# Fraud investigation in the extra virgin olive oil supply chain

## Identification of vulnerable points and development of novel fraud detection methods

Jing Yan

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**Jing Yan**

**Thesis**

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# Chapter 1

General introduction

### 1.1. Olive oil

Olives are a typical crop of the Mediterranean region. According to Eurostat data (Eurostat, 2019), olive tree farms are found in eight EU Member States: Spain (2.5 million hectares), Italy (1.1 million hectares), Greece (671 thousand hectares), Portugal (300 thousand hectares), Croatia (17 thousand hectares), France (15 thousand hectares), Cyprus (10 thousand hectares) and Slovakia (1 thousand hectares). In total, the European olive tree farms are slightly under 5 million hectares, more than half of which are in Spain and most of which are devoted to growing olives for olive oil production (Rossi, 2017). The International Olive Council (IOC) reported that world olive oil production is 2.9 million tonnes (t) in the 2017-2018 crop year. The joint production of four European countries (Spain, Italy, Greece and Portugal) takes the lead, which is approximately 1.8 million t (IOC, 2018a). The European Union is the main economy of the consumption of olive oil, which accounts for 53% of the world market, followed by the United States, which accounts for 10% (IOC, 2018b). Furthermore, a rapid increase in the demand for olive oil has been noticed in India, China and Japan.

Olive oil is extracted from olive fruit via a mechanical or chemical process (Ou et al., 2015). It consists mainly of oleic acid and small amounts of other fatty acids (FAs). Furthermore, olive oil is an important constituent of the diet in the Mediterranean countries. It has also been reported to prevent some pathologies, e.g. cardiovascular disease, neurodegeneration, obesity, metabolic syndrome, diabetes and diverse types of cancer (Battino et al., 2019; Borzi et al., 2019; Escrich et al., 2006; Gill et al., 2005; Owen et al., 2004). Therefore, the perceived nutritional value and health benefits of olive oil have also increased its market demand.

### 1.2. Olive oil regulations

The olive oil quality is affected by the planting environment (temperature, location and soil quality), the irrigation practice, the quality of olive fruit (ripeness, cultivar, storage time and mechanical damage) and the processing methods (traditional cold press and refining process) (Boselli et al., 2009). Therefore, Codex (2015) and IOC (2016) have released standards to identify different quality grades of olive oils. In **Figure 1.1**, olive oils and olive pomace oils are classified into six and three grades, respectively. Due to the differences of the acidity values, other physico-chemical and organoleptic characteristics (**Table 1.1 and 1.2**), virgin olive oils, obtained by mechanical means, are classified into four types, including extra virgin olive oil (EVOO), virgin olive oil (VOO), ordinary virgin olive oil (OVOO) and lampante virgin olive oil (LVOO) (Codex, 2015; IOC, 2016). However, LVOO is unfit for consumption (**Figure 1.1**) due to the high levels of acidity, unpleasant flavour and sometimes an unnatural colour. Therefore, to be qualified for consumption, it must be submitted to a series of refining processes (chemical or physical methods). This processed oil is called refined olive oil (ROO). Olive pomace oils are the oils obtained from the olive pomace by chemical or physical treatments

(IOC, 2016). It can be classified into three categories according to the physico-chemical characteristics (**Table 1.1 and 1.2**), including crude olive pomace oil (COPO), refined olive pomace oil (ROPO) and olive pomace oil (OPO).

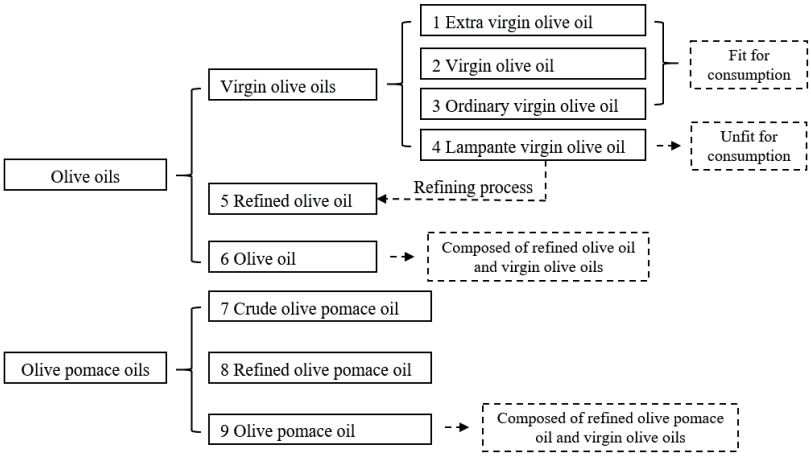


**Figure 1.1.** Categories of olive oils and olive pomace oils based on the level of quality according to Codex and IOC standards.

Concerning the purity and quality criteria of olive oils and olive pomace oils, the national and international standards, including CODEX STAN 33-1981 (Codex, 2015) and those from the IOC (COI/T.15/No3) (IOC, 2016), European Commission (Reg (EEC) 2568/91) (European Commission, 2013), USA Standards (75 FR 22363) (United States Department of Agriculture, 2010), China (ICS 67.200.10) (General Administration of Quality Supervision Inspection and Quarantine, 2009) and Australia (AS 5264-2011) (Australian Standard Committee FT-034, 2011), appear to be slightly different (**Table 1.1 and 1.2**). Generally, the criteria of national standards are stricter than those of the international standards. To take the organoleptic detection of the defect as an example (**Table 1.1**), the threshold values for the median of the defect in olive oils and olive pomace oils of the Australian standard are lower than those of the IOC and Codex standards. Moreover, the threshold peroxide values in olive oils and olive pomace oils of the Chinese standard are half of those of the IOC and EU standards. The K270 value provides useful information on the degree of oxidation of the oil. The higher the K270 value, the higher the oxidation of the oil. In **Table 1.1**, the K270 threshold values of olive pomace oils (≤ 2.00) are higher than those of ROO (1.25), OO (≤ 1.15) and virgin olive oils (≤ 0.30) according to the IOC standard. In **Table 1.2**, the wax values of (E)VOO (≤ 150 mg/kg) is lower than those of OVOO (≤ 250 mg/kg), ROO (≤ 350 mg/kg) and OO (≤ 350 mg/kg). Furthermore, olive pomace oils show the highest values of wax, which is higher than 350 mg/kg. Therefore, in decreasing order of quality and purity according to the six national and international standards referred to above, olive oils can be ranked as follows: EVOO, VOO, OVOO, ROO, OO, ROPO, OPO, COPO and LVOO.

**Table 1.1.** Quality criteria of olive oils and olive pomace oils based on six (inter)national standards.

| Quality Criteria | EVOO [a] | VOO | OVOO | LVOO | ROO | OO | COPO | ROPO | OPO | Standards [b] |
|---|---|---|---|---|---|---|---|---|---|---|
| Median of the fruity attribute (Mf) | > 0 | > 0 | - | - | - | - | - | - | - | All |
| Median of defect (Md) | 0 | ≤ 2.50 | ≤ 6.00 | - | - | - | - | - | - | Codex |
| | 0 | ≤ 3.50 | ≤ 6.00 | > 6.00 | - | - | - | - | - | IOC |
| | 0 | ≤ 3.50 | - | > 3.50 | - | - | - | - | - | EU |
| | 0 | ≤ 2.50 | - | > 2.50 | - | - | - | - | - | USDA/China |
| | 0 | ≤ 2.50 | - | > 2.50 | ≤ 2.50 | ≤ 2.50 | - | ≤ 2.50 | ≤ 2.50 | AUS |
| Free acidity (% m/m expressed in oleic acid) | ≤ 0.80 | ≤ 2.00 | ≤ 3.30 | - | ≤ 0.30 | ≤ 1.00 | - | ≤ 0.30 | ≤ 1.00 | Codex |
| | ≤ 0.80 | ≤ 2.00 | ≤ 3.30 | > 3.30 | ≤ 0.30 | ≤ 1.00 | - | ≤ 0.30 | ≤ 1.00 | IOC |
| | ≤ 0.80 | ≤ 2.00 | - | > 2.00 | ≤ 0.30 | ≤ 1.00 | - | ≤ 0.30 | ≤ 1.00 | EU/USDA/AUS |
| | ≤ 0.80 | ≤ 2.00 | - | > 2.00 | ≤ 0.30 | ≤ 1.00 | - | - | - | China |
| Peroxide value (mEq O2/kg oil) | - | ≤ 20 | - | - | ≤ 5 | ≤ 15 | - | ≤ 5 | ≤ 15 | Codex |
| | ≤ 20 | ≤ 20 | ≤ 20 | - | ≤ 5 | ≤ 15 | - | ≤ 5 | ≤ 15 | IOC |
| | ≤ 20 | ≤ 20 | - | - | ≤ 5 | ≤ 15 | - | ≤ 5 | ≤ 15 | EU/USDA |
| | ≤ 10 | ≤ 10 | - | - | ≤ 2.5 | ≤ 7.5 | - | ≤ 2.5 | ≤ 7.5 | China |
| | ≤ 20 | ≤ 20 | - | > 20 | ≤ 5 | ≤ 15 | - | ≤ 5 | ≤ 15 | AUS |
| Absorbance in ultra-violet ($K_{1cm}^{1\%}$) | | | | | | | | | | |
| $K_{268}$ or $K_{270}$ | ≤ 0.22 | ≤ 0.25 | ≤ 0.30 | - | ≤ 1.10 | ≤ 0.90 | - | ≤ 2.00 | ≤ 1.70 | Codex |
| | ≤ 0.22 | ≤ 0.25 | - | - | ≤ 1.10 | ≤ 0.90 | - | ≤ 2.00 | ≤ 1.70 | EU/USDA/China |
| | ≤ 0.22 | ≤ 0.25 | ≤ 0.30 | - | ≤ 1.25 | ≤ 1.15 | - | ≤ 2.00 | ≤ 1.70 | IOC |
| | ≤ 0.22 | ≤ 0.25 | - | > 0.25 | ≤ 1.10 | ≤ 0.90 | - | ≤ 2.00 | ≤ 1.70 | AUS |
| $K_{232}$ | ≤ 2.50 | ≤ 2.60 | - | - | - | - | - | - | - | Codex/IOC/EU/USDA/China |
| | ≤ 2.50 | ≤ 2.60 | - | ≤ 2.60 | - | - | - | - | - | AUS |
| Delta-K | ≤ 0.01 | ≤ 0.01 | ≤ 0.01 | - | ≤ 0.16 | ≤ 0.15 | - | ≤ 0.20 | ≤ 0.18 | Codex/IOC |
| | ≤ 0.01 | ≤ 0.01 | - | - | ≤ 0.16 | ≤ 0.15 | - | ≤ 0.20 | ≤ 0.18 | EU/USDA/China |
| | ≤ 0.01 | ≤ 0.01 | - | > 0.01 | ≤ 0.16 | ≤ 0.15 | - | ≤ 0.20 | ≤ 0.18 | AUS |
| Moisture and volatile matter (% m/m) | ≤ 0.20 | ≤ 0.20 | ≤ 0.20 | ≤ 0.30 | ≤ 0.10 | ≤ 0.10 | ≤ 1.50 | ≤ 0.10 | ≤ 0.10 | IOC |
| | ≤ 0.20 | ≤ 0.20 | ≤ 0.20 | ≤ 0.20 | ≤ 0.10 | ≤ 0.10 | - | ≤ 0.10 | ≤ 0.10 | Codex |
| | ≤ 0.20 | ≤ 0.20 | - | ≤ 0.30 | ≤ 0.10 | ≤ 0.10 | ≤ 1.50 | ≤ 0.10 | ≤ 0.10 | China/AUS |
| | ≤ 0.20 | ≤ 0.20 | - | - | ≤ 0.10 | ≤ 0.10 | ≤ 1.50 | ≤ 0.10 | ≤ 0.10 | USDA |
| Fatty acid ethyl esters (mg/kg) | ≤ 35 | - | - | - | - | - | - | - | - | IOC |
| | ≤ 30 | - | - | - | - | - | - | - | - | EU |
| Trace metals (mg/kg) | | | | | | | | | | |
| Iron | ≤ 3 | ≤ 3 | ≤ 3 | ≤ 3 | ≤ 3 | ≤ 3 | - | ≤ 3 | ≤ 3 | All |
| Copper | ≤ 0.1 | ≤ 0.1 | ≤ 0.1 | ≤ 0.1 | ≤ 0.1 | ≤ 0.1 | - | ≤ 0.1 | ≤ 0.1 | All |

[a] EVOO, extra virgin olive oil; VOO, virgin olive oil; OVOO, ordinary virgin olive oil; LVOO, lampante virgin olive oils; ROO, refined olive oil; OO, olive oil; COPO, crude olive pomace oil; ROPO, refined olive pomace oil; OPO, olive pomace oil.

[b] Codex = CODEX STAN 33-1981 (Codex, 2015); IOC = COI/T.15/No3 (IOC, 2016); EU = Commission Implementing Regulation (EU) 2019/1604 of 27 September 2019 amending Regulation (EEC) No 2568/91 (European Commission, 2019); US Standards = 75 FR 22363 (United States Department of Agriculture, 2010); China = ICS 67.200.10 (General Administration of Quality Supervision Inspection and Quarantine, 2009); AUS = AS 5264-2011 (Australian Standard Committee FT-034, 2011).

