and the retention time.

Each compound was injected again into the gas chromatograph at a concentration level of 10 mg/L, when Q1 was adjusted to the m/z of the parent ion and Q3 to the full scan analysis (m/z from 50 to m/z of the parent ion plus 10) to determine the two fragments with the highest intensity. Successive injections of each compound were performed at different collision energy (CE) values each time to determine the optimum CE for each compound.

2.7. Method Validation

2.7.1. Linearity

Linearity was evaluated across a series of five points of the spiked model wine with each compound. The concentration range was between 10 and 2000 $\mu\text{g/L}$ (except for 2-phenyl-ethanol, where the concentration range was between 1 and 200 $\mu\text{g/L}$).

For the statistical evaluation of linearity, an internal standard was used. More specifically, the nominal value of each standard was placed on the x-axis and the ratio of the area of the standard to the area of the internal standard was placed on the y-axis.

2.7.2. Repeatability and Recovery

For the estimation of repeatability and recovery, six identical samples were analyzed at two different concentration levels, 10 and 500 $\mu\text{g/L}$. Each sample was produced by spiking the model wine with a mixture of all compounds. The concentration of each compound in each sample was calculated using the calibration curve plotted for each compound.

2.7.3. Reproducibility

For the calculation of reproducibility, a standard addition procedure was applied to red wine. The concentration level chosen for adding volatile compounds was 500 $\mu\text{g/L}$. Using this procedure, six samples were produced for six consecutive days. Each sample was analyzed on the day of its extraction. All the wines produced by standard addition as well as the original wine produced before the standard addition, were analyzed in duplicate.

2.7.4. Limit of Detection and Limit of Quantification

A mixture of all compounds was injected at different concentrations to calculate LOQs (S/N:10) and LODs (S/N:3).

2.8. Sensory Analysis

Twenty-four participants were recruited, and the twelve with the best performance were selected for the sensory panel. The selection process consisted of 38 sessions that covered theory; introduction to triangle tests, ageusia and anosmia tests; introduction to

ranking tests; discrimination between levels of a stimulus; and determination of a threshold for the basic attributes of taste [32]. Each test was accompanied by measurable goals that the candidates had to achieve to proceed to the next stage.

The second cycle of training involved 10 sessions, which included initiation and training for 50 odors and recognition of these odors in the sensory laboratory according to relevant guidelines [33].

The following cycles of training consisted of triangle tests with different levels of fruity aroma, barrel aroma, and wine defects in five sessions. Ranking tests for fruity odor and barrel odor were conducted in two sessions. Training in the use of a continuous scale was conducted by analyzing samples of wine and synthetic wines spiked with aroma compounds.

The sensory panel analyzed 25 commercial wines from the Nemea region. The sensory attributes were fruity odor, barrel odor, and flavor. The scale was continuous from 0 to 10.

Latin-square design and three-digit codes were used to present the samples. Specialized software for sensory analysis generated the presentation design and the code for each sample. The data analysis was performed using this software.

The analyses took place in a sensory laboratory that had been constructed according to relevant guidelines [34].

3. Results

3.1. Method Development Sample Preparation

MS Parameters

For each compound, at least two pairs of fragments (for Quadrupole 1 and Quadrupole 3) were chosen. The pair with the higher intensity was used for quantitation, and the pair with the lower intensity was used for qualification purposes.

For the compounds acetovanillone, isoeugenol, ethyl-cinnamate, eugenol, and whiskey lactone, three pairs were chosen, since the second and the third pair of fragments had similar intensity.

For each compound, except isobutyl-acetate and whiskey lactone, the first fragment was common to both pairs, while the second fragment ("Q3") was different. Isobutyl-acetate and whiskey lactone had different first fragments in each pair.

The collision energy for each compound ranged between 5 and 15 eV. The retention times ranged between 7.8 and 25.8 min.

Some compounds had the same or a very similar m/z to the first fragment. More specifically, Group 1 consisted of ethyl-caproate, ethyl-caprylate, ethyl-decanoate, and ethyl-dodecanoate. Group 2 consisted of ethyl-3-hydroxybutyrate and ethyl-butyrate. Group 3 consisted of geraniol and linalool. Group 4 consisted of eugenol and isoeugenol. Each compound had a significantly different retention time compared to other compounds in the same group. As a result, each compound could be quantified and qualified with high confidence.

Cis-whiskey lactone and *trans*-whiskey lactone exhibited significantly different retention times, leading to excellent peak resolution. For quantification purposes, the areas under the respective peaks were summed, and a new concentration-versus-area curve was generated for whiskey lactone.

3.2. Method Validation

3.2.1. Linearity

Table 1 shows the statistical data of linearity (R^2) for each compound. The coefficient of determination (R^2) values for all compounds were higher than 0.98. More specifically, the R^2 values ranged from 0.9827 to 1.0000.

Four compounds had R^2 values between 0.9827 and 0.9895; six compounds had R^2 values between 0.990 and 0.995; and thirty compounds had R^2 values higher than 0.995.

Twelve of these thirty compounds had R^2 values higher than 0.999.

3.2.2. Repeatability—Recovery

Table 1 shows the average recovery, the standard deviation (SD), and the relative standard deviation (RSD%) for each compound at two concentration levels: 10 and 500 µg/L. At the 10 µg/L level, the recovery range for all compounds was between 84.6% and 116%.

The RSD% was below 20% for all target compounds except decyl-aldehyde (20.5%) and 2-phenyl-ethyl-acetate (22.5%). The RSD% ranged from 4.3% to 22.5%.

At the 500 µg/L level, the recovery range for all compounds was between 81.2% and 120%, with the exception of β-ionone (183%). The RSD% was below 20% for all compounds except decyl-aldehyde (16%) and ethyl-butylate (16%). The RSD% ranged from 3.5% to 16%.

3.2.3. Reproducibility

The concentration of each compound was calculated by subtracting the concentration before the standard addition from the concentration after the standard addition. The mean recovery, the standard deviation, and the relative standard deviation were calculated for each compound based on the analyses of the six samples.

The combined reproducibility uncertainty was less than 20% for all the target compounds (except for citral, which had a combined reproducibility uncertainty as high as 36.2%), ranging from 4.2% to 17.0%, as shown in Table 1. The combined uncertainty for a confidence level of 95% was between 8.4% and 34.0% (except for citral: 72.4%).

3.2.4. Limit of Detection

The results are presented in Table 1. Limits of detection ranged from 0.015 up to 1.5 µg/L.

3.3. Analysis of Wines

The method developed during this study was applied to analyze 39 volatile compounds of 25 commercial wines from AOC Nemea. Each sample was analyzed in triplicate.

The compounds quantified at concentrations higher than the limit of quantification (LOQ) were categorized into four groups: total phenols (2-methoxy-4-vinylphenol, ethyl-phenol), total acetates (2-phenyl-ethyl-acetate, isoamyl-acetate, isobutyl-acetate), total esters (ethyl-2-methyl-butylate, ethyl-3-hydroxybutylate, ethyl-butylate, ethyl-caproate, ethyl-caprylate, ethyl-decanoate, ethyl-dodecanoate, ethyl-isobutylate, ethyl-isovalerate), and oak compounds (acetovanillone, ethyl-guaiacol, vanillin, whiskey lactone). Total phenols and oak compounds are theoretically related to the barrel aroma, while total acetates and total esters are theoretically related to the fruity aroma. Phenyl-ethanol and 4-vinyl-phenol were excluded from this categorization because they have rose-like and defective aromas, respectively. The range of concentration and the average for each compound are presented in Table 3.

Table 3. The aromatic compounds detected over the LOQ, the range of concentration for all Nemea samples, the average concentration, and the odor threshold for each compound.

| Compound | Range of Concentration (µg/L) | Average (µg/L) | Odor Threshold (µg/L) |
|------------------------|-------------------------------|----------------|-----------------------|
| 2-phenyl-ethanol | 8073–94,723 | 41,076 | 14,000 |
| Acetates | | | |
| 2-phenyl-ethyl-acetate | 9.6–125 | 37.2 | 250 |
| Isoamyl-acetate | 103–1490 | 356 | 30 |
| Isobutyl-acetate | 14.2–78.3 | 39.7 | 1600 |
| Total acetates | 121–1692 | 422 | |

Table 3. Cont.

| Compound | Range of Concentration (µg/L) | Average (µg/L) | Odor Threshold (µg/L) |
|-------------------------|-------------------------------|----------------|-----------------------|
| Esters | | | |
| Ethyl-2-methylbutyrate | 10.6–54.1 | 22.7 | 18 |
| Ethyl-3-hydroxybutyrate | 167–612 | 399 | 20,000 |
| Ethyl-butyrate | 52.7–324 | 136 | 20 |
| Ethyl-caproate | 60.2–352 | 160 | 14 |
| Ethyl-caprylate | 45.6–276 | 137 | 5 |
| Ethyl-decanoate | 9.3–115 | 32.8 | 200 |
| Ethyl-dodecanoate | 9.0–10.2 | 9.6 | 800 |
| Ethyl-isobutyrate | 38–345 | 142 | 15 |
| Ethyl-isovalerate | 11.1–114 | 39.1 | 3 |
| Total esters | 372–1892 | 1036 | |
| Oak Compounds | | | |
| Acetovanillone | 26.4–85.8 | 61.6 | 1000 |
| Ethyl-guaiacol | 14.1–89.4 | 39.8 | 33 |
| Vanillin | 16.2–276 | 80.8 | 200 |
| trans-Whiskey lactone | 20.1–208 | 93.1 | 67 |
| cis-Whiskey lactone | 20.8–746 | 244 | 790 |
| Total “Oak” Compounds | 26–1176 | 479 | |
| Phenols | | | |
| 2-methoxy-4-vinylphenol | 21.7–963 | 53.4 | 40 |
| 4-vinyl-phenol | 41.4–1119 | 269 | 180 |
| Ethyl-phenol | 20.9–228 | 87.8 | 140 |
| Total Phenols | 20.9–1382 | 266 | |

The results for each sample for the compound groups are presented in Table 4.