**Table 1.2.** Purity criteria of olive oils and olive pomace oils based on six (inter)national standards.

| Purity Criteria | EVOO [a] | VOO | OVOO | LVOO | ROO | OO | COPO | ROPO | OPO | Standards [b] |
|---|---|---|---|---|---|---|---|---|---|---|
| Wax (mg/kg) | ≤ 150 | ≤ 150 | ≤ 250 | ≤ 300 | ≤ 350 | ≤ 350 | > 350 | > 350 | > 350 | IOC |
| | ≤ 250 | ≤ 250 | ≤ 250 | - | ≤ 350 | ≤ 350 | - | > 350 | > 350 | Codex |
| | ≤ 150 | ≤ 150 | - | ≤ 300 | ≤ 350 | ≤ 350 | > 350 | > 350 | > 350 | EU |
| | ≤ 250 | ≤ 250 | - | ≤ 300 | ≤ 350 | ≤ 350 | > 350 | > 350 | > 350 | China/USDA/AUS |
| Total sterols (mg/kg) | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | - | ≥ 1800 | ≥ 1600 | Codex |
| | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 1000 | ≥ 2500 | ≥ 1800 | ≥ 1600 | EU/IOC/China/USDA/AUS |
| ECN 42 | ≤ \|0.2\| | ≤ \|0.2\| | ≤ \|0.2\| | ≤ \|0.3\| | ≤ \|0.3\| | ≤ \|0.3\| | ≤ \|0.5\| | ≤ \|0.5\| | ≤ \|0.5\| | Codex/China |
| | ≤ \|0.2\| | ≤ \|0.2\| | ≤ \|0.2\| | ≤ \|0.3\| | ≤ \|0.3\| | ≤ \|0.3\| | ≤ \|0.6\| | ≤ \|0.5\| | ≤ \|0.5\| | EU/IOC/USDA/AUS |
| Stigmastadiene (mg/kg) | ≤ 0.15 | ≤ 0.15 | ≤ 0.15 | - | - | - | - | - | - | Codex |
| | ≤ 0.05 | ≤ 0.05 | ≤ 0.05 | ≤ 0.50 | - | - | - | - | - | EU/ |
| | ≤ 0.05 | ≤ 0.05 | ≤ 0.10 | ≤ 0.50 | - | - | - | - | - | IOC/ |
| | ≤ 0.15 | - | - | - | - | - | - | - | - | China |
| | ≤ 0.15 | ≤ 0.15 | ≤ 0.15 | ≤ 0.50 | - | - | - | - | - | USDA |
| | ≤ 0.10 | ≤ 0.10 | ≤ 0.10 | ≤ 0.50 | - | - | - | - | - | AUS |
| Fatty acid composition (% m/m methyl esters) | -Myristic Acid (C14:0) ≤ 0.05<br>-Palmitoleic Acid (C16:1) 0.3 - 3.5<br>-Palmitic Acid (C16:0) 7.5 - 20.0<br>-Heptadecanoic Acid (C17:0) ≤ 0.3<br>-Heptadecenoic Acid(C17:1) ≤ 0.3<br>-Oleic Acid (C18:1) 55.0 - 83.0<br>-Stearic Acid (C18:0) 0.5 - 5.0<br>-Linoleic Acid (C18:2) 3.5 - 21.0<br>-Linolenic Acid (C18:3) ≤ 1.5<br>-Arachidic Acid (C20:0) ≤ 0.6<br>-Gadoleic Acid (C20:1) ≤ 0.4<br>-Behenic Acid (C22:0) ≤ 0.2<br>-Lignoceric Acid (C24:0) ≤ 0.2 | | | | | | | | | All |
| Trans fatty acids (% m/m) | ≤ 0.05 | ≤ 0.05 | ≤ 0.05 | - | ≤ 0.20 | ≤ 0.20 | - | ≤ 0.40 | ≤ 0.40 | Codex/China |
| C18:1 T | ≤ 0.05 | ≤ 0.05 | ≤ 0.05 | 0.10 | ≤ 0.20 | ≤ 0.20 | 0.20 | ≤ 0.40 | ≤ 0.40 | EU/IOC/USDA/AUS |
| Trans fatty acids (% m/m) | ≤ 0.05 | ≤ 0.05 | ≤ 0.05 | - | ≤ 0.30 | ≤ 0.30 | - | ≤ 0.35 | ≤ 0.35 | Codex/China |
| C18:2 T + C18:3 T | ≤ 0.05 | ≤ 0.05 | ≤ 0.05 | 0.10 | ≤ 0.30 | ≤ 0.30 | 0.10 | ≤ 0.35 | ≤ 0.35 | EU/IOC/USDA/AUS |
| 2-glyceryl monopalmitate (% m/m) C16:0 ≤ 14% | ≤ 0.9 | ≤ 0.9 | ≤ 0.9 | ≤ 0.9 | ≤ 0.9 | ≤ 0.9 | ≤ 1.4 | ≤ 1.4 | ≤ 1.2 | IOC |
| | ≤ 0.9 | ≤ 0.9 | - | ≤ 0.9 | - | - | | | | USDA |
| 2-glyceryl monopalmitate (% m/m) C16:0 > 14% | ≤ 1.0 | ≤ 1.0 | ≤ 1.0 | ≤ 1.1 | ≤ 1.1 | ≤ 1.0 | ≤ 1.4 | ≤ 1.4 | ≤ 1.2 | IOC |
| | ≤ 1.0 | ≤ 1.0 | - | ≤ 1.1 | - | - | | | | USDA |
| 2-glyceryl monopalmitate (% m/m) | ≤ 1.5 | ≤ 1.5 | ≤ 1.5 | ≤ 1.5 | ≤ 1.8 | ≤ 1.8 | ≤ 2.2 | ≤ 2.2 | ≤ 2.2 | AUS |
| Unsaponifiable matter content (g/kg) | ≤ 15 | ≤ 15 | ≤ 15 | ≤ 15 | ≤ 15 | ≤ 15 | ≤ 30 | ≤ 30 | ≤ 30 | Codex/IOC/China/USDA |

[a] EVOO, extra virgin olive oil; VOO, virgin olive oil; OVOO, ordinary virgin olive oil; LVOO, lampante virgin olive oils; ROO, refined olive oil; OO, olive oil; COPO, crude olive pomace oil; ROPO, refined olive pomace oil; OPO, olive pomace oil.

[b] Codex = CODEX STAN 33-1981 (Codex, 2015); IOC = COI/T.15/No3 (IOC, 2016); EU = Commission Implementing Regulation (EU) 2019/1604 of 27 September 2019 amending Regulation (EEC) No 2568/91 (European Commission, 2019); US Standards = 75 FR 22363 (United States Department of Agriculture, 2010); China = ICS 67.200.10 (General Administration of Quality Supervision Inspection and Quarantine, 2009); AUS = AS 5264-2011 (Australian Standard Committee FT-034, 2011).

## 1.3. Olive oil processing

The major difference between EVOO and lower grade olive oils (ROO and POO, including COPO, ROPO and OPO in this study) is mainly due to the extraction process. The extraction process can be divided into three typical extraction methods, including mechanical extraction, olive pomace extraction and refining process (Gunstone, 2011; Peri, 2014). The effect of the different extraction methods on the quality and stability of olive oils has been investigated.

### *1.3.1. Mechanical extraction*

**Figure 1.2** shows the four main steps of the mechanical extraction process, including washing, crushing, malaxation and pressing/centrifugation, which is a slight modification of the extraction method of Gunstone (2011).

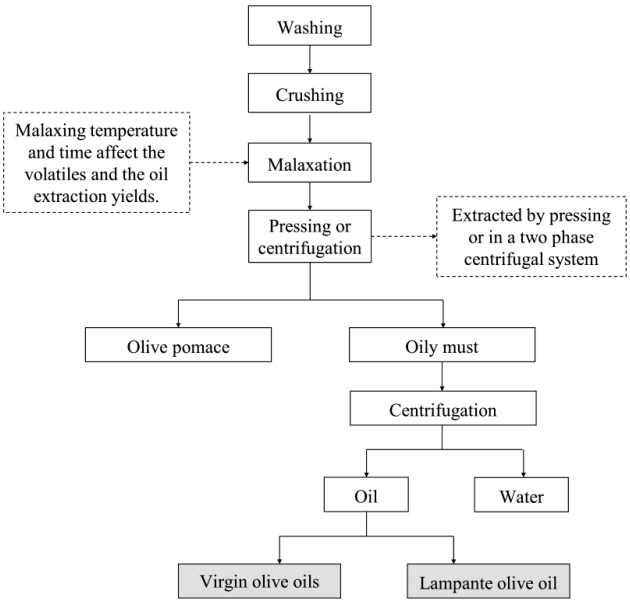


**Figure 1.2.** Flow chart for olive oil mechanical extraction (adapted from Gunstone (2011)).

Previous studies have reported that the olive oil extraction affects the quality of virgin olive oils, especially the organoleptic characteristics (Gunstone, 2011; Ranalli & Serraiocco, 1996). To provide hygienic oils, the washing operation removes all the contaminants, including dust, soil, sand, stone fragments and any mineral or metallic contaminants (Di Giovacchino et al., 2002b). Crushing increases the breakage of olives, which in turn improves the extraction yield of olive oil. It can be done using granite millstones or metallic crushers. However, these

two methods result in different quality of oils. The content of total phenolic compound in the oil obtained by a metallic crusher is higher than that obtained by the granite millstones (Di Giovacchino et al., 2002b). Whereas the application of the granite millstones contributes to a high concentration of total volatile compounds (Angerosa & Di Giacinto, 1995).

With regard to the malaxation treatment (slowly churning milled oil crops), the temperature and time of malaxation will affect the final quality of oils. High temperature generally increases phenol contents (Fregapane & Salvador, 2013) and decreases volatile content (Ranalli et al., 2001). On the other hand, a longer malaxation time results in relatively low phenol contents and high contents of oil volatiles (Angerosa et al., 2001; Di Giovacchino et al., 2002a). Furthermore, the increasing of the malaxation temperature or time contributes to the oil yield and the effect of temperature is larger than that of time (Inarejos-Garcia et al., 2009). In addition, oils obtained by a two-phase continuous centrifugation system exhibited a higher content of total volatile compounds (Ranalli & Serraiocco, 1996).

Overall, the above indicate that the crushing, malaxation and separation methods of the mechanical extraction can affect the quality of virgin olive oils and especially the organoleptic properties.

### *1.3.2. Olive pomace extraction*

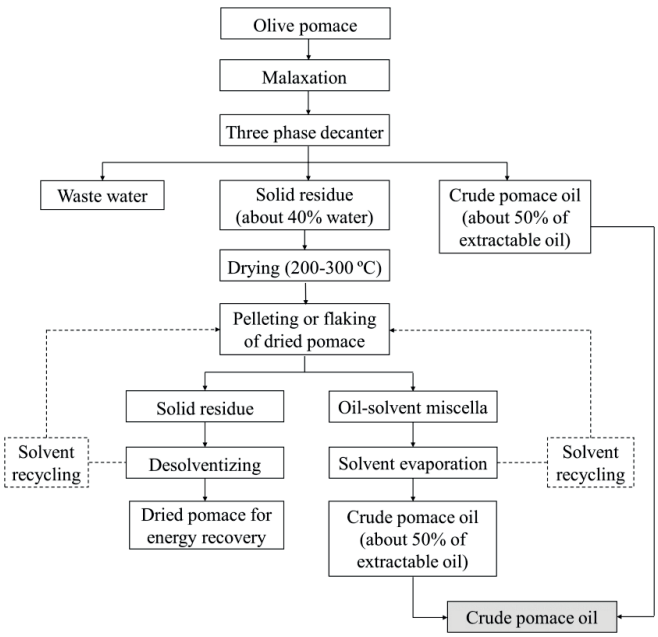


**Figure 1.3.** Flow chart for crude olive pomace oil extraction (adapted from Peri (2014)).

Generally, olive pomace contains on average 5-8% of residual oil after mechanical extraction (Gogus & Maskan, 2006). The major extraction operations to obtain COPO are shown in **Figure 1.3** (Peri, 2014), but the extraction operations were slightly modified.

Peri (2014) reported that about half of the residual oil in olive pomace can be extracted by malaxation and three-phase decanter separation, another half of the residual oil can be obtained by solvent extraction. Drying of pomace and pelleting/flaking of dried pomace are operated to increase the efficiency of the solvent extraction. Subsequently, the crude olive pomace oil is obtained using n-hexane, which is generally used as a good extraction solvent for oil. Ultimately, COPO has higher contents of unsaponifiable substances and free FAs due to the drying and the application of the solvent in comparison to other olive oils (Gogus & Maskan, 2006).

### *1.3.3. Refining process*

The COPO contains higher levels of unsaponifiable substances and free FAs than other olive oils, due to the treatments of drying and the application of the solvent (Gogus & Maskan, 2006). Moreover, COPO is not an edible vegetable oil and can only be used for soap production or other industrial application. Therefore, both COPO and LVOO must be refined to become edible. In fact, EVOO is only a minority part of olive oil production and olive oil refining is a very important sector of the food industry for economic gain (Antonopoulos et al., 2006). **Figure 1.4** presents the refined olive oil extraction process. Five major operations (settling, neutralisation, winterisation, bleaching and deodorisation) and their removed substances of each step are listed.

**Settling.** Generally, the filtering treatment of the solvent extraction makes the COPO clear. Therefore, only LVOO needs settling to get rid of cloudiness and sediment (Peri, 2014). After settling for a minimum of four weeks at a constant temperature (20-25 °C), phosphatides, moisture, impurities and some waxes can be removed (Antonopoulos et al., 2006).

**Neutralisation.** This operation aims to eliminate the free FAs, phosphatides, chlorophyll pigments, pesticides, aromatic hydrocarbons and metals (Antonopoulos et al., 2006). To achieve this goal, more than 10-20 % of the solvents need to be added to the oil at a suitable temperature (65-90 °C). These solvents include sodium hydroxide, potassium hydroxide or calcium hydroxide (Antonopoulos et al., 2006; Peri, 2014).

**Winterisation.** The objective of this treatment is to remove any compound that might cause a cloudy or murky oil, it mainly eliminates waxes and saturated triacylglycerols (Ruiz-Méndez et al., 2013). It is carried out by cooling down the oil at 5-8 °C and it results in crystallisation in 24-48 h.

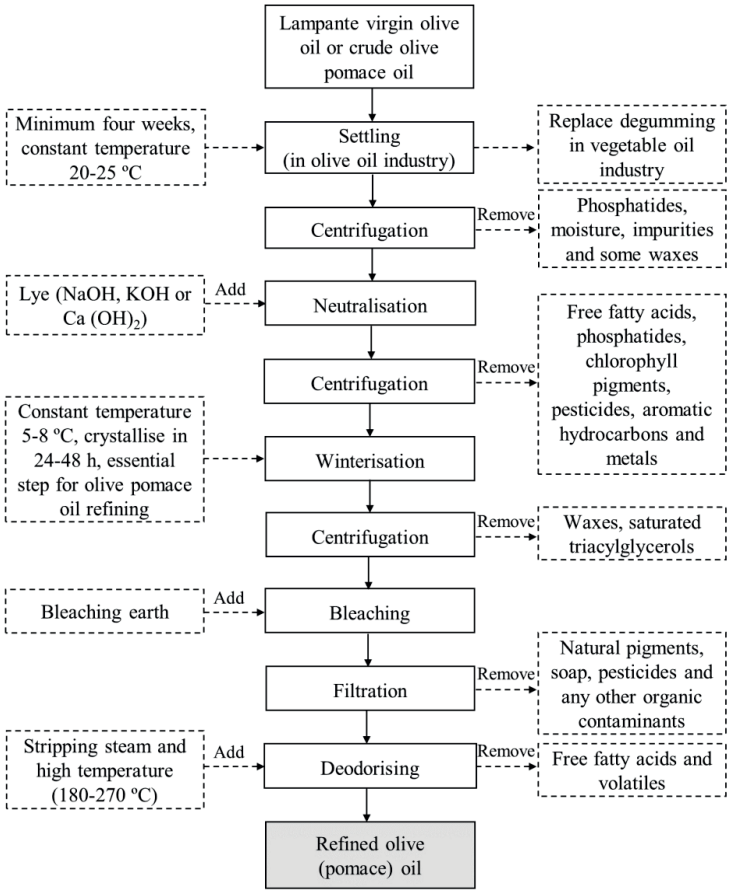


**Figure 1.4.** Flow chart for refined olive oil extraction (adapted from Peri (2014)).

**Bleaching.** This operation mainly aims to remove natural pigments such as carotene, xanthophyll and chlorophyll. In addition, soaps, pesticides and any other organic contaminants are eliminated as well (Antonopoulos et al., 2006). To achieve this goal, 0.5-1.5% of bleaching earth is added to the oil, at a temperature of 90-110 °C for about half an hour, (Peri, 2014).

**Deodorisation.** Deodorisation allows for removing undesirable odours and free FAs. It is carried out by distilling under vacuum with stripping gas at a temperature of 180-270 °C. Subsequently, the oil is cooled down under vacuum to prevent it from deterioration (Ruiz-Méndez et al., 2013).

The refining process aims to eliminate the undesirable components and to extend the shelf life of oils (Fragaki et al., 2005). This operation produces an edible oil with bland flavour and odour, light colour, stability to oxidation and is suitable for frying. However, the refining procedure not only eliminates the unpleasant flavour, but also removes the substances that

contribute to the healthy properties of olive oils. Furthermore, some special compounds will still exist after the refining process. For instance, the absence of cis-phytol after the refining process can be used as a marker for non-refined edible oils (Vetter et al., 2012). The concentration of fatty acid alkyl esters has been used to distinguish EVOO from mildly refined low quality olive oil (Caponio et al., 2011). Furthermore, the content of stigmasta-3,5-diene (formed from β-sitosterol) is strongly dependent on the temperature used during the deodorisation operation (Leon-Camacho et al., 2004). Therefore, studies have proposed stigmasta-3,5-diene, which does not exist in EVOO, as a potential marker of EVOO/ROO blending (Leon-Camacho et al., 2004).