Table 4. The average concentration of each category of compounds, the fruity aroma (sensory attribute), the barrel aroma (sensory attribute), and the flavor intensity (sensory attribute).

| Sample Code | Concentration (µg/L) | | | | | Sensory Result (0–10) | | |
|-------------|-----------------------|---------------|----------------|--------------|---------------|-----------------------|--------------|------------------|
| | 2-phenyl-ethanol | Total Phenols | Total Acetates | Total Esters | Oak Compounds | Fruity Aroma | Barrel Aroma | Flavor Intensity |
| KK001 | 7.1 × 10 ⁴ | 20.9 | 445.1 | 962.1 | 392 | 4.9 | 1.7 | 4.3 |
| KK002 | 8.8 × 10 ⁴ | 265 | 285.9 | 995 | 1175.8 | 5.1 | 3.4 | 4.2 |
| KK003 | 6.0 × 10 ⁴ | 157.9 | 474.4 | 1255.3 | 437.2 | 4 | 3.4 | 4.3 |
| KK004 | 9.5 × 10 ⁴ | 1382 | 472.7 | 995.4 | 629 | 3.4 | 1.4 | 2.7 |
| KK005 | 7.6 × 10 ⁴ | 198.2 | 562.6 | 1078.9 | 545 | 3.2 | 4.3 | 4 |
| KK006 | 5.5 × 10 ⁴ | 99.7 | 402 | 809 | 551.1 | 3.7 | 4.8 | 4 |
| KK007 | 4.7 × 10 ⁴ | 41.7 | 315.6 | 911.7 | 1124.2 | 4.8 | 4.1 | 4.1 |
| KK008 | 4.7 × 10 ⁴ | NA | 1692.6 | 1892.6 | 502.7 | 5.9 | 2.6 | 4 |
| KK009 | 7.7 × 10 ⁴ | 385.3 | 297 | 1698.5 | 431.9 | 4.7 | 2.7 | 3.7 |
| KK010 | 8.4 × 10 ⁴ | NA | 315.4 | 815.7 | 279 | 4.3 | 1.9 | 3.2 |
| KK011 | 2.8 × 10 ⁴ | 824.2 | 302.4 | 885.7 | 525.6 | 5.6 | 2.5 | 4.2 |
| KK012 | 2.8 × 10 ⁴ | 38.3 | 367.7 | 1433.8 | 532.8 | 3.8 | 4.8 | 4.1 |
| KK013 | 2.6 × 10 ⁴ | 43.2 | 387.5 | 1098.4 | 318.5 | 4.4 | 4.8 | 3.9 |
| KK014 | 2.8 × 10 ⁴ | 211.6 | 423.1 | 577.4 | 287.3 | 3.7 | 4.1 | 3.6 |
| KK015 | 2.2 × 10 ⁴ | 637.8 | 299.5 | 1087.7 | 958.6 | 4.7 | 3.1 | 3.5 |
| KK016 | 2.3 × 10 ⁴ | 212.1 | 422.2 | 984 | 310.4 | 5.5 | 3.3 | 4.2 |
| KK017 | 2.2 × 10 ⁴ | NA | 121.1 | 703.2 | 26.4 | 3.7 | 0 | 3.3 |
| KK018 | 1.5 × 10 ⁴ | 42.4 | 262.9 | 752.3 | 200.3 | 4.1 | 3.3 | 4.2 |
| KK019 | 2.1 × 10 ⁴ | 84.6 | 213.7 | 606.4 | 338.1 | 4.6 | 3.3 | 4 |
| KK020 | 2.9 × 10 ⁴ | 21.7 | 612.6 | 1297.8 | 291.3 | 5.3 | 3.3 | 4.8 |
| KK021 | 2.1 × 10 ⁴ | 271.4 | 315.8 | 1344 | 595.9 | 3.3 | 3.6 | 3.4 |

Table 4. Cont.

| Sample Code | Concentration (µg/L) | | | | Sensory Result (0–10) | | | |
|-------------|----------------------|---------------|----------------|--------------|-----------------------|--------------|--------------|------------------|
| | 2-phenyl-ethanol | Total Phenols | Total Acetates | Total Esters | Oak Compounds | Fruity Aroma | Barrel Aroma | Flavor Intensity |
| KK022 | 1.7×10^4 | 508.1 | 271.8 | 879 | 404.5 | 3.7 | 4.5 | 3.7 |
| KK023 | 2.7×10^4 | 66.8 | 743.8 | 1374.4 | 444.9 | 4.6 | 3.7 | 3.6 |
| KK024 | 8.1×10^3 | 80.6 | 220.8 | 1085.9 | 540.1 | 4.8 | 3.6 | 4.2 |
| KK025 | 1.2×10^4 | NA | 326.9 | 372.8 | 123.3 | 4.9 | 2.6 | 4.2 |

For the evaluation of the sensory analysis results the odor active value (OAV) of each compound was calculated. The OAV is calculated using the aroma detection threshold. In general, the detection threshold is the minimum value of a sensory stimulus that can be perceived [35]. More specifically, the aroma detection threshold is the lowest concentration at which a compound can be detected by a sensory panel. The OAV is calculated as

$$\text{OAV} = C/T,$$

where C is the concentration of the compound and T is the detection threshold of that compound [36].

To calculate the OAV, we used the detection thresholds published in other studies in which each compound’s aroma perception by the panel is described, which helps to determine its contribution to the wine’s aromatic profile [37–44].

OAV-barrel was calculated by summing up the OAVs of phenols and oak compounds; OAV-fruity was calculated by summing up the OAVs of acetates, esters, and phenyl-ethanol. The percentages of OAV-barrel and OAV-fruity were also calculated. The results are presented in Table 5.

Table 5. The odor active value (OAV) of each category of compounds; the sum OAV of fruity, the sum OAV of barrel, the percentage OAV of fruity, and the percentage OAV of barrel for each sample are shown.

| Code | OAV | | | | | OAV Sum | | | OAV % | |
|-------|------------------|----------------|--------------|------------|-----------------|---------|--------|-------|---------|---------|
| | 2-phenyl-ethanol | Total Acetates | Total Esters | Phenol Sum | “Oak” Compounds | Fruity | Barrel | SUM | Fruity% | Barrel% |
| KK001 | 5.1 | 12 | 47.3 | 0.1 | 2.1 | 64.4 | 2.2 | 66.6 | 96.7 | 3.3 |
| KK002 | 6.3 | 6.9 | 64.8 | 1.5 | 5.9 | 77.9 | 7.4 | 85.3 | 91.3 | 8.7 |
| KK003 | 4.3 | 13.6 | 70 | 2 | 2.3 | 87.9 | 4.2 | 92.1 | 95.4 | 4.6 |
| KK004 | 6.8 | 12.5 | 61.9 | 8.8 | 4.4 | 81.1 | 13.2 | 94.3 | 86 | 14 |
| KK005 | 5.4 | 15 | 53.6 | 1.1 | 2.8 | 74.1 | 3.9 | 78 | 95 | 5 |
| KK006 | 3.9 | 10.3 | 41.3 | 0.7 | 3.6 | 55.6 | 4.3 | 59.9 | 92.8 | 7.2 |
| KK007 | 3.3 | 7.6 | 52.1 | 0.3 | 4.1 | 63.1 | 4.4 | 67.5 | 93.5 | 6.5 |
| KK008 | 3.4 | 50.2 | 115.7 | NA | 3.1 | 169.3 | 3.1 | 172.4 | 98.2 | 1.8 |
| KK009 | 5.5 | 7.1 | 123.9 | 2.2 | 2.1 | 136.5 | 4.3 | 140.8 | 96.9 | 3.1 |
| KK010 | 6 | 8.1 | 39.6 | NA | 1.2 | 53.7 | 1.2 | 55 | 97.8 | 2.2 |
| KK011 | 2 | 8.9 | 48.9 | 6.2 | 3.9 | 59.8 | 10.1 | 69.9 | 85.6 | 14.4 |
| KK012 | 2 | 11.1 | 98.2 | 1 | 2.7 | 111.3 | 3.6 | 114.9 | 96.8 | 3.2 |
| KK013 | 1.9 | 11.3 | 81.1 | 1.1 | 1.6 | 94.4 | 2.7 | 97.1 | 97.2 | 2.8 |
| KK014 | 2 | 12.4 | 30.2 | 2 | 1.1 | 44.6 | 3.1 | 47.6 | 93.6 | 6.4 |
| KK015 | 1.6 | 8.9 | 61.1 | 4.5 | 4.1 | 71.6 | 8.7 | 80.2 | 89.2 | 10.8 |
| KK016 | 1.6 | 12.9 | 61.9 | 3.1 | 1.4 | 76.4 | 4.4 | 80.9 | 94.5 | 5.5 |
| KK017 | 1.5 | 3.4 | 61.3 | NA | 0 | 66.2 | 0 | 66.3 | 100 | 0 |
| KK018 | 1 | 8.3 | 45.6 | 0.2 | 0.8 | 55 | 1 | 56 | 98.2 | 1.8 |
| KK019 | 1.5 | 6.4 | 37.6 | 0.5 | 1.6 | 45.6 | 2.2 | 47.7 | 95.5 | 4.5 |
| KK020 | 2.1 | 17.9 | 94.9 | 0.5 | 1.5 | 115 | 2 | 117 | 98.2 | 1.8 |
| KK021 | 1.5 | 8.9 | 96.9 | 3.2 | 3.6 | 107.3 | 6.8 | 114.1 | 94 | 6 |

Table 5. Cont.

| Code | 2-phenyl-ethanol | OAV | | | | OAV Sum | | | OAV % | |
|-------|------------------|----------------|--------------|------------|-----------------|---------|--------|------|---------|---------|
| | | Total Acetates | Total Esters | Phenol Sum | “Oak” Compounds | Fruity | Barrel | SUM | Fruity% | Barrel% |
| KK022 | 1.2 | 7.6 | 48.6 | 4.3 | 3 | 57.5 | 7.4 | 64.9 | 88.6 | 11.4 |
| KK023 | 1.9 | 21.5 | 97.1 | 1.7 | 3.7 | 120.5 | 5.4 | 126 | 95.7 | 4.3 |
| KK024 | 0.6 | 6.8 | 75.1 | 1.2 | 2.8 | 82.5 | 4 | 86.5 | 95.4 | 4.6 |
| KK025 | 0.9 | 9.9 | 20.5 | NA | 0.6 | 31.2 | 0.6 | 31.9 | 98.1 | 1.9 |

The range of OAV-fruity was 31.2–169.3; the range of OAV-barrel was 0–13.2. The percentage of OAV-fruity ranged from 85.6% to 100%; the percentage of OAV-barrel ranged from 0% to 14.4%.