### 1.4. Olive oil fraud incidents

Food fraud has gained tremendous recognition and concern in recent years. Since the beginning of the new millennium, many food fraud scandals have emerged, such as the addition of Sudan I, which is a genotoxic and carcinogenic compound, to chilli products to enhance the colour and improve the appearance of the product (EU, 2003); the addition of melamine to infant formula, which resulted in illness and death (China Daily, 2008); the addition of horsemeat in beef, which resulted in a crisis of consumer trust, reduction of red meat consumption and boycotting of beef products (O'Mahony, 2013; Stanciu, 2015); and the contamination of eggs by fipronil, which resulted in a serious economic shock to the poultry industry in Europe and heightened both public concern regarding the use of fipronil (van der Merwe et al., 2019). These scandals caused a large number of problems, such as damage to the reputation of food companies, the collapse of food markets, erosion of consumer confidence, management and/or employees being fired, prosecutions and imprisonments (Silvis et al., 2017). Furthermore, due to the globalisation of the production and distribution of food, food fraud incidents have a global impact (Spink & Moyer, 2011; Spink et al., 2017).

Olive oil has particular organoleptic and nutritional properties, which allows olive oil access to a relatively high price market compared with other vegetable oils (European Commission, 2012). Therefore, it is considered as one of the most frequently reported fraudulent commodities based on the three global food fraud databases over the period 2008-2013 (Weesepoel & van Ruth, 2015). In some cases, besides economic fraud, olive oil fraud shows a direct link to public health problems and even deaths. In Morocco in 1959, the adulteration of olive oil with lubricating oil resulted in 10,000 people ill (Travers, 1962). In Spain in 1981, over 20,000 people were poisoned by the consumption of denatured rapeseed oil labelled as olive oil (Lipp, 2012; Mueller, 2011; Wood et al., 1994).

EVOO has recently gained in popularity due to its premium quality (**section 1.2**) and reputation as a healthy and delectable oil, which makes EVOO prone to adulterations. There are three typical methods of EVOO fraud (Yan et al., 2018): 1) the blending with less costly

vegetable oils; 2) the replacement with vegetable oil with addition of chemical compounds (e.g. chlorophyll); 3) the replacement with lower olive oil grades, which are ROO and POO. EVOO fraud incidents happened frequently in recent years, especially in Mediterranean countries, which are the leading producers of olive oil. Furthermore, the adulteration of EVOO with lower grade olive oils is one of the most common adulteration practices. For instance, several mislabelled EVOO were reported as blended with ROO or POO to increase profits (Aguilera et al., 2005). In 2007, an Italian operation led to the arrest of 33 suspects who labelled olive pomace oil as EVOO and exported it to the US (Smith, 2017). In 2015, Italian police investigated top Italian olive oil producers for allegedly passing off lower-quality products as "100% extra virgin" (Squires, 2015).

### 1.5. Olive oil fraud prevention

In order to control the risk of food fraud and prevent food fraud incidents, food fraud prevention must be a prioritised enterprise risk control method (Spink et al., 2016a). To build an optimal food fraud prevention system, a focus on prevention based on a review of the fraud opportunity is needed (Spink et al., 2016a). Therefore, food fraud vulnerability assessments (FFVA) are an essential activity to prevent food fraud. FFVA can be employed to identify the systems' weaknesses or flaws that create opportunities for undesirable incidents (Spink et al., 2017).

Several FFVA tools, including Vulnerability Assessment and Critical Control Point, NSF Fraud Protection Model (NSF, 2014), USP Food Fraud Mitigation Guidance (USP, 2016), Food Fraud Initial Screening (FFIS) model (Spink et al., 2016b), CARVER + Shock tool (FDA, 2009) and SSAFE FFVA tool (SSAFE, 2016), have been established to help companies and regulators anticipate the fraud vulnerability in the food supply chain. **Table 1.3** shows the comparison of these six FFVA tools. Specific vulnerability assessment tools were developed to meet specific needs in terms of the aim of each tool. Some of the tools intended to think like a criminal and to characterise the most attractive or vulnerable targets for food fraud. Specifically, FFIS as an early-stage model could give a quick fraud vulnerability assessment, but only broad vulnerabilities can be identified (Spink et al., 2016b). Therefore, this initial screening model will be followed by another FFVA tool to identify the specific vulnerabilities, such as the SSAFE or USP FFVA. With regard to the mechanisms, compared to other tools, the SSAFE tool gives more details of the target by using 50 indicators and it is the only science-based tool (based on the study of criminal behaviour and criminal decision-making (PwC, 2015)). Moreover, the scope of the application of these FFVA tools are different. Therefore, it is important to choose an appropriate tool for FFVA, which will affect the ability to quantify the likelihood of a vulnerability in a given situation.

**Table 1.3.** Comparison of six food fraud vulnerability assessment tools (adapted from Manning and Soon (2016)).

| | Aims | Mechanisms | Scope of application |
|---|---|---|---|
| Vulnerability assessment and critical control point (VACCP) (Manning & Soon, 2016) | To assess how exposed organisation or premise is to food fraud incidents. | Qualitative assessments (likelihood × impact) of threats. | It can be applied in the wider supply chain. |
| NSF fraud protection model (NSF, 2014) | To help organisations 'think like a criminal'.<br>To provide a clear visual representation of relative product risk, provide a pro-active approach to anticipating the potential for fraud. | Built on a four quadrant Boston Consulting Group matrix.<br>Top right – products most attractive to fraudster.<br>Bottom left – products least attractive to fraudster.<br>Size of the circle – perceived difficulty of undertaking the particular fraud. | The model delivers a working framework to food companies and regulators to better anticipate which product lines are most/least likely to be targeted by fraudsters and why, whether they have been attacked previously. |
| USP Food fraud mitigation guidance (USP, 2016) | To help users develop and implement their own fraud management system to deal specifically with intentional EMA of food ingredients. | Four steps:<br>1-3) Characterise the overall fraud vulnerabilities of an ingredient by the assessing nine factors contributing to fraud occurrence and five potential impacts when fraud does occur.<br>4) Provide guidance on how to use the outcome of the first three steps to develop a mitigation strategy. | Can be applied by any user responsible for ensuring the integrity of food ingredients. |
| Food fraud initial screening model (FFIS) (Spink et al., 2016b) | To quickly review the broad risks. | Four process steps:<br>1) Define the scope and basic terms.<br>2) Review incidents and suspicious activity.<br>3) Conduct the FFIS for health hazards and enterprise-wide risks.<br>4) Plot the Food Fraud risks on the Corporate Risk Map. | Can be applied across all food risks, all product risks and across all enterprise-wide activities. |
| CARVER + Shock tool (FDA, 2009) | To think like an attacker by identifying the most attractive targets for attack.<br>To assess the vulnerabilities within a system or infrastructure to an attack. | Based on seven attributes which are scored on a scale of 1 to 10. Lower vulnerability – lower values (e.g., 1 or 2), whereas, higher vulnerability – higher values (e.g., 9 or 10).<br>1) Criticality - measure of public health and economic impacts of an attack.<br>2) Accessibility – ability to physically access and egress from target.<br>3) Recuperability – ability of system to recover from an attack.<br>4) Vulnerability – ease of accomplishing attack.<br>5) Effect – amount of direct loss from an attack as measured by loss in production.<br>6) Recognisability – ease of identifying target.<br>7) Shock – the combined health, economic and psychological impacts of an attack. | Can be applied to evaluate the potential vulnerabilities of various food supply chains and assess the potential vulnerabilities of individual facilities or processes. |
| SSAFE food fraud vulnerability assessment tool (SSAFE, 2016; van Ruth et al., 2017) | To think like a criminal to fight food fraud and to identify the vulnerable points in the supply chain. | Based on 50 indicators which are ranked low, medium or high vulnerability. These 50 indicators can be classified into three elements:<br>1) Opportunities (suitable target).<br>2) Motivations (motivated offender).<br>3) Control measures (guardianship). | It can help food companies to perform a vulnerability assessment and prepare a control plan to reduce food fraud risks. It can be applied to different products, business sizes and regions. |

The free online SSAFE FFVA tool aims to strengthen internal controls of companies and reduce opportunities and motivations to adulterate food (Food Safety Magazine, 2016). It provides guidance on how to use this tool. Therefore, people who may not have specific knowledge on food fraud and vulnerability could also use it smoothly (Manning & Soon, 2019). So far, it has been successfully applied to assess the fraud vulnerability in the spices supply chain (Silvis et al., 2017), the Dutch milk supply chain (Yang et al., 2019) and five other food supply chains (van Ruth et al., 2018).

## 1.6. Olive oil fraud detection methods

A food fraud prevention system and mitigation strategies can be applied to prevent food fraud, whereas, potential detection techniques are applied to find/discover food fraud and to limit its impact. As mentioned in **section 1.4**, there are three typical types of EVOO fraud. For each of these variants, analytical detection methods have been developed. The analytical tools can be divided into three groups, which are (a) confirmatory techniques, (b) laboratory-based screening techniques and (c) handheld screening techniques (**Table 1.4**).

### *1.6.1. Confirmatory techniques*

EVOO authentication is mainly based on chemical properties by using chromatography techniques, including high-resolution gas chromatography (GC) and high-performance liquid chromatography (HPLC), which are also classical approaches to identify EVOO adulteration. For the chemical characterisation, major and minor components in oils are analysed using appropriate analysis techniques.

Triacylglycerols (TAGs), as the most abundant group of compounds (Velasco & Dobarganes, 2002), have been applied for the discrimination of EVOO from other vegetable oils using HPLC. For instance, HPLC-atmospheric pressure chemical ionisation-tandem mass spectrometry was applied to analyse the TAGs for the detection of olive oil adulteration with soybean oil (Fasciotti & Netto, 2010). Furthermore, it was found that the more saturated TAGs (OOO, POO and SOO. Where P, palmitoyl; S, stearoyl; O, oleoyl) are predominant in olive oil and less saturated TAGs (LLnLn, LLLn, LLL+OLLn and OLL+OOLn. Where O, oleoyl; L, linoleoyl; Ln, linonenoyl) mainly originate from soybean oil (Fasciotti & Netto, 2010). Elhamdy and Elfizga (1995) also reported that the addition of less than 1% of linoleic-rich vegetable oils to olive oil was detected qualitatively and quantitatively by reversed-phase HPLC.

Apart from the major compounds, minor compounds (phospholipids, phenolic, wax, squalene, sterols, chlorophylls, carotenoids and metals) can also be used as effective biomarkers to evaluate the authenticity of EVOO. For instance, liquid chromatography-mass spectrometry (LC-MS) has been used for the quantification of squalene in oils, the amount of squalene in hazelnut oil is around 20 times lower than in olive oil (Benitez-Sanchez et al., 2003). Carbon

stable isotopes analysed by high resolution GC were used to identify olive oil adulteration with the cheaper pomace olive oil at a concentration lower than 5% (Angerosa et al., 1997). Furthermore, GC-MS was applied to analyse 4,4-dimethylsterols for the detection of EVOO adulteration with hazelnut oil at a concentration less than 4% (Damirchi et al., 2005).

In total, chemical analysis has been considered as a powerful and useful tool to conduct qualitative and quantitative analyses of complex and plant-derived biomarker compounds (Aparicio & Aparicio-Ruiz, 2000; Flores et al., 2006). Generally, the methods of conventional analysis are complex (tedious chemical treatment), time-consuming and expensive. Therefore, there is a trend to develop rapid and non-destructive methods for EVOO authentication.

### *1.6.2. Laboratory based screening techniques*

To increase efficiency and to save time, a variety of screening techniques and chemometric procedures have emerged as powerful tools for the authentication of EVOO with the advantages of non-destructive, rapidness, high-throughput and sensitivity test. Among these rapid, non-destructive screening techniques, infrared (IR) techniques could be considered as widely used tools for EVOO authentication. **Table 1.4** shows that near infrared spectroscopy (NIR) (Kasemsumran et al., 2005), Fourier transform-NIR (FT-NIR) (Azizian et al., 2015), mid-IR (MIR) (Gurdeniz & Ozen, 2009) and FT-MIR (Rohman et al., 2017) have been successfully applied to discriminate EVOO from other vegetable oils. Moreover, they can detect EVOO adulteration with other vegetable oils at levels above 10%. Raman spectroscopy is another widely used screening technique which could distinguish EVOO from lower grade olive oils (Yang & Irudayaraj, 2001; Zou et al., 2009). Except for these two techniques, there are several screening techniques that can also be used for EVOO authentication, including nuclear magnetic resonance (Fragaki et al., 2005), ultraviolet-visible spectroscopy (Torrecilla et al., 2010), synchronous fluorescence spectroscopic (Kunz et al., 2011), electrospray ionisation-MS (Alves et al., 2014), direct analysis in real time-time of flight-MS (Vaclavik et al., 2009), differential scanning calorimetry (Karbasian et al., 2015), real-time polymerase chain reaction (Kumar et al., 2011), headspace-MS (Pena et al., 2005), proton transfer reaction-MS (Araghipour et al., 2008), proton transfer reaction-time of flight-MS (Taiti & Marone, 2017), electronic-nose systems (Oliveros et al., 2002) and electronic-tongue systems (Apetrei & Apetrei, 2014).

**Table 1.4.** Representative examples of three types of analytical approaches for olive oil authentication.

| Analytical approaches | Parameter [a] | Instrumental measurement [b] | Data treatment [c] | Main finding [d] | Reference |
|---|---|---|---|---|---|
| Confirmatory techniques | TAG | HPLC-APCI-MS/MS | PCA | Detection commercial blend containing 15% (w/w) of olive oil in soybean oil | (Fasciotti & Netto, 2010) |
| | TAG | RP-HPLC | - | Addition of less than 1% of linoleic-rich vegetable oils to olive oils can be detected easily | (Elhamdy & Elfizga, 1995) |
| | FAME | GC | ANOVA | Discrimination between EVOO and other vegetable oils | (IOC, 2017a) |
| | FAAE | GC-FID | ANOVA | Differentiate EVOO and mildly deodorised olive oils | (Jabeur et al., 2015) |
| | Wax esters | GC | ANOVA | Differentiate EVOO from OPO | (IOC, 2003) |
| | Stigmastadienes | GC-FID | ANOVA | Successful differentiate EVOO from ROO and OPO | (IOC, 2017c) |
| | TAG + ΔECN42 | HPLC | ANOVA | Differentiate seed oils from olive oils | (IOC, 2017b) |
| | 4,4-Dimethylsterols | GC-MS | ANOVA | Detection of EVOO adulteration with hazelnut oil at concentration less than 4% | (Damirchi et al., 2005) |
| | 2-Glyceryl monopalmitate | GC | ANOVA | Differentiate EVOO from esterified oils | (IOC, 2017d) |
| | Diacylglycerol | GC-FID | ANOVA | Detection of VOO adulteration with mild refined oil | (Perez-Camino et al., 2001) |
| | Isobaric lignans | HPLC-DAD-ToF/MS | ANOVA | Differentiate EVOO from ROO, OPO | (Cecchi et al., 2017) |
| | Carbon stable isotopes | HRGC | ANOVA | Detection of olive oil adulteration with the cheaper pomace olive oil at concentration lower than 5% | (Angerosa et al., 1997) |
| | Tocopherols | HPLC | ANOVA | The ratio of a-/(b+c)-tocopherol concentration proposed as a screening marker of the authenticity of EVOO | (Chen et al., 2011) |
| | Squalene | HPLC-MS | ANOVA | Differentiate olive oils from hazelnut oil, the amount of squalene in hazelnut oil is around 20 times lower than in olive oils | (Benitez-Sanchez et al., 2003) |
| Laboratory based screening techniques | Dielectric properties | LCR meter | PCA, PLS | Discriminate the olive oils adulterated with other vegetable oils at levels below 5% | (Hu et al., 2010) |
| | Acoustic properties | Ultrasonic Interferometer | - | Assessment the EVOO adulteration in vegetable oils | (Kumari et al., 2017) |
| | - | NIR | PCA, PLS-DA | Rapid discrimination and quantification of olive oils adulteration with vegetable oils | (Kasemsumran et al., 2005) |
| | - | FT-NIR | PLS | Prediction of EVOO FA composition with satisfactory accuracy and identification of the kind and amount of adulterant vegetable oils in EVOO | (Azizian et al., 2015) |
| | - | MIR | PCA, PLS-DA | Detection adulteration of EVOO with other types of vegetable oils. Detection limit for adulteration is determined as 10% | (Gurdeniz & Ozen, 2009) |
| | - | FT-MIR | DA, PLS, PCR | Successfully classify EVOO and EVOO adulterated with grape seed oil, soybean oil and walnut oil | (Rohman et al., 2017) |
| | - | Raman | PCA | Distinguish the genuine olive oils from the olive oils containing 5% or more of other edible oils, such as soybean oil, rapeseed oil, sunflower seed oil, or corn oil | (Zou et al., 2009) |
| | - | FT-Raman | PLS | Rapid determination of adulteration in EVOO with OPO | (Yang & Irudayaraj, 2001) |
| | $^{31}$P NMR spectra | NMR | HCA, DA | Detection of EVOO adulteration as low as 5% w/w for refined and lampante olive oil | (Fragaki et al., 2005) |
| | - | UV-Vis | PLS | Detection of EVOO adulteration with the lower grade olive oils at concentration less than 10% | (Torrecilla et al., 2010) |
| | - | Synchronous fluorescence spectroscopic | PLS | Detection of EVOO adulteration with sunflower oil | (Kunz et al., 2011) |
| | - | ESI-MS | PLS | Rapid quantify adulteration in EVOO with inexpensive vegetable oils (soybean, corn, sunflower and canola oils) | (Alves et al., 2014) |
| | - | MALDI-ToF MS | - | Detection of EVOO adulteration with hazelnut oil by analysis of phospholipids | (Calvano et al., 2012) |
| | Polar compounds | DART-ToF MS | LDA | Detection of EVOO adulteration with above 6% of hazelnut oil | (Vaclavik et al., 2009) |
| | Thermal properties | DSC | - | Differentiate EVOO from ROO | (Karbasian et al., 2015) |
| | DNA | Real-time PCR | ANOVA | Rapid detection of EVOO adulteration with above 5% of canola or sunflower oil | (Kumar et al., 2011) |
| | Volatile compounds | Headspace-MS | SIMCA, PLS, CA, PCR | Successful identify the adulteration of 7 and 15% hazelnut oil in ROO and VOO, respectively | (Pena et al., 2005) |
| | Volatile compounds | PTR-MS | PLS-DA | Geographical origin classification of olive oils | (Araghipour et al., 2008) |
| | Volatile compounds | PTR-ToF-MS | PLS-DA | Discriminate EVOO from non-EVOO | (Taiti & Marone, 2017) |
| | Volatile compounds | Electronic nose | LDA, QDA, ANN | Fast detection of adulteration of VOOs | (Oliveros et al., 2002) |
| | Sensory properties | Electronic tongue | PLS-DA, PLSR | Rapid detection of EVOO adulterations with percentages lower than 10% of sunflower oil, soybean oil and corn oil | (Apetrei & Apetrei, 2014) |