The sensory analysis scores for the fruity aroma attribute ranged from 3.2 to 5.9 on a continuous 10-point scale. The sensory analysis scores for the barrel aroma attribute ranged from 0 to 4.8 on the same scale. The results are presented in Table 4. The analytical responses for total acetates, total esters, total phenols, and oak compounds using GC-MS/MS and the sensory analysis results for the fruity aroma, barrel aroma, and flavor intensity were analyzed by means of PCA to investigate the correlations between these categories. The biplot was constructed with the first two PCs, which explained 50.2% of the total variance. In addition, partial least squares regression (PLSR) analysis was conducted to identify correlations among the aforementioned parameters and provide an additional validation measure for the PCA.

4. Discussion

The developed method analyzes different categories of aromatic compounds. The selection of two or three pairs of fragments for each compound ensures a high confidence for quantification and identification. In the cases where a few compounds have similar pairs of fragments, the retention time differentiates them. The linearity of concentrations was satisfactory for all compounds. Recoveries were between 70% and 120% for 38 out of 39 compounds. The sole exception was β -ionone, which exhibited enhanced recovery at the high control level of 500 $\mu\text{g/L}$. The reproducibility showed excellent results for 38 out of 39 compounds. The only exception was citral, which showed an increased combined reproducibility uncertainty.

LODs and LOQs were determined at very low concentrations, similar to or lower than those reported in other studies. More specifically, the limits of quantification (LOQs), when compared to other methods for the determination of aromatic compounds using GC-MS/MS, exhibited similar values; for instance, the LOQ for eugenol was consistent with those obtained from alternative methods [28] or slightly lower, ranging from 2 to 66 times lower [31]. However, when compared to methods utilizing GC-FID, the LOQs were significantly lower, ranging from 5 to 560 times lower [29]. Similarly, in comparison to methods employing GC-MS, the LOQs were lower also, spanning a range of 7 to 500 times lower [27,30].

The chromatographic analysis time is less than 30 min, which is remarkably short, similar to that in other studies that utilized GC-MS or GC-MS/MS [27,28,30,31]. Furthermore, it is significantly shorter than the time required for methods employing GC-FID [29], thereby enhancing the analytical capacity of a laboratory and enabling the daily evaluation of multiple samples.

The identification is performed using at least two pairs of fragments, which increases the certainty of the detection. Most of the methods already developed use “Wax”-type chromatographic columns, which are very specific and have a limited range of applications in an analytical laboratory. The second most common type of column is polarity’s “type-5”. Since GC-MS/MS instruments are often used for pesticide analysis in private-sector laboratories, this study used a column specific for pesticide analysis, so that the same

GC-MS/MS configuration can be used for different types of analysis without changing columns or settings. The injection volume of the sample is small, to protect the GC-MS/MS consumables and extend their lifetime.

Previous studies on aroma determination in wines have focused on specific aroma categories, such as compounds associated with a fruity character, wine defects, esters, and smoky aromas [27–31]. However, these investigations often examined a limited set of compounds. In this study, we present a novel method that encompasses a broader range of aroma compounds, spanning all the aforementioned categories. Specifically, we employ gas chromatography–tandem mass spectrometry (GC-MS/MS) to analyze a comprehensive panel of volatile compounds. Remarkably, our developed method can identify 39 distinct compounds, significantly surpassing the scope of previous studies.

The principal component analysis (PCA) we conducted aimed to identify correlations among the categories total acetates, total esters, total phenols, and oak compounds using gas chromatography–tandem mass spectrometry (GC-MS/MS), and the sensory analysis results for the fruity aroma, barrel aroma, and flavor intensity yielded notable results. The eigenvectors indicate that Principal Component 1 depends on the total acetates, total esters, fruity aroma sensory parameters, and flavor intensity (see Table 6). On the other hand, Principal Component 2 depends on total phenols, oak compounds, and barrel aroma sensory parameters (see the biplot in Figure 1).

Table 6. The eigenvectors for PC1 and PC2.

| | Prin1 | Prin2 |
|------------------|----------|----------|
| Phenol Sum | −0.21102 | 0.56998 |
| Total Acetates | 0.51338 | 0.03573 |
| Total esters | 0.48203 | 0.27627 |
| “Oak” Compounds | 0.15779 | 0.64906 |
| Fruity Aroma | 0.46956 | −0.14097 |
| Barrel Aroma | 0.18523 | 0.27852 |
| Flavor Intensity | 0.4241 | −0.2807 |