| Analytical approaches | Parameter [a] | Instrumental measurement [b] | Data treatment [c] | Main finding [d] | Reference |
|---|---|---|---|---|---|
| Handheld screening techniques | - | 473-nm Laser-Induced Fluorescence | PCA, SVM, ANN, PLSR | Successfully differentiate olive oils from rapeseed, peanut and blend oils | (Mu et al., 2016) |
| | - | Portable E-nose | SVM | Detection of EVOO adulterations with sunflower oil | (Wojnowski et al., 2017) |
| | - | Handheld microPHAZIR NIR | PCA, SIMCA | Commercial EVOO authenticity: Out of 88 commercial products labelled EVOO, 39 (44%) were classified as belonging to the class of authentic EVOO to the class of authentic EVOO | (Karunathilaka et al., 2017) |
| | - | Portable Raman | PLSR | Rapid and accurate prediction sunflower adulteration in EVOO | (Tiryaki & Ayvaz, 2017) |

[a] TAG, triacylglycerol; FAME, fatty acid methyl esters, FAAE, fatty acid alkyl esters.

[b] HPLC-APCI-MS/MS, high-performance liquid chromatography-atmospheric pressure chemical ionisation-tandem mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography; FID, flame ionisation detector; GC-MS, gas chromatography-mass spectrometry; HRGC, high resolution gas chromatography; LCR, inductance, capacitance and resistance; FT-NIR, Fourier transform near-infrared spectroscopy; FT-MIR, Fourier transform mid-infrared spectroscopy; NMR, nuclear magnetic resonance; UV-Vis, ultraviolet-visible spectroscopy; ESI-MS, electrospray ionisation-mass spectrometry; MALDI-ToF MS, matrix assisted laser desorption/ionisation-time of flight mass spectrometry; DART-ToF MS, direct analysis in real time-time of flight mass spectrometry; DSC, differential scanning calorimetry; PCR, polymerase chain reaction; PTR-ToF MS, Proton Transfer Reaction-time of flight mass spectrometry.

[c] ANN, artificial neural network; ANOVA, analysis of variance; CA, cluster analysis; HCA, hierarchical cluster analysis; LDA, linear discriminant analysis; PCA, principal component analysis; PLS-DA, partial least squares discriminant analysis; PLSR, partial least squares regression; PCR, principal component regression; QDA, quadratic discriminant analysis; SIMCA, soft independent modelling of class analogy; SVM, support vector machine;

[d] EVOO, extra virgin olive oil; ROO, refined olive oil; OPO, olive pomace oil; FA, fatty acid.

Dielectric spectroscopy as a rapid, low cost and non-destructive technique has been considered as a promising analysis method for food authentication too. The relationship between the dielectric properties and chemical properties has been investigated. Researchers found that the dielectric properties of oils were affected by the degree of unsaturation or the type of oils (Berardinelli et al., 2013; Hu et al., 2008). Moreover, Hu et al. (2010) found that olive oil adulterated with different types of vegetable oils at levels of adulteration below 5% could be identified based on dielectric spectroscopy.

Ultrasound as a fast and inexpensive technique has been widely applied in food industry (Awad et al., 2012): 1) to accelerate the reaction (Ince et al., 2001); 2) to decontaminate (Jose et al., 2014); 3) and to extract compounds (Jerman et al., 2010). Generally, it is possible to apply ultrasonic techniques to predict density, viscosity, degree of homogenization of a mixture and concentration of solid particles in a liquid (Dukhin & Goetz, 2009; McIntosh et al., 2016). However, only a few ultrasound applications have focused on the characterisation of food products, such as for determining the fat content of dairy products (Dukhin et al., 2005). Kumari et al. (2017) determined links between chemical changes (polymerisation) and ultrasonic properties (e.g. velocity, acoustic impedance). Hence ultrasonic technique may have potential to measure certain changes in properties after mixing of different oils.

### *1.6.3. Handheld screening techniques*

Except for the conventional laboratory analysis, some portable, palm-size devices have appeared and may have potential for EVOO authentication. The most important advantage of these rapid analyses is that they can provide analytical results at the sampling location and select a small number of suspect samples to be sent to the laboratory for confirmatory analysis. Therefore, these techniques have received much attention in food quality control, especially in regard to in-situ applications. For instance, 473 nm laser-induced fluorescence spectroscopy with the help of multivariate analysis was applied to detect and quantify adulteration of EVOO (Mu et al., 2016). Except for this method, a portable electronic-nose system (Wojnowski et al., 2017), handheld MicroNIR (Karunathilaka et al., 2017) and portable Raman spectroscopy (Tiryaki & Ayvaz, 2017) have been applied to discriminate EVOO from other vegetable oils (**Table 1.4**).

### *1.6.4. Statistical methods*

Many of the methods described in the previous sections result in analytical fingerprints. After these fingerprint analyses, a large dataset of test samples is generated. Because of the many chemical or physical features included in a fingerprint, it is challenging to elucidate information directly. In order to explore such a large dataset for its ability to discriminate EVOO from all other vegetable oils, multivariate analysis tools have been widely used (**Table 1.4**). Furthermore, the combination of chemical/physical analysis and multivariate data analysis leads to methods that are harder to circumvent by fraudsters. The multivariate analysis methods

include principal component analysis (Zou et al., 2009), discriminant analysis, linear discriminant analysis, hierarchical cluster analysis, support vector machine, artificial neural network, principal components regression (Rohman et al., 2017), soft independent modelling of class analogy, partial least square regression (Rohman et al., 2017), partial least square discriminant analysis (Torrecilla et al., 2010) and many more.

### 1.7. Knowledge gap

EVOO fraud comes in many forms. Fraud detection methods have received considerable attention. However, when a shift of thinking from fraud detection to fraud prevention is required, we need to learn to think as a criminal to combat fraud. By determining criminals' most likely courses of action, fraud vulnerabilities could be addressed and the weakest spots in the EVOO supply chain could be pinpointed out. In addition, it could also provide guidance on how to apply the latest detection methods efficiently to mitigate vulnerabilities and ensure EVOO authenticity. Nevertheless, to date, the mapping or identification of fraud vulnerabilities in the EVOO supply chain has not received any attention yet.

The literature review study in the previous sections highlighted that the majority of the studies conducted in the area of EVOO fraud aim to discriminate EVOO from other botanical vegetable oils with various analytical procedures. There are also some studies available on the identification of the botanical, geographical and production system origin of EVOO. However, only a few studies attempted to discriminate EVOO from its lower grade counterparts (Cecchi et al., 2017; Karbasian et al., 2015; Torrecilla et al., 2010). That is, because it is one of the more difficult EVOO adulteration issues due to the highly similar gross composition of these oils. Therefore, it is important to test more techniques in order to enrich the strategies to combat EVOO fraud.

Fraudsters try to catch up and circumvent the analytical methods (van Ruth & Granato, 2017). This has led to an increase in the use of fingerprint methods (complex data + advanced statistical techniques) to avoid the risk of being circumvented by fraudsters. The application of screening methods on-site could further support the detection of EVOO frauds. With this, large numbers of oil samples can be scanned on-site when a quick response is required. So far, the application of on-site screening methods to discriminate EVOO from its lower grade counterparts has received little attention.

Considering the identified gaps above, in this study, we focus on EVOO adulteration with lower olive oil grades and address (a) the vulnerabilities in the EVOO supply chain network and (b) fraud detection methods. These methods include (b1) confirmatory methods in which a search was carried out for new markers and (b2) rapid laboratory-based and on-site screening methods.

### 1.8. Research aim

The main objectives of this thesis are to develop strategies to combat fraud in the EVOO supply chains through knowledge about weak spots and underlying risk factors and the development of novel detection methods.

The detailed objectives are:

(a) To understand the differences and similarities in fraud vulnerability between actors in the EVOO supply chain; to elucidate the characteristics of high vulnerability actors and to identify risk factors contributing to fraud vulnerability (**Chapter 2**).

(b) To develop confirmatory and rapid screening fraud detection methods to assess the authenticity of EVOO based on its chemical and physical properties and to evaluate the performance of these techniques (**Chapters 3-6**).

### 1.9. Outline of this thesis

The outline of this thesis is presented in **Figure 1.5**. **Chapter 1** provides a general introduction on the theoretical information on the topics covered in this study.

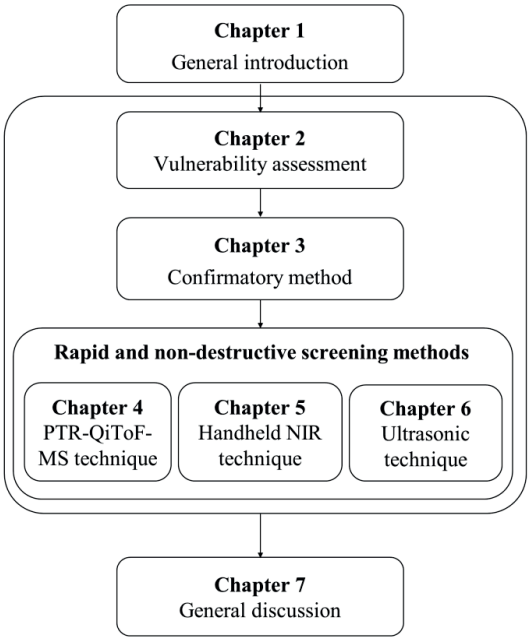


**Figure 1.5.** Schematic presentation of the PhD project. NIR, near infrared spectroscopy; PTR-QiToF-MS, proton-transfer-reaction quadrupole ion guide time-of-flight mass spectrometry.

In **Chapter 2**, the weak spots and underlying risk factors of various places in the EVOO supply chain are investigated. The fraud vulnerability of 28 companies across the EVOO supply chain is examined using the SSAFE FFVA tool. Amongst these companies, seven are olive oil producers, seven are business-to-business companies, seven are food manufacturers and seven are retailers. Moreover, the similarities and differences in fraud vulnerabilities according to group characteristics (the role, the scale and the location of the company) are evaluated.

**Chapter 3** aims to explore the processing derived contaminants 2- and 3-monochloropropanediol esters and glycidyl esters for the discrimination of processing grades of olive oils as potential discriminatory markers. A confirmatory method to differentiate different grade olive oils by the processing derived contaminants is developed. Oil samples are analysed using GC coupled to tandem MS. These processing derived contaminants are evaluated as potential markers to distinguish EVOO from lower grade olive oils, as well as other vegetable oils. Moreover, the sensitivity and the detection efficiency of this method is evaluated.

Subsequently, three rapid and non-destructive techniques are introduced. **Chapter 4** deals with the development of a simple, rapid but robust analytical and chemometric procedure based on proton-transfer-reaction quadrupole ion guide time-of-flight mass spectrometry (PTR-QiToF-MS) fingerprints to distinguish EVOO from other olive oil grades (ROO and POO). The volatile organic compounds (VOCs) of the olive oil grades and other vegetable oils are analysed using an advanced laboratory-based screening technique, PTR-QiToF-MS. A robust one-class classification approach is applied to distinguish EVOO from other olive oil grades, as well as other vegetable oils. The performance of this approach is evaluated and some distinct VOCs associated with the identity of EVOO are pinpointed.

**Chapter 5** explores a rapid and non-destructive handheld NIR technique to distinguish EVOO from lower grade olive oils and examines the underlying causes for the spectral differences of the oils. The spectral features of olive oil grades are examined by handheld NIR. The performance of classification models for sorting the olive oil grades is evaluated. Furthermore, compositional analyses, including analysis of FAs, chlorophylls, carotenoids and moisture contents as well as chromatic coordinates, are conducted to explore the underlying causes for the spectral differences of the olive oils.

**Chapter 6** focuses on physical properties of the oils and aims to develop a novel, rapid and non-destructive method for the discrimination of the oils. The ultrasonic properties of the oils are analysed by a developed ultrasonic pulse echo system. To understand the underlying causes for the differences in the ultrasonic properties of the oils, viscosities, densities and FA compositions are measured. In addition, the correlations between sound properties and physical traits (such as their viscosity and density) are investigated, as well as the correlation between physical traits and FA compositions.

Finally, the findings of **Chapters 2-6** are integrated in the General discussion (**Chapter 7**). This chapter presents the vulnerable points in the EVOO supply chain and evaluates the confirmatory and screening methods. In addition, fraud prevention strategies for the EVOO supply chain and the limitation of this thesis are proposed in this final chapter.