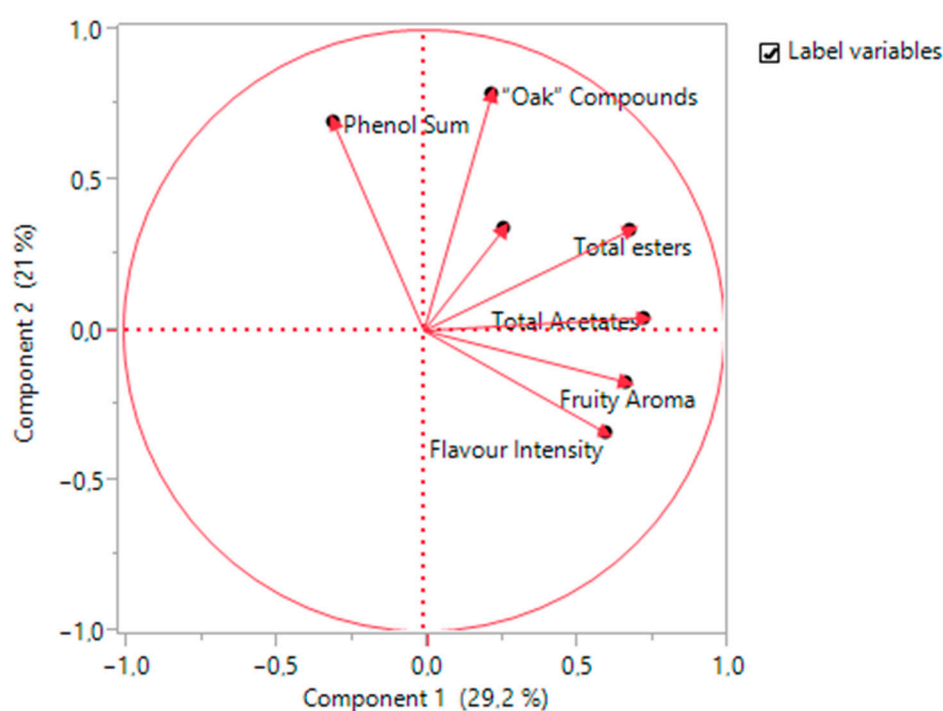


Figure 1. PCA for total acetates, total esters, total phenols, and oak compounds using GC-MS/MS and the sensory analysis results for the fruity aroma, barrel aroma, and flavor intensity.

The correlations among the analytical parameters revealed that total acetates and total esters are more strongly correlated with the sensory parameter of the fruity aroma, while oak compounds are more strongly correlated with the sensory parameter of the barrel aroma (see Table 7).

Table 7. Correlations between the GC-MS/MS and sensory analytical parameters.

| | Phenol Sum | Total Acetates | Total Esters | “Oak” Compounds | Fruity Aroma | Barrel Aroma | Flavor Aroma |
|-----------------|------------|----------------|--------------|--------------------|--------------|--------------|--------------|
| Phenol Sum | 1 | −0.1507 | −0.0656 | 0.3032 | −0.0409 | −0.0114 | −0.3239 |
| Total Acetates | −0.1507 | 1 | 0.6042 | 0.0138 | 0.3627 | 0.0097 | 0.0884 |
| Total esters | −0.0656 | 0.6042 | 1 | 0.2399 | 0.2023 | 0.1194 | 0.0547 |
| “Oak” Compounds | 0.3032 | 0.0138 | 0.2399 | 1 | 0.1123 | 0.2869 | −0.0006 |
| Fruity Aroma | −0.0409 | 0.3627 | 0.2023 | 0.1123 | 1 | −0.1643 | 0.494 |
| Barrel Aroma | −0.0114 | 0.0097 | 0.1194 | 0.2869 | −0.1643 | 1 | 0.3771 |
| Fl Aroma | −0.3239 | 0.0884 | 0.0547 | −0.0006 | 0.494 | 0.3771 | 1 |

The data from the partial least squares (PLS) analysis indicate that total acetates and total esters are strongly associated with the fruity sensory attribute, while oak compounds exhibit a strong association with the barrel sensory attribute (see Table 8).

Table 8. Partial least squares model coefficients for centered and scaled data.

| Coefficient | Phenol Sum | Total Acetates | Total Esters | Oak Compounds |
|--------------|------------|-------------------|-----------------|------------------|
| Fruity Aroma | 0.2683 | 0.5049 | 0.3520 | 0.3753 |
| Barrel Aroma | 0.2388 | 0.1787 | 0.2590 | 0.4881 |

These correlations provide solid evidence that the analytical method using GC-MS/MS produces results analogous to human responses, as acetates and esters are associated with a fruity aroma and oak compounds with a barrel aroma, according to the extensive literature.

The PCA provided significant insights into the relationship between the price of retail wines and the fruity and barrel aromas. Specifically, among the 25 wines analyzed, four samples were located in the +PC1, +PC2 region (upper right quadrant in the PCA, see the biplot in Figure 2). This indicates a higher fruity aroma and higher barrel aroma than the average. The average retail price of these products was EUR 11.1. Five samples were located in the +PC1, −PC2 region (lower right quadrant in the PCA, see the biplot in Figure 2). This indicates a higher fruity aroma and a lower barrel aroma than the average. The average retail price of these products was EUR 10.6. Five samples were located in the −PC1, +PC2 region (upper left quadrant in the PCA, see the biplot in Figure 2). This indicates a lower fruity aroma and a higher barrel aroma than the average. The average retail price of these products was EUR 14.7. Six samples were located in the −PC1, −PC2 region (lower left quadrant in the PCA, see the biplot in Figure 2). This indicates a lower fruity aroma and a lower barrel aroma than the average. The average retail price of these products was EUR 8.5. The remaining five samples were not distinctively placed in any of the four PCA quadrants. These results suggest that the wineries in the Nemea region price the wines characterized by oak volatiles as more expensive than the other wines, probably because these wines were matured in new oak barrels, and thus, the cost of production was higher. This conclusion agrees with other studies conducted on wines from other countries where the fruit and oak character of wine is considered a higher quality indicator by winemakers [2].