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# Chapter 2

## Food fraud: Assessing fraud vulnerability in the extra virgin olive oil supply chain

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**Abstract**

As a high value commodity on the market, extra virgin olive oil (EVOO) is a suitable target for fraudsters. To understand differences in perceived fraud vulnerability between tier groups across the EVOO supply chain and to disclose underlying factors, the perceived fraud vulnerability of 28 companies was examined using the SSAFE food fraud vulnerability assessment tool. Amongst these companies were seven olive oil producers, seven business-to-business companies, seven food manufacturers and seven retailers. The similarities and differences in perceived fraud vulnerabilities according to group characteristics (the role, the scale and the location of the company) were evaluated. Non-parametric tests and multiple correspondence analysis were applied for data exploration. An in-depth fraud vulnerability assessment of the EVOO supply chain was provided. Eight fraud factors related to opportunities and motivations scored high in the supply chain indicating their importance as fraud drivers and enablers. Four factors related to control measures are perceived as greatest vulnerability in the EVOO supply chain. Then, the vulnerability to fraud in the EVOO supply chain across all actors is perceived as high level on average. In decreasing contribution to the overall perceived fraud vulnerability, the fraud factor categories were ranked as follow: technical opportunities, a lack of managerial controls, a lack of technical controls, economic drivers, cultural and behavioural drivers and opportunities in time and place. Among the tier groups, the retailers and B2B companies experienced higher levels of perceived vulnerability than olive oil producers and food manufacturers due to the additional vulnerability related to the opportunities in time and place and greatest lack of control measures. Furthermore, the perceived fraud vulnerability of the company was not only determined by the tier group, but also impacted by the scale and location of the company.

**Keywords:** B2B company; FFVA tool; food fraud; food manufacturer; olive oil producer; retailer.

## 2.1. Introduction

Olive oil is the oil obtained from olive fruit after appropriate processing. The nutritional value and health benefits of olive oil make it a valuable commodity and consequently it is sold at a high price on the market. Increasing prices and relatively low consumer capabilities to detect inauthentic olive oil create an appealing crime opportunity for fraudsters (Food Standards Agency, 2016). Its liquid state also permits easy blending and mixing with inferior or cheaper oils (NSF, 2014). Therefore, olive oil is considered as one of the most frequently reported fraudulent commodities based on the three global food fraud databases from 2008 to 2013 (Weesepoel & van Ruth, 2015). Obviously, olive oil fraud is not a new threat as its adulteration has been reported since earlier years. In Morocco, olive oil was adulterated with lubricating oil used in jet engines, which left 10,000 people ill (Travers, 1962). While in 1981, over 20,000 people were poisoned from toxic oil syndrome that resulted from the sale of denatured rapeseed oil labelled as olive oil in Spain (Lipp, 2012; Mueller, 2011). These olive oil fraud incidents have resulted in actual public health risks.

Extra virgin olive oil (EVOO), as the premium quality olive oil, is particularly susceptible to be adulterated with cheaper vegetable oils (Jabeur et al., 2014), or lower quality olive oils (Karbasian et al., 2015). Moreover, Forbes stressed that probably 80% of the Italian EVOO on the market is fraudulent commodities (Rodriguez, 2016). In recent years, many EVOO fraud incidents have been exposed (Lord et al., 2017). An Italian operation led to the arrest of 33 suspects who exported fake EVOO to the USA (Smith, 2017). In 2019, the Europol-coordinated operation arrested 20 fraudsters and seized 150,000 litres of low-quality oils that had been adulterated with colorants to make them appear like EVOO (Taylor, 2019). Moreover, the risks of fraud may only be increasing due to the growing globalisation (Manning & Smith, 2015) and more competitive markets. To discern the EVOO fraud, laboratory-based analytical techniques on both targeted and non-targeted methods have been developed (Bajoub et al., 2018). These techniques are applied to identify the EVOO fraud incidents that have occurred and may reduce their impact, but they do not contribute much to prevention of further incidents. This actually requires a shift in thinking from “identification” and “mitigation” to “risk assessment” and “prevention” to prevent future food fraud.

For preventing EVOO fraud, Lord et al. (2017) proposed a conceptual and analytical framework informed by a situational understanding of the nature of the activities and behaviours involved in the fraud in the EVOO supply chain. Additionally, the Global Food Safety Initiative (GFSI, 2017) defined food fraud vulnerability as a “susceptibility or exposure to a food fraud risk, which is regarded as a gap or deficiency that could place consumer health at risk if not addressed”. Food fraud vulnerability assessment (FFVA) is employed to identify the weaknesses or flaws that creates opportunities for undesirable events (Spink et al., 2017). Several FFVA tools, including Vulnerability Assessment and Critical Control Points, NSF Fraud Protection Model (NSF, 2014), Food Fraud Mitigation Guidance (USP, 2016), Food

Fraud Initial Screening Model (Spink et al., 2016), CARVER + Shock tool (FDA, 2009) and SSAFE FFVA tool (SSAFE, 2016), have been established to help companies and regulators anticipate fraud vulnerability in the food supply chain.

The free online FFVA tool, developed by the non-profit SSAFE organisation in partnership with Wageningen University, VU University Amsterdam, PricewaterhouseCoopers, and food industry leaders around the world, aims to help strengthen internal controls of companies while reducing opportunities and motivations to adulterate food for economic gain (Food Safety Magazine, 2016). It has been successfully applied to assess the perceived fraud vulnerability in the spices chain (Silvis et al., 2017), the Dutch milk supply chain (Yang et al., 2019) and five other supply chains (van Ruth et al., 2018).

EVOO fraud incidents were frequently reported (Rodriguez, 2016; Smith, 2017; Taylor, 2019). However, it is currently unknown which factors contribute to the vulnerability of this chain and whether the differences in fraud vulnerability between actors exist, if so, what the characteristics of these company are. Therefore, the aims of this study are to evaluate the perceived fraud vulnerability of tier groups (olive oil producers, business-to-business (B2B) companies, food manufacturers and retailers) in the EVOO supply chain using the SSAFE FFVA tool and to identify risk factors contributing to the perceived vulnerability. Furthermore, the relationship between the perceived fraud vulnerability and particular fraud factors on the one hand, and company characteristics on the other hand will also be examined to pinpoint weaker groups.

## 2.2. Materials and methods

### *2.2.1. Interviewed companies*

Twenty-eight European companies were interviewed, seventeen of which were located in the Netherlands and eleven in Mediterranean countries. Based on their obligation on the market, the companies were divided into four tier groups, including seven olive oil producers, seven B2B companies, seven food manufacturers and seven retailers. Among the seven food manufacturers, EVOO is added as ingredient to tomato sauces (3), pastries (3) and crackers (1). With regard to the scale of the companies, sixteen were small scale companies (< 50 staff), five were medium scale companies (< 250 staff) and seven were large scale companies (> 250 staff). **Table 2.1** shows the characteristics of the 28 companies. The typical roadmap of the EVOO supply chain from farm to table is presented in Supplementary **Figure S2.1**.

### *2.2.2. Adaptation of the FFVA tool to the EVOO supply chain*

Before onset of the interview, slight changes were made to the FFVA tool (Supplementary **Table S2.1**) to make it suitable for the EVOO supply chain investigation. For

example, the questions related to "food counterfeiting" (Q6/7) were not considered since counterfeiting is not typical EVOO fraud type. Because B2B companies and retailers only act as an intermediary to transfer EVOO to their consumers, all the questions about the raw material and processing lines (Q2/3/8/32/33) were eliminated. Two olive oil producers produced also the olives themselves, their questions about suppliers were changed to customers. In total, there were 48 questions for olive oil producers and food manufacturers, but there were only 43 questions for B2B companies and retailers. For group comparison, only the 43 questions were considered. Furthermore, the numbering of the questions was kept the same as the SSAFE FFVA tool to facilitate comparability between the obtained results and other studies that used the same tool.



**Table 2.1.** Characteristics of the 28 companies assessed in the study.

| Tiers | Company scale | Company location [a] | Product |
|---|---|---|---|
| Olive oil producers | Small | Mediterranean (Spain) | EVOO (Organic) [b] |
| | Small | Mediterranean (Portugal) | EVOO (Organic) |
| | Small | Mediterranean (Portugal) | EVOO (Conventional) |
| | Small | Mediterranean (Greece) | EVOO (Organic) |
| | Small | Mediterranean (Greece) | EVOO (Conventional) |
| | Small | Mediterranean (France) | EVOO (Organic) |
| | Large | Mediterranean (Spain) | EVOO (Conventional) |
| B2B [c] companies | Small | The Netherlands | EVOO (Organic) |
| | Small | The Netherlands | EVOO (Organic) |
| | Small | The Netherlands | EVOO (Conventional) |
| | Small | The Netherlands | EVOO (Conventional) |
| | Small | The Netherlands | EVOO (Conventional) |
| | Small | The Netherlands | EVOO (Conventional) |
| | Small | The Netherlands | EVOO (Conventional) |
| Food manufacturers | Medium | The Netherlands | Crackers [d] |
| | Medium | The Netherlands | Tomato sauce |
| | Medium | The Netherlands | Pastry |
| | Medium | Mediterranean (Italy) | Tomato sauce |
| | Medium | Mediterranean (Spain) | Pastry |
| | Large | Mediterranean (Spain) | Pastry |
| | Large | Mediterranean (Spain) | Tomato sauce |
| Retailers | Small | The Netherlands | EVOO (Organic) |
| | Small | The Netherlands | EVOO (Organic) |
| | Small | The Netherlands | EVOO (Organic) |
| | Large | The Netherlands | EVOO (Organic) |
| | Large | The Netherlands | EVOO (Conventional) |
| | Large | The Netherlands | EVOO (Conventional) |
| | Large | The Netherlands | EVOO (Conventional) |

[a] Eleven Mediterranean countries consist of five Spain, two Greece, two Portugal, one Italy and one France; [b] EVOO refers to extra virgin olive oil, the 21 EVOO products consist of 10 organic EVOO and 11 conventional EVOO products; [c] B2B refers to business-to-business; [d] EVOO was used as an ingredient in the products of food manufacturers (tomato sauce, pastry and crackers).

### *2.2.3. Data collection*

Twenty-eight companies carried out the same food fraud vulnerability assessment using the SSAFE FFVA tool (SSAFE, 2016). The adapted questionnaires were sent to the interviewees in advance to prepare the respondents for the face-to-face interviews (11 participants), skype interviews (2 participants) and telephone interviews (5 participants). The duration of these interviews was between 1 to 1.5 h each. During the interview, the interviewer asked the questions, explained the questions if necessary and recorded answers and extra explanations given by interviewees. For the other ten interviews, the questionnaire and an explanation of the questionnaire were sent to the interviewees through email and a 10-minute telephone interview was conducted for cases where the interviewee requested additional explanations for certain questions.

### *2.2.4. Statistical analyses*

#### *2.2.4.1. Frequency calculation*

Three descriptions are provided to each question (SSAFE, 2016). These three descriptions are converted into low, medium and high vulnerability level, and are represented by a score of 1, 2 and 3, respectively. Therefore, the low-medium-high vulnerability frequencies were calculated from the answers of interviewees for 48 fraud factors/questions (Q) (43 factors were answered by 28 interviewees and 5 factors were answered by 14 interviewees), for the three key elements (opportunities, Q1-11; motivations, Q12-31; control measures, Q32-50) and for the six fraud factor categories (technical opportunities, Q1-5; opportunities in time and place, Q8-11; economic drivers, Q12-14, Q19-20,Q26, Q30-31; cultural and behavioural drivers, Q15-18, Q21-25, Q28-29; technical controls, Q32-37, Q42-44, Q50; managerial controls, Q38-41, Q45-49). Afterwards, these three types of frequencies were calculated for the four tier groups (olive oil producers, B2B companies, food manufacturers and retailers).

The frequency of perceived vulnerability levels for each fraud factor/key element/fraud factor category (Fi) was determined by Eq. (1):

$$\mathrm{Fi} = \frac{\mathrm{Sij}}{\mathrm{Gj}} \quad , \quad (1)$$

where Fi is the frequency of score i (i = 1, 2, 3), Sij is the number of observations which get score i in group j (j = 43 or 48 individual questions, three individual key elements and six individual fraud factor categories) and Gj is the total number of observations in group j.

#### *2.2.4.2. Univariate analysis*

Non-parametric Kruskal-Wallis tests were applied for group comparisons due to the ordinal data in this study. The pairwise comparisons were carried out using the Mann-Whitney U tests. Mean ranks were applied to compare the perceived fraud vulnerability of the factors

between groups. The higher the mean rank value, the higher the perceived fraud vulnerability. These data analysis methods were performed using SPSS statistic 23 software (IBM, Chicago, IL, USA).

*2.2.4.3. Cluster analysis*

The score data were subjected to multiple correspondence analysis (MCA) to investigate the association between groups. This was performed using R 3.4.3 software (R Foundation for Statistical Computing, Vienna, Austria).



## 2.3. Results and discussion

### *2.3.1. Descriptive exploration*

*2.3.1.1. Degree of perceived fraud vulnerability of the EVOO supply chain*

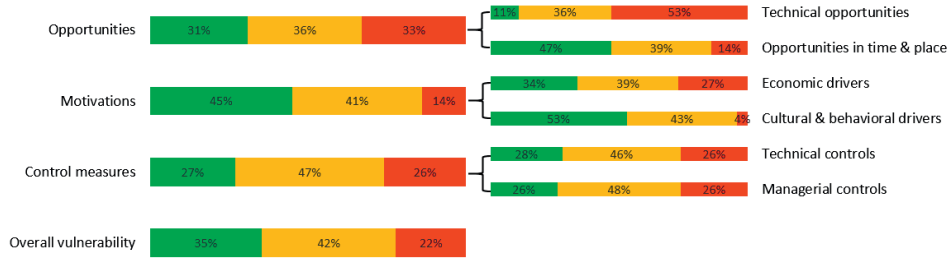


**Figure 2.1.** Low-medium-high vulnerability frequencies of the answers of 28 interviewees for the three key elements and the six fraud factor categories. Green, orange and red describe low, medium and high vulnerability frequency, respectively.

Perceived fraud vulnerability is dependent on the opportunities and motivations of fraudsters to commit fraud, as well as the presence or lack of suitable food fraud control measures (van Ruth et al., 2017). Therefore, these three key elements were assessed to identify the perceived fraud vulnerability of the EVOO supply chain. The frequencies of the different answering options cumulated for the key elements and reflecting low, medium and high perceived vulnerability are presented as green, orange and red bars, respectively, in **Figure 2.1**. The frequencies of individual questions are presented in **Figure 2.2**. The more prevalent the red (high vulnerability) and orange colour (medium vulnerability), the higher the perceived vulnerability. Only 35% of all questions were rated low vulnerability (**Figure 2.1**). The orange-red (medium-high vulnerability) colour dominates in the upper and lower parts of **Figure 2.2**, whereas green (low vulnerability) is the predominant colour in the middle part. The results indicate that most of the factors related to the opportunities and control measures were assigned high vulnerability.

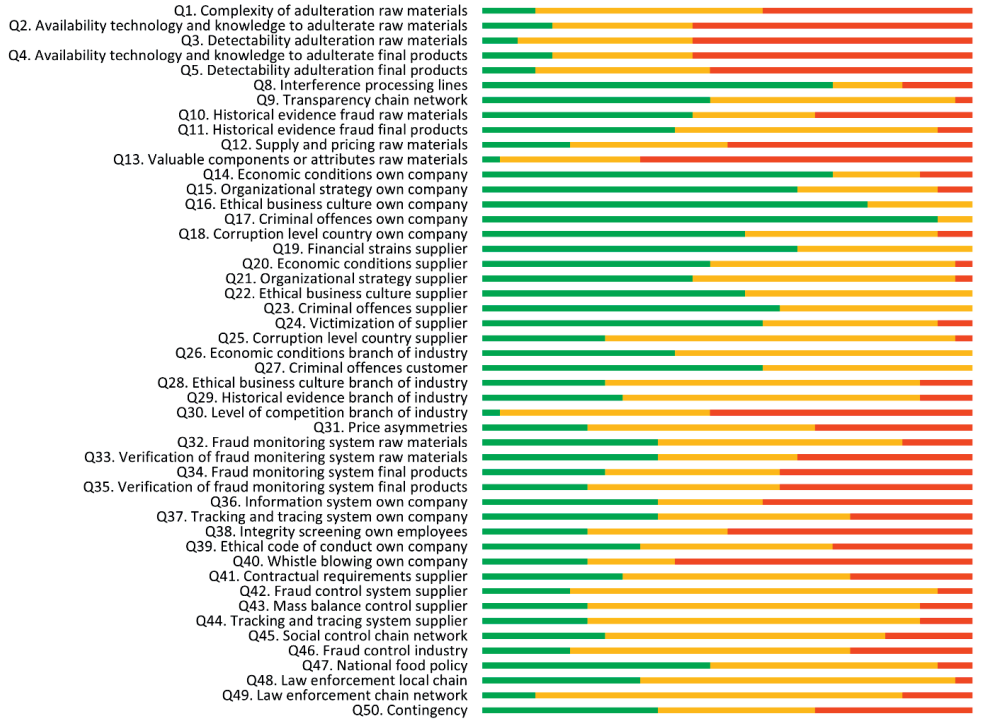


**Figure 2.2.** Overview of low-medium-high vulnerability frequencies of the answers for 48 fraud factors. Amongst these fraud factors, 43 were answered by 28 interviewees and 5 were answered by 14 interviewees. Green, orange and red refer to low, medium and high level of vulnerability, respectively.