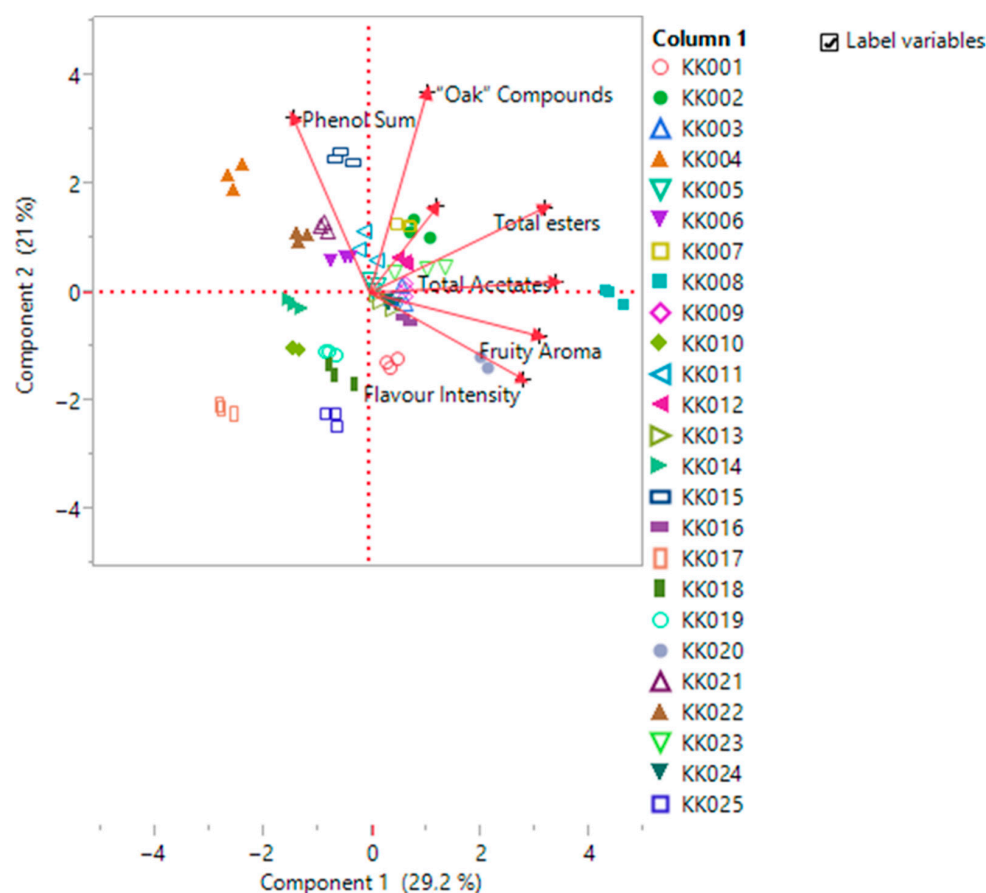


Figure 2. Biplot of PCA for PC1 and PC2.

5. Conclusions

In this study, we developed and validated a new simple method for determining the complex aroma compounds of wine. This analytical method uses sample preparation by means of liquid–liquid extraction using dichloromethane and using phenol as an internal standard. The method employs gas chromatography–tandem mass spectrometry (GC–MS/MS) for the quantification and identification of the volatile compounds. The developed method was validated for 39 aroma compounds.

The validation procedure used criteria such as the linearity, repeatability, reproducibility, recovery, limit of detection, and limit of quantitation. The validation established that the developed method is linear, repeatable, reproducible, accurate, and sensitive.

The method was then applied to determine the complex aroma compounds in 25 commercial wines of the Agiorgitiko variety. The results of the GC–MS/MS and sensory analysis evaluation were correlated by means of PCA. The correlations between the parameters showed that the newly developed method using GC–MS/MS produces similar results to the human response.

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Appendix A

Table A1. List of standards, Chemical Abstracts Service Number (CAS Registry Number), molecular formula, relative formula mass (Mr), purity, and company.

| Name | CAS Registry Number | Molecular Formula | Mr | Purity | Company |
|---------------------------------|---------------------|--|--------|---------------|--------------------------------------|
| 2-Methoxy-4-methylphenol | 93-51-6 | C ₈ H ₁₀ O ₂ | 138.16 | 99.6% | Sigma Aldrich (St. Louis, MO, USA) |
| 2-Methoxy-4-vinylphenol | 7786-61-0 | C ₉ H ₁₀ O ₂ | 150.17 | 99.6% | Sigma Aldrich (St. Louis, MO, USA) |
| 2-Phenethyl acetate | 103-45-7 | C ₁₀ H ₁₂ O ₂ | 164.2 | 99.5% | Honeywell Fluka (Charlotte, NC, USA) |
| 2-Phenylethanol | 60-12-8 | C ₈ H ₁₀ O | 122.16 | 99.3% | Sigma Aldrich (St. Louis, MO, USA) |
| 3-(Methylthio)propionaldehyde | 3268-49-3 | C ₄ H ₈ SO | 104.17 | 97.2% | Sigma Aldrich (St. Louis, MO, USA) |
| 4-Ethylguaiacol | 2785-89-9 | C ₉ H ₁₂ O ₂ | 152.19 | 98.0% | Sigma Aldrich (St. Louis, MO, USA) |
| 4-Ethylphenol | 123-07-9 | C ₈ H ₁₀ O | 122.17 | 99.2% | Acros Organics (Geel, Belgium) |
| 4-Vinylphenol solution 10 wt. % | 2628-17-3 | C ₈ H ₈ O | 120.15 | 96.0% | Sigma Aldrich (St. Louis, MO, USA) |
| Acetovanillone | 498-02-2 | C ₉ H ₁₀ O ₃ | 166.17 | 98.0% | Sigma Aldrich (St. Louis, MO, USA) |
| β-ionone | 79-77-6 | C ₁₃ H ₂₀ O | 192.3 | 97.1% | Honeywell Fluka (Charlotte, NC, USA) |
| Benzyl-acetate | 140-11-4 | C ₉ H ₁₀ O ₂ | 150.17 | 99.9% | Sigma Aldrich (St. Louis, MO, USA) |
| Citral | 5392-40-5 | C ₁₀ H ₁₆ O | 152.23 | 96.0% | Sigma Aldrich (St. Louis, MO, USA) |
| Citronellol | 106-22-9 | C ₁₀ H ₂₀ O | 156.27 | 95.0% | Acros Organics (Geel, Belgium) |
| Damascenone natural | 23696-85-7 | C ₁₃ H ₁₈ O | 190.28 | 1.1–1.4 wt. % | Sigma Aldrich (St. Louis, MO, USA) |
| Decyl aldehyde | 112-31-2 | C ₁₀ H ₂₀ O | 156.27 | 98.5% | Acros Organics (Geel, Belgium) |
| Ethyl 2-methylbutyrate | 7452-79-1 | C ₇ H ₁₄ O ₂ | 130.19 | 99.3% | Acros Organics (Geel, Belgium) |
| Ethyl 3-hydroxybutyrate | 5405-41-4 | C ₆ H ₁₂ O ₃ | 132.16 | 99.6% | Sigma Aldrich (St. Louis, MO, USA) |
| Ethyl butyrate | 105-54-4 | C ₆ H ₁₂ O ₂ | 116.16 | ≥98.0% | Honeywell Fluka (Charlotte, NC, USA) |
| Ethyl caproate | 123-66-0 | C ₈ H ₁₆ O ₂ | 144.21 | 99.7% | Acros Organics (Geel, Belgium) |
| Ethyl caprylate | 106-32-1 | C ₁₀ H ₂₀ O ₂ | 172.26 | 99.2% | Sigma Aldrich (St. Louis, MO, USA) |
| Ethyl cinnamate trans | 103-36-6 | C ₁₁ H ₁₂ O ₂ | 176.21 | 99.7% | Acros Organics (Geel, Belgium) |
| Ethyl decanoate | 110-38-3 | C ₁₂ H ₂₄ O ₂ | 200.32 | 99.7% | Sigma Aldrich (St. Louis, MO, USA) |
| Ethyl dodecanoate | 106-33-2 | C ₁₄ H ₂₈ O ₂ | 228.37 | 99.7% | Sigma Aldrich (St. Louis, MO, USA) |
| Ethyl isobutyrate | 97-62-1 | C ₆ H ₁₂ O ₂ | 116.16 | 99.3% | Honeywell Fluka (Charlotte, NC, USA) |
| Ethyl isovalerate | 108-64-5 | C ₇ H ₁₄ O ₂ | 130.18 | 99.7% | Honeywell Fluka (Charlotte, NC, USA) |
| Ethyl vanillin | 121-32-4 | C ₉ H ₁₀ O ₃ | 166.17 | 97.0% | Acros Organics (Geel, Belgium) |
| Eugenol | 97-53-0 | C ₁₀ H ₁₂ O ₂ | 164.2 | 99.9% | Acros Organics (Geel, Belgium) |
| Geraniol | 106-24-1 | C ₁₀ H ₁₈ O | 154.25 | 99.0% | Acros Organics (Geel, Belgium) |
| Guaiacol | 90-05-1 | C ₇ H ₈ O ₂ | 124.14 | 99.5% | Sigma Aldrich (St. Louis, MO, USA) |
| Hexanal | 66-25-1 | C ₆ H ₁₂ O | 100.16 | ≥97.5% | Sigma Aldrich (St. Louis, MO, USA) |
| Hexylacetate | 142-92-7 | C ₈ H ₁₆ O ₂ | 144.21 | ≥98.5% | Honeywell Fluka (Charlotte, NC, USA) |
| Isoamyl acetate | 123-92-2 | C ₇ H ₁₄ O ₂ | 130.19 | ≥99.0% | Acros Organics (Geel, Belgium) |
| Isobutyl acetate | 110-19-0 | C ₆ H ₁₂ O ₂ | 116.16 | ≥98.5% | Honeywell Fluka (Charlotte, NC, USA) |
| Isoeugenol | 97-54-1 | C ₁₀ H ₁₂ O ₂ | 164.2 | 99.3% | Sigma Aldrich (St. Louis, MO, USA) |
| Linalool | 78-70-6 | C ₁₀ H ₁₈ O | 154.25 | 98.5% | Acros Organics (Geel, Belgium) |
| Rose oxide | 16409-43-1 | C ₁₀ H ₁₈ O | 154.25 | 99.9% | Honeywell Fluka (Charlotte, NC, USA) |
| Thymol | 89-83-8 | C ₁₀ H ₁₄ O | 150.22 | 99.9% | Sigma Aldrich (St. Louis, MO, USA) |
| Vanillin | 121-33-5 | C ₈ H ₈ O ₃ | 152.15 | 99.5% | Acros Organics (Geel, Belgium) |
| Whiskey lactone | 39212-23-2 | C ₉ H ₁₆ O ₂ | 156.22 | 99.4% | Sigma Aldrich (St. Louis, MO, USA) |

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