**Opportunities.** All factors related to the technical opportunities (Q1-5) were rated high vulnerability (orange-red > 80%). This indicates that it is perceived as easy to adulterate the EVOO. It supports the fact that liquid adulteration with another liquid is common and physically the easiest to perform (NSF, 2014). Additionally, the detection and confirmation of the EVOO fraud are difficult and require advanced laboratory analysis. The European Commission (2013) and International Olive Council (IOC, 2016) have established both laboratory analytical methods and organoleptic criteria for the EVOO characterisation. Organoleptic evaluation is particularly precise and not replaceable with laboratory analysis, but it seems to lack reproducibility for commercial EVOO, which is likely due to differences in sensory sensibility between the different IOC panel labs (Circi et al., 2017). Moreover, food fraud as an intentional act is difficult to be determined, because potential adulterants are unconventional, where the current conventional detection systems are not testing food for these contaminants as they simply do not know what to test for (Djekic et al., 2018). Therefore, technical opportunities are perceived as important drivers to commit fraud (high vulnerability), demonstrated with high frequencies of answering options reflecting medium (orange, 36%) and high (red, 53%) vulnerability (**Figure 2.1**). On the contrary, the fraud vulnerability of opportunities in time and place is perceived as relatively low level (orange-red = 53%).

**Motivations.** Forty-five percent of the factors related to motivations are perceived as low vulnerability based on the green colour in **Figure 2.1** and **2.2**. However, three factors (Q12/13/30) were assessed as high vulnerability. When the participants were asked about the supplying and pricing of raw materials (Q12), the majority (82%) commented that the price of raw materials varied depending on the harvest quantity and quality of olive fruit, as well as the geographical origin. This is in agreement with Santini et al. (2018), who reported that the average price of EVOO in Italy grew by 74% from 2011 to 2016. The Food Standards Agency (2016) also showed that the average price of EVOO increased by nearly 10% between December 2014 and June 2015 as olive harvests in Spain and Italy suffered due to a widespread infestation of the olive tree stock by the *Xylella fastidiosa* bacteria. Moreover, 96% of those who were interviewed indicated that special attributes or components mainly determine the value of EVOO (Q13), such as organic characteristic, high level of antioxidants and special organoleptic properties. These findings are in accordance with a previous study which found Dutch consumers willing to pay for organic olive oil due to its production system (Kalogeras et al., 2009). Another study also reported that nutrition and taste of EVOO were some of the top reasons for most American consumers' purchasing decisions (Wang et al., 2013). Furthermore, 96% of the respondents reported that there is a growing competition across the EVOO supply chain (Q30). This is in line with Santini et al. (2018) who stated that the olive oil business is highly competitive in Italy due to internal (competition between companies: price difference and oil quality) and external (the international competition: new producing countries emerging) impacts. As such, small scale companies have no competitive pricing advantage compared to other companies. Therefore, they must understand the typical features of their olive oil and establish a strong marketing strategy to promote them as unique and distinctive traits of the product to gain market share (Santini et al., 2018). These three factors are all associated with economic drivers, which supports the fact that the economically motivated adulteration is one of the major types of food fraud (Moore et al., 2012). Consequently, economic drivers (orange-red = 66%) contributed more than cultural and behavioural drivers (orange-red = 47%) to the perceived fraud vulnerability.

**Control measures.** Adequate control measures were merely lacking, considering the 27% of low vulnerability answers to this group of questions (**Figure 2.1**). Control measures can be divided into technical (hard) controls and managerial (soft) controls. Hard controls refer to those aimed at the detection of fraud by generating data and actual information on the prevalence of adulterated products, while soft controls are more preventive in nature and aim at reducing opportunities and or motivations in the management system and the chain environment (van Ruth et al., 2017). In terms of the technical (hard) controls, the fraud factor related to "fraud control system of suppliers" (Q42, red-orange = 82%) was assigned to high vulnerability (**Figure 2.2**). It indicates that most of the suppliers of the interviewed companies lacked a well-established fraud control system. More than half of the participants (16 out of 28) are small scale companies, it is reported that small companies lack any resources to implement

a fraud mitigation plan (Levitt, 2016; Silvis et al., 2017). Furthermore, three factors related to the managerial (soft) controls were assigned to high vulnerability (**Figure 2.2**). Over 80% of the participants and their suppliers lacked a specific whistle blowing system (Q40) and guidelines for fraud mitigation (Q46). A possible explanation for this might be that there exist barriers to whistleblowing. For instance, whistleblowing was viewed as treason or betrayal in Italy (Dungan et al., 2015; Osterhaus & Fagan, 2007). Another possible explanation is that countries have limited or no legal frameworks to protect whistleblowers (Transparency International's Secretariat, 2013), therefore, the whistleblowers may run the risk of being pursued and sanctioned (Motarjemi, 2018). On the other hand, this result is contrary to Soon and Manning (2017) who reported that small and medium scale companies (21 out of 28 in this study) can more readily implement a whistle blowing protocol within their organisation and with their suppliers. In addition, 89% of the interviewees stated that fraud-related enforcement practices are not aligned across the international EVOO supply chain (Q49). This is in accordance with a previous study (Corini & van der Meulen, 2018), which indicated that the enforcement of food fraud in the EU member states are different. The EU food law for the prevention of food fraud, Article 19 (European Parliament & European Union Council, 2019), requires food business operators to withdraw and recall food products when they consider food products not to be in compliance with food safety requirements. Greece, the Netherlands and Portugal considered Article 19 applicable to the horsemeat scandal, while Ireland and Italy argued that Article 19 requirements had not been met, so they did not recall the involved food products (van der Meulen, 2015a). Taking all the above into consideration, we can conclude that there is a great lack of food fraud control measures in the EVOO supply chain. It is likely to be related to the implementation of regulations (Havinga, 2014; van der Meulen, 2015b) and the food regulatory arrangements by companies (Havinga, 2012).

Overall, the EVOO supply chain is perceived as high vulnerable to food fraud (orange-red = 64%, **Figure 2.1**), compared with the other food supply chains (Silvis et al., 2017; van Ruth et al., 2018; Yang et al., 2019). Eight fraud factors related to opportunities (Q1-5) and motivations (Q12/13/30) scored high in the supply chain indicating their importance as fraud drivers and enablers. Four factors (Q40/42/46/49) related to control measures are perceived as greatest lacking in the EVOO supply chain. Technical opportunities were rated the most vulnerable to fraud, followed by the lack of managerial (soft) controls, the lack of technical (hard) controls, economic drivers, cultural and behavioural drivers and opportunities in time and place.

*2.3.1.2. Exploring clusters in perceived fraud vulnerability patterns for all companies*

The FFVA data were subjected to MCA to explore the similarities in perceived fraud vulnerability patterns across the 28 companies. The first two dimensions of the MCA are shown in **Figure 2.3**. They explained 29% of the total variance. The scores appear to be considerably influenced by the role of the company (**Figure 2.3a**) in the supply chain, as well as the scale

(**Figure 2.3b**) and location (**Figure 2.3c**) of the company. In order to illustrate the association between groupings and the fraud factors, the loadings plot is shown in **Figure 2.4**.

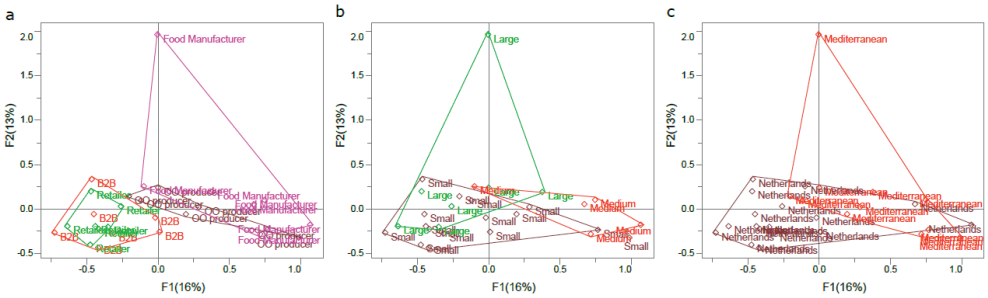


**Figure 2.3.** Scores plots of multiple correspondence analysis on the responses of 28 interviewees a) four tier groups: olive oil producers, business-to-business (B2B) companies, food manufacturers, retailers; b) three groups according to the scale of the companies: small, medium and large; c) two groups according to the location of the companies: the Netherlands and Mediterranean countries.

The retailers and B2B companies (left side along F1) are separated from the olive oil producers and food manufacturers (right side along F1) in **Figure 2.3a**. The retailers and B2B groups show relatively high scores for opportunities, motivations and control measures (**Figure 2.4**). In contrast, the olive oil producers and food manufacturers are widely spread and overlap with each other in **Figure 2.3a**. They show relatively low scores for opportunities, motivations and control measures (**Figure 2.4**). The results indicate that B2B companies and retailers are generally more vulnerable to fraud than the other two tier groups, which means that they are perceived as more likely to be victims of fraud. For food manufacturers, the companies that produce tomato sauce are separated from the other two types of businesses (pastry and crackers) (Supplementary **Figure S2.2**). Furthermore, the tomato sauce companies present high scores for opportunities, motivations and control measures (Supplementary **Figure S2.2** and **S2.3**), indicating that EVOO as an ingredient of the semi-solid product (tomato sauce) is perceived as more susceptible to be adulterated than that of the other two solid products (pastry and crackers).

Three groups of companies of different scales (size) widely spread and overlap with each other in **Figure 2.3b**. The small scale companies grouped in the left lower quadrant and presented relatively high scores for the three key elements, large scale companies mainly grouped in the left quadrants and mainly showed medium scores. Whereas the medium scale companies grouped in the left quadrants and they appeared to be less vulnerable to fraud than the other companies (**Figure 2.4**). The results is in line with the previous study reporting that small businesses suffer fraud more frequently than large organisations (Doody, 2009).

With regard to the location of the companies, the Netherlands group overlaps with a certain number of the Mediterranean group in **Figure 2.3c**. Moreover, the Netherlands group show relatively high scores for the three key elements (**Figure 2.4**). This means that companies in the Netherlands perceives themselves as more vulnerable to fraud. This higher perceived

vulnerability could be due to the fact that the Dutch companies are primarily B2B companies and retailers and they are also primarily small scale companies (**Table 2.1**). Another possible reason is that the level of food safety in the Netherlands is high, but the opportunities to commit food fraud have increased, because fraudsters are trying their utmost to remain out of the line of sight of the supervisory authority (NVWA, 2018a). furthermore, olive oil is always imported from the south of Europe, which results in a longer chain and more opportunity to commit fraud.

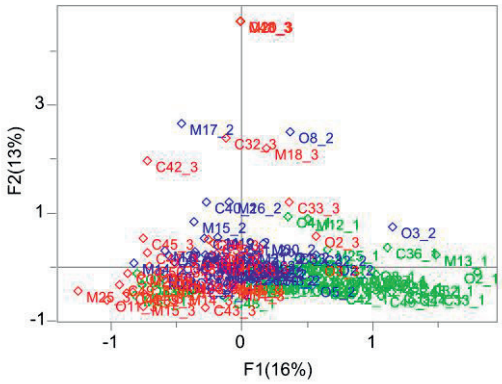


**Figure 2.4.** Loadings plot of multiple correspondence analysis on the responses of 28 interviewees. Higher, intermediate and lower vulnerability scores are coloured red, blue and green, respectively. O refers to opportunities; M refers to motivations; C refers to control measures; numbers after the letter refer to question number_vulnerability level.

Additionally, the differences and similarities between the conventional and organic EVOO supply chains were investigated. It is interesting to notice that these two supply chains are widely spread and overlap with each other in Supplementary **Figure S2.4**, which means that there is no big difference between these two supply chains. It reveals that the organic property did not contribute to additional perceived fraud vulnerability. This finding is consistent with that of Barbieri et al. (2015) who found that organic farming information did not affect consumers' preferences for EVOO, which means that the organic property did not trigger fraud.

These findings indicate that the assignment to the clusters is not only due to the role of the company in the supply chain, but it is also determined by the scale and location of the company. Retailers and B2B companies have a similar perceived fraud vulnerability pattern. And they are also more vulnerable to food fraud than the other two tier groups.

*2.3.1.3. Exploring perceived fraud vulnerability patterns according to the role of the companies*

The low-medium-high vulnerability frequencies of answering options across the four tier groups (olive oil producers, B2B companies, food manufacturers and retailers) are presented in **Figure 2.5**. The perceived fraud vulnerability patterns of individual tier groups (Supplementary **Figure S2.5**) are broadly similar to the overall perceived fraud vulnerability of

the EVOO supply chain (**Figure 2.2**). The upper and lower parts (opportunities and control measures) are dominated by the orange-red colour, whereas in the middle part (motivations), green is the predominant colour.


**Figure 2.5.** Low-medium-high vulnerability frequencies of answers for the three key elements (opportunities, motivations and control measures) and the six fraud factor categories (technical opportunities, opportunities in time & place, economic drivers, cultural & behavioural drivers, technical controls and managerial controls) of the fraud vulnerability assessments for the four tier groups. Green, orange and red refer to low, medium and high level of vulnerability, respectively. B2B refers to business-to-business.

**Opportunities.** Technical opportunities (orange-red > 80%) were assigned to high vulnerability by the four tier groups. The B2B companies and retailers assigned opportunities in time and place (Q9/10/11) to high vulnerability (orange-red = 71%), whereas the olive oil producers and food manufacturers rated them with a low vulnerability (orange-red = 39%). The EVOO supply chain was described by the B2B companies and retailers as a complex supply chain that lacks transparency, with short-term relationships and no information exchange (Q9). According to Soon et al. (2019), several sources could help companies increase the information exchange in the supply chain, including their own internal experts, guidelines provided by the different assessment methods, food safety certification bodies, professional memberships and networking with their suppliers and consumers. Furthermore, to solve traceability issues and enhance transparency, RFID and blockchain technologies (Galvez et al., 2018) could be applied in the supply chain. Moreover, when questioned about the historical fraud incidents (Q10/11), the olive oil producers (small scale companies) lack of access to the food fraud databases, which may result in a difficulty understand of the historic incidents (Manning & Soon, 2019). In addition, with regard to food manufacturers, compared with the frequently reported EVOO fraud incidents (Smith, 2017; Taylor, 2019), an EVOO fraud incident seldom reported for food that use EVOO as an ingredient. Consequently, B2B companies and retailers are generally more vulnerable to commit fraud. Furthermore, they are perceived more likely to be victims of fraud due to the increased opportunities in time and place.

**Motivations.** The factors related to economic drivers are perceived as medium-high vulnerability level by the olive oil producers, B2B companies and retailers. This is in agreement

with Moore et al. (2012) who found that fraud conducted for economic gain by food producers, processors, distributors, or retailers is gaining attention and the economic motivation is the main reason for food fraud. However, food manufacturers assigned the economic drivers to the lowest vulnerability compared to the other three tier groups. A possible reason is that the addition of a small amount of EVOO as a flavour additive does not lead to large price difference. This, in turn, seems to be less motivated to commit fraud. With regard to the cultural and behavioural drivers, the retailers (orange-red = 61%) assigned these drivers to be more vulnerable to commit fraud than the other three tier groups (orange-red < 50%). This is mainly due to a lack of confidence in their suppliers (Supplementary **Figure S2.5**). Similarly, the lack of confidence in their suppliers also appeared in the milk supply chain (Yang et al., 2019). The result reveals a weak interaction between the companies and their suppliers. Furthermore, a lack of information and knowledge sharing with other tier groups (including their suppliers) may increase food vulnerability (Soon et al., 2019). Consequently, the retailers (orange-red = 64%) assigned high vulnerability scores to fraud factors associated with motivations, followed by olive oil producers (orange-red = 57%), B2B companies (orange-red = 54%) and eventually the food manufacturers (orange-red = 44%).

**Control measures.** Most of the retailers and B2B companies (orange-red > 80%) stated that they predominantly lack technical controls (i.e. a fraud monitoring system, tracking and tracing system and contingency plan). Only ~60% of the responses coming from the olive oil producers and food manufacturers are in line with this statement. It is possible that the specialised trained counter-fraud staff is rarely employed within the food industry (Soon et al., 2019), which results in a weak fraud control system. Moreover, B2B companies (orange-red = 87%) were more vulnerable to commit fraud due to a lack of managerial controls. All the B2B companies in this study were small scale companies, while small businesses do not have the resources to map out the dangers of food fraud in their supply chain (Levitt, 2016; Silvis et al., 2017). Hence, a lack of resources may lead to companies being more vulnerable to fraud. Therefore, the food manufacturers (green = 39%) and olive oil producers (green = 37%) have more adequate control measures in place, followed by the retailers (green = 17%) and the B2B companies (green = 13%). This result is in line with the perceived fraud vulnerability assessment of the other supply chains (van Ruth et al., 2018).

Overall, the perceived fraud vulnerability varied between tier groups. Since B2B companies are in the middle part of the supply chain (Supplementary **Figure S2.1**), they are also more likely to pass the potential fraud on to their customers (food manufacturers and retailers) (van Ruth et al., 2018). With regard to the medium-high vulnerability frequencies of responses of each tier group (**Figure 2.5**), the decreasing order in perceived fraud vulnerability is retailers (orange-red = 73%), B2B companies (orange-red = 69%), olive oil producers (orange-red = 61%) and food manufacturers (orange-red = 55%). Similar to the other food supply chain (Silvis et al., 2017; van Ruth et al., 2018; Yang et al., 2019), the companies in the middle and end of the supply chain (B2B companies and retailers) are more vulnerable to fraud

than the companies at the beginning of the supply chain (producers). There are two possible reasons. Firstly, because of the increased complexity of the food supply chain, when more members (the intermediaries between producers and consumers: wholesalers, distributors and retailers) involve in the supply chain, more uncertainties accrue (Lamarre & Pergler, 2009; Ting et al., 2014) and the likelihood of food fraud increases (Manning & Smith, 2015). Thus, B2B companies and retailers are more vulnerable to fraud. Secondly, considering their reputation and that they are easily traced through the origin place on the package, the olive oil producers and food manufacturers are more likely to present a positive outlook. Therefore, when questioned about the likelihood of being affected by food fraud, they possibly say that "It won't happen to us" (Soon et al., 2019; Weinstein, 1984).

### *2.3.2. Statistical evaluation: Effect of group characteristics on perceived fraud vulnerability*

#### *2.3.2.1. The role of the companies*

**Table 2.2.** Fraud factors of the FFVA demonstrating significantly different perceived fraud vulnerability between tier groups.

| Question number | Fraud factor | Food manufacturers (n = 7) | Olive oil producers (n = 7) | B2B companies (n = 7) | Retailers (n = 7) |
|---|---|---|---|---|---|
| Q22 | Ethical business culture (supplier) | 10 $^{c}$ | 14 $^{b}$ | 12 $^{bc}$ | 22 $^{a}$ |
| Q24 | Victimisation (supplier) | 9 $^{b}$ | 17 $^{a}$ | 14 $^{ab}$ | 19 $^{a}$ |
| Q36 | Information system (own company) | 7 $^{a}$ | 12 $^{a}$ | 20 $^{b}$ | 20 $^{b}$ |
| Q37 | Tracking and tracing system (own company) | 9 $^{a}$ | 13 $^{a}$ | 16 $^{ab}$ | 21 $^{b}$ |
| Q39 | Ethical code of conduct (own company) | 9 $^{a}$ | 12 $^{ab}$ | 20 $^{b}$ | 16 $^{ab}$ |

Different superscript letters (a, b, c) in a row indicate significant differences from each other (Kruskal-Wallis tests and Mann-Whitney U tests, $p < 0.05$). Higher, intermediate and lower vulnerability scores are coloured red, orange and green, respectively. FFVA refers to food fraud vulnerability assessment; B2B refers to business-to-business.

Five factors revealing significant differences (Mann-Whitney U test, $p < 0.05$) between tier groups are shown in **Table 2.2**, including two motivation related fraud factors (Q22, the ethical business culture of suppliers; Q24, victimisation of suppliers) and three internal control measures related fraud factors (Q36, information system; Q37, tracking and tracing system; Q39, ethical code of conduct). Specifically, retailers show less confidence in their suppliers than other three tier groups (Q22/24). This is in line with previous research, which showed that external frauds occurred at their suppliers were reported more frequently than internal frauds occurred at the companies themselves due to less stringent control and preventive measures (Manning, 2016) and increased awareness of food fraud in the supply chain (Soon et al., 2019). In addition, all retailers and B2B companies showed a significantly greater lack of internal control measures (Q36/37/39) than the other two tier groups. This is in line with van Ruth et al. (2018) who reported that B2B companies (wholesalers) and retailers also lacked these internal control measures in the other food supply chains.

### *2.3.2.2. The scale of the companies*

**Table 2.3.** Fraud factors of the FFVA demonstrating significantly different perceived fraud vulnerability between groups according to the scale of the company.

| Question number | Fraud factor | Medium (n = 5) | Small (n = 16) | Large (n = 7) |
|---|---|---|---|---|
| Q22 | Ethical business culture (supplier) | 8 $^{b}$ | 14 $^{ab}$ | 20 $^{a}$ |
| Q36 | Information system (own company) | 7 $^{a}$ | 17 $^{b}$ | 14 $^{ab}$ |
| Q47 | National food policy | 10 $^{a}$ | 13 $^{a}$ | 22 $^{b}$ |

Different superscript letters (a, b) in a row indicate significant differences from each other (Kruskal-Wallis tests and Mann-Whitney U tests, $p < 0.05$). Higher, intermediate and lower vulnerability scores are coloured red, orange and green, respectively. FFVA refers to food fraud vulnerability assessment.

Three fraud factors showed statistically significantly different perceived vulnerability (Mann-Whitney U test, $p < 0.05$) between companies due to the different scale of the companies (**Table 2.3**). Small and large scale companies are perceived to be more vulnerable to fraud based on these three factors. Large scale companies (mean rank = 20) show significantly higher perceived vulnerability in the factor related to motivation (Q22, the ethical business culture of the suppliers) than medium scale companies (mean rank = 8). Since the significant difference in this factor was also found between tier groups (**Table 2.2**), both the role of the companies in the supply chain and the scale of the companies contributed to the score of this factor. While small scale companies showed greatest lack of information system on mass balance flow (Q36, mean rank = 17). Most of them stated that they only have a basic administrative system with limited information or no specific information on mass balances of incoming materials and final products. Moreover, there is no systematic analysis of mass flow data throughout the companies. In addition, large scale companies stated that there only exists a general national food policy without specific legislative requirements for food fraud mitigation (Q47, mean rank = 22). These large scale companies are primarily located in the Netherlands and the result is in agreement with the facts. The food policy of the Netherlands provides details on food safety control system (NVWA, 2017, 2018b). Furthermore, the Food Confidence Task Force (The Dutch Minister for Agriculture & The Dutch Minister of Health Welfare and Sport, 2013) defined a set of criteria for quality schemes that strengthen the private safeguarding of food safety and integrity. Although some general requirements are included in the EU General Food Law, there is no explicit framework in place to target food fraud (mitigation) in Dutch food policy. There is even no consensus on a uniform definition of food fraud in EU legislations (European Parliament, 2016; Manning & Soon, 2019).

### *2.3.2.3. The location of the companies*

The significant differences (Mann-Whitney U test, $p < 0.05$) between the companies in the Netherlands and Mediterranean countries are presented in **Table 2.4**. The corruption level of the Netherlands (mean rank = 12) is significantly lower than that of the Mediterranean countries (mean rank = 19) resulting from the motivation related fraud factor Q18. This is in

agreement with the Transparency International (2018) reporting that the corruption perception index of the Netherlands (index value = 82) is higher than that of the Mediterranean countries (index value < 50). The lower the index value, the higher the corruption level. On the other hand, the corruption perception at the country level may activate the corruption perception at the industry level (Gounev & Bezlov, 2010), which in turn results in food fraud, e.g. horsemeat scandal. However, the fraud vulnerability of the Dutch companies is perceived as higher level than that of the companies in Mediterranean countries due to greatest lack of internal controls (Q36/39). Since Dutch companies are primarily B2B companies and retailers and they are also primarily small scale companies (**Table 2.1**), the significant differences in these two fraud factors were also found between the tier groups (**Table 2.2**).

**Table 2.4.** Fraud factors of the FFVA demonstrating significantly different perceived fraud vulnerability between groups according to the location of the company.

| Question number | Fraud factor | Mediterranean countries (n = 11) | The Netherlands (n = 17) |
|---|---|---|---|
| Q18 | Corruption level country (own company) | 19 [a] | 12 [b] |
| Q36 | Information system (own company) | 9 [a] | 18 [b] |
| Q39 | Ethical code of conduct (own company) | 10 [a] | 17 [b] |

Different superscript letters (a, b) in a row indicate significant differences from each other (Mann-Whitney U tests, $p < 0.05$). Higher and lower vulnerability scores are coloured red and green, respectively. FFVA refers to food fraud vulnerability assessment.

Taken together, it is evident that there are differences in perceived fraud vulnerability due to the scale and location of the companies, and those two are intertwined. Therefore, the perceived fraud vulnerability is affected by the role, the scale and the location of the companies. In addition, B2B companies and retailers are all located in the Netherlands, whereas the olive oil producers are located in Mediterranean countries. This intertwining affects the results but are factual when it comes to olive oil producers. There are simply no olive trees growing in the Netherlands. However, in future research, B2B companies and retailers in the south of Europe could be examined as well.

## 2.4. Conclusions and outlook

An in-depth perceived fraud vulnerability assessment of the EVOO supply chain was provided to understand the differences in perceived fraud vulnerability between tier groups. Eight fraud factors related to opportunities (Q1-5) and motivations (Q12/13/30) scored high in the supply chain indicating their importance as fraud drivers and enablers. Four fraud factors (Q40/42/46/49) related to control measures are perceived as greatest lacking in the EVOO supply chain. Furthermore, the perceived fraud vulnerability varied between tier groups. The decreasing order in perceived fraud vulnerability is: retailers, B2B companies, olive oil producers and food manufacturers. The retailers and B2B companies are accompanied by

additional vulnerability due to opportunities in time and place and greatest lack of control measures. In addition, the variations of the perceived fraud vulnerability across the EVOO supply chain were determined not only by the role of the company, but also by the scale and the location of the company.

The knowledge from the current study can be used to pinpoint the weaker spots in the chain in order to develop and implement data-driven control measures. For instance, once the weaker spots were identified, then the potential situational mechanisms for reducing and preventing food fraud in the EVOO supply chain could be generated (Lord et al., 2017). For individual actors, it is recommended to select suppliers with care, be part of a transparent chain and implement adequate control measures in order to reduce fraud vulnerability. Future research could focus on an extension of actors. This could concern a larger diversity of countries including those from outside the EU, but also a greater variety of nodes in the chain, e.g. olive growers and food service industry actors.

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## Supplementary materials

**Table S2.1.** The questionnaire of the SSAFE food fraud vulnerability assessment tool.

| No. | Question |
|---|---|
| 1 | In your opinion and experience, is it simple or complex to adulterate EVOO [a]? |
| 2 | Is the technology and knowledge to adulterate raw material (EVOO) generally available? (Only for producers and food manufacturers) |
| 3 | How easily can adulteration of raw material (EVOO) be detected and with what kind of methods? (Only for producers and food manufacturers) |
| 4 | How available is the technology and knowledge to enable the adulteration of your final product? |
| 5 | How easily would adulteration of your final products be detected and what kind of methods are available? |
| 8 | How would you describe the production lines / processing activities of your company? (Only for producers and food manufacturers) |
| 9 | How would you describe your part of the food supply chain? |
| 10 | Have fraudulent incidents of EVOO been reported? |
| 11 | Have fraudulent incidents of similar final products been reported? |
| 12 | How would you define the supply and pricing of EVOO? |
| 13 | Do special attributes or components determine the value of EVOO? |
| 14 | How would you describe the economic condition of your company? |
| 15 | What are the characteristics of the business strategy of your company? |
| 16 | How would you describe the ethical business culture of your company? |
| 17 | Has your company been involved in criminal offences previously? |
| 18 | How would you rate the corruption level (according to the Transparency International Corruption Perception Index) in the countries where your company is active? (www.transparency.org/cpi) |
| 19 | How would you describe the financial strains imposed by your company on your direct supplier(s)? |
| 20 | How would you describe the economic health of your direct supplier(s)? |
| 21 | What are the characteristics of the business strategy of your direct supplier(s)? |
| 22 | How would you describe the ethical business culture of your direct supplier(s)? |
| 23 | Has your direct supplier(s) been involved in criminal offences previously? |
| 24 | Has your direct supplier(s) been a victim of food fraud committed by their suppliers, customers or other parties? |
| 25 | How would you rate the corruption level (according to the Transparency International Corruption Perception Index) in the countries where your direct supplier(s) and customers are active? |
| 26 | How would you describe the economic health across your sector of the food supply chain (i.e. your company and your direct competitors)? |
| 27 | Has your customer(s) been involved in criminal offences previously? |
| 28 | How would you describe the ethical business culture across your sector of the food supply chain (i.e. your company and your direct competitors)? |
| 29 | How common are criminal offences across your sector of the food supply chain? (i.e. your company and your direct competitors)? |
| 30 | How would you rate the level of competition across your sector of the food supply chain (i.e. your company and your direct competitors)? |
| 31 | Are there price differences as a result of regulatory differences across countries? |
| 32 | How would you rate your company's raw material (EVOO) monitoring control systems' ability to detect fraud? (Only for producers and food manufacturers) |
| 33 | Are the fraud monitoring tasks of raw material (EVOO) control system verified in your company? (Only for producers and food manufacturers) |
| 34 | How would you describe the fraud related parts of your final product monitoring control system of your company? |
| 35 | Are the fraud monitoring tasks of your final product control system verified in your company? |
| 36 | How extensive is the information system for internal control of mass balance flows in your company? |
| 37 | How extensive is the tracking & tracing system of your company? |
| 38 | Is integrity screening of employees' common procedure in your company? |
| 39 | Is there an ethical code of conduct or guideline in place and embedded in your company? |
| 40 | Is there a whistle blowing system (system for reporting assumed fraudulent activities) in place in your company? |
| 41 | Do contractual requirements with your direct suppliers include elements that limit opportunities for fraud? |

| No. | Question |
|---|---|
| 42 | What features the fraud control system of your direct supplier(s)? |
| 43 | How extensive is the information system for control of mass balance flows of your direct supplier(s)? |
| 44 | How extensive is the traceability system of your direct supplier(s)? |
| 45 | How would you describe the social control and transparency of actions across your supply chain? |
| 46 | How well established is guidance for fraud prevention and control across your sector of the food supply chain? (i.e. your company and your direct competitors) |
| 47 | How would you describe your national food policy? (i.e. country-level) |
| 48 | How well are fraud prevention laws enforced locally? |
| 49 | How well are fraud related laws enforced across your international supply chain? |
| 50 | Does your company have fraud contingency measures in place? |

[a] EVOO, extra virgin olive oil.



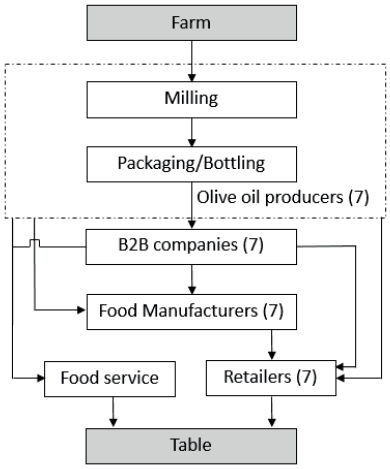


**Figure S2.1.** Roadmap of the extra virgin olive oil supply chain from farm to table. B2B refers to business-to-business.

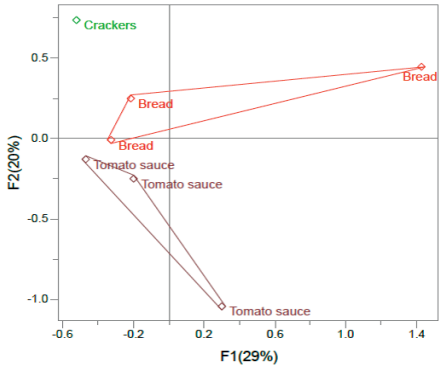


**Figure S2.2.** Scores plot of multiple correspondence analysis on the responses of seven food manufacturers.

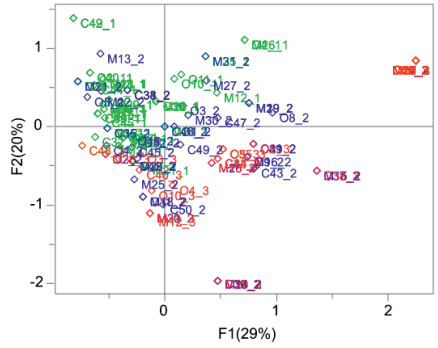


**Figure S2.3.** Loadings plot of multiple correspondence analysis on the responses of seven food manufacturers. Higher, intermediate and lower vulnerability scores are coloured red, blue and green, respectively. O refers to opportunities; M refers to motivations; C refers to control measures; numbers after the letter refer to question number_vulnerability level.

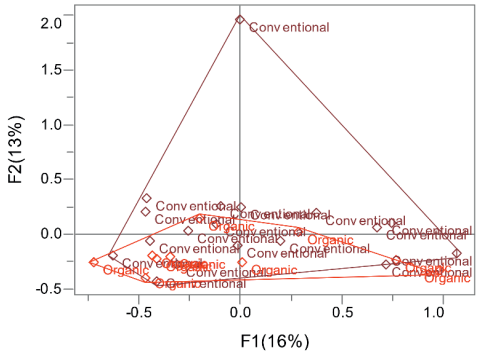


**Figure S2.4.** Scores plot of multiple correspondence analysis on the responses of 10 organic EVOO companies and 18 conventional EVOO companies. EVOO refers to extra virgin olive oil.

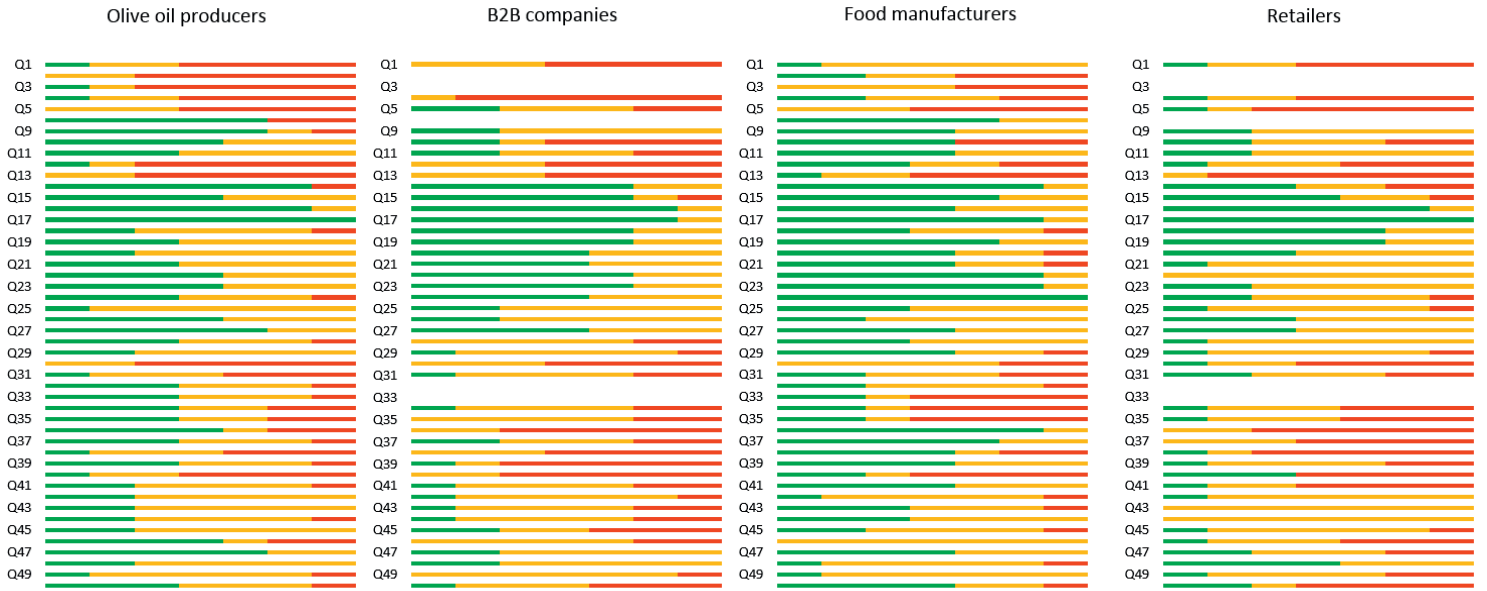


**Figure S2.5.** Overview of low-medium-high vulnerability frequencies of the answers for individual fraud factors of the fraud vulnerability assessments for the four tier groups. Green, orange and red refer to low, medium and high level of vulnerability, respectively. B2B refers to business-to-business.

# Chapter 3

## Discrimination of processing grades of olive oil and other vegetable oils by monochloropropanediol esters and glycidyl esters

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**Abstract**

In this study, the processing derived contaminants 2- and 3-monochloropropanediol (2- and 3-MCPD) esters and glycidyl esters (GEs) were analysed in 84 oil samples by gas chromatography-tandem mass spectrometry for the discrimination of processing grades of olive oils as a potential authentication tool. Concentrations of 2- and 3-MCPD esters and GEs varied in the ranges 0-6 mg/kg, 0-1.5 mg/kg, and 0-1 mg/kg oil, respectively. The concentrations of the three compounds in lower grade olive oils were significantly higher ($p < 0.05$) than that in EVOO. A similar difference was observed for other refined and cold-pressed vegetable oils. The limit of fraud detection of lower grade oils in EVOO was 2% when using 3-MCPD esters, 5% for 2-MCPD esters and 13-14% for GEs based on calculations of virtual mixtures of the current sample set. Especially the MCPD esters appear very specific and promising for the detection of lower processing grade oils in EVOO.

**Keywords**: Authenticity; fraud; GC-MS/MS; processing contaminants.

### 3.1. Introduction

In recent decades, the popularity of olive oil has seen a rise worldwide due to its perceived health benefits. This holds especially for extra virgin olive oil (EVOO). The easiness to adulterate EVOO, the difficulty of detection, discrepancy between supply and demand, economic drivers, as well as cultural and behavioural risk factors and lack of control measures contribute to the susceptibility of EVOO to fraud. Three typical olive oil frauds have been reported: (a) the most common way is to blend with other vegetable oils (Jabeur et al., 2014), (b) replacement with vegetable oil with addition of chemical compounds to disguise the adulteration (Roca et al., 2010) and (c) the replacement of EVOO with lower olive oil grades. The latter may include refined olive oil (ROO) (Karbasian et al., 2015) and pomace olive oil (POO) (Škevin et al., 2011), as well as soft deodorised oils (Aparicio-Ruiz et al., 2017).



Since EVOO adulteration is a serious issue, it is desirable to explore innovative and reliable methods to reveal EVOO fraud. In order to guarantee food quality and expose olive oil fraud issues, many analytical strategies have been reported over the last few decades (Luykx & Van Ruth, 2008). The chemical methods are widely used to identify olive oil adulteration and are based on single or multiple markers (Tres & van Ruth, 2011). Those markers can be divided into two groups. The first one comprises the major components of olive oils, such as triglycerides (Jabeur et al., 2014), fatty acids (Škevin et al., 2011), waxes and sterols (Aparicio & Aparicio-Ruiz, 2000). The second group consists of minor components of olive oils, which includes chlorophylls (Roca et al., 2010), carotenoids (Moyano et al., 2008), phenolic compounds (Lerma-Garcia et al., 2008) and squalene (Ben Mansour et al., 2015).

The detection of ROO in EVOO remains a challenge though. Although one would usually look for the reduction of the desirable EVOO compounds, the naturally present variation of those compounds would still result in a relatively wide acceptable range. Since many compounds are removed during the refining process, very few unique characteristics are left. The only ones to be considered are compounds that are formed in the refining process and are persistent throughout all the steps in the refining process. Monochloropropanediol (MCPD) esters and glycidyl esters (GEs) may be that kind of compounds, but few studies have looked into these compounds in olive oils so far.

MCPD esters are minor compounds derived from diacylglycerols and are chlorinated through refining processes and special treatments. Free 3-MCPD was reported first in acid-hydrolysed vegetable proteins (Velisek et al., 1980). It was also detected at an early stage in rapeseed oil adulterated with aniline and refined with hydrochloric acid (Gardner et al., 1983). GEs are minor compounds formed from monoacylglycerols in the refining process. Although many studies looked into the formation of MCPD esters and GEs, a definite and generally accepted mechanism has not been established yet (Zhao et al., 2016). Some studies have focused on factors that influence the formation of those compounds. According to Weißhaar (2008), temperature, pressure, water activity and other technical parameters during the refining

process trigger the formation of 3-MCPD esters. Deodorisation, a process which requires usually high temperatures, result in considerable formation of 3-MCPD esters and GEs especially when the temperature reaches up to 230 ºC (Hrncirik & van Duijn, 2011). Zelinkova et al. (2006) reported also an increase during prolonged heating of rapeseed oils at 230˚C, but in contrary a reduction under the same conditions for olive oils.

MCPD and glycidol are not harmless compounds. Various toxicological studies revealed their toxicity. The International Agency for Research on Cancer (IARC) characterises 3-MCPD as possibly carcinogenic to humans, based on cancer incidents caused by 3-MCPD in laboratory animals (IARC, 2012). The European Food Safety Authority (EFSA, 2016) concluded that the critical effect of 3-MCPD is kidney toxicity and glycidol has potential genotoxic and carcinogenic effects. Meanwhile, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2016) concluded that glycidol is a genotoxic compound. Based on those research data, the EFSA (2016) established a tolerable daily intake (TDI) of 0.8 µg/kg body weight (bw) per day for the sum of free 3-MCPD and 3-MCPD esters. More recently the JECFA (2016) announced a regulatory maximum TDI of 4 µg/kg bw per day for free 3-MCPD and 3-MCPD esters. In view of insufficient toxicokinetic data, no health-based guidance value could be established for 2-MCPD. Due to genotoxicity and carcinogenicity, it is not appropriate to establish a health-based guidance value for glycidol. Therefore, the margin of exposure (MoE) approach was applied, MoE estimates were calculated by dividing the reference point of T25 10.2 mg/kg bw per day by the exposure levels (EFSA, 2016; JECFA, 2016). MCPD and glycidol may be useful for discrimination of processing grades of oils, but we have to consider that there is also an unusual dark side to this group of markers.

In the current study, we aim to explore the processing derived contaminants MCPD esters and GEs for the discrimination of processing grades of olive oils as potential authentication tool. The results will be compared to the levels of these compounds in some other vegetable oils.

## 3.2. Materials and Methods

### *3.2.1. Samples*

Eighty-four oil samples, the authenticity of which was confirmed in various preliminary tests, which are based on fatty acid compositional fingerprinting, volatile organic compound fingerprinting and spectroscopic tests measuring K232, K268 and ΔK values (IOC, 2015), were selected from a pool of 400 oil samples. The large oil set was supplied by many olive oil producers, traders and retailers in the EU. For the current study, the selected set of 84 samples comprised 30 EVOO samples (Origin: 6 Greece, 14 Italy, 6 Spain, 4 EU), 18 ROO samples (Origin: 4 Italy, 5 Spain, 9 EU), 16 POO samples (Origin: 6 Italy, 6 Spain, 4 EU), 8 cold pressed vegetable oil samples (C-VEGE) (Origin: 8 EU) and 12 refined vegetable oil samples (R-VEGE)

(Origin: 1 South America, 1 Africa, 10 EU). C-VEGE consisted of 7 cold-pressed rapeseed oils and 1 sunflower oil. R-VEGE consisted of 3 refined rapeseed oils, 4 peanut oils and 5 sunflower oils.

Blends of one EVOO and one ROO sample, as well as one EVOO and one POO sample were manually prepared (mix1 and mix2) and comprised 10%, 20%, 30%, 40% and 50% of ROO or POO in EVOO.

Prior to analysis, samples were stored in capped bottles, which were kept in the dark at room temperature until analysis.



### *3.2.2. Reagents and standards*

**Reagents:** tetrahydrofuran, anhydrous; methanol, analytical grade; n-heptane, analytical grade; acetone, analytical grade; toluene, analytical grade; sulfuric acid (purity ≥ 95%); sodium hydrogen carbonate (purity≥ 99%); sodium sulphate (purity ≥ 99%); phenylboronic acid (PBA) (purity ≥ 97%); sodium bromide (purity ≥ 99.5%), all reagents upon were purchased from Sigma-Aldrich. Ultra-pure water obtained from a Millipore Milli-Q purification system (Millipore Co., Bedford, MA, USA). 1,2,3,4-tetrachloronaphthalene (1,2,3,4-TCN) 10 μg/mL in iso-octane were obtained from Agilent.

**Standards:** 1,2-bis-palmitoyl-3-chloropropanediol [PP-3-MCPD] (98%, CAS: 51930-97-3), 1,3-dipalmitoyl-2-chloropropanediol [PP-2-MCPD] (98%, CAS: 169471-41-4) and glycidyl palmitate [Gly-P] (98%, CAS: 7501-44-2) were all purchased from Toronto Research Chemicals (Toronto, Canada). The stock solutions of 1 mg/mL in toluene were prepared for those three-native standards PP-3-MCPD, PP-2-MCPD and Gly-P.

1,2-dipalmitoyl-3-chloropropanediol-d5 [PP-3-MCPD-d5] (1.0 mg/mL in Toluene) (99.5%, CAS: 1185057-55-9), 1,3-dipalmitoyl-2-chloropropanediol-d5 [PP-2-MCPD-d5] (1.0 mg/mL in Toluene) (97.4%, CAS: 1426395-62-1) and glycidyl palmitate-d5 [Gly-P-d5] (1.0 mg/mL in Toluene) (99.5%, CAS: 1794941-80-2) were purchased from Chiron AS (Trondheim, Norway), supplied by Campro Scientific (Veenendaal, The Netherlands).

For preparation of the working solutions for the calibration curve, a modified method of the AOCS Cd29a_13 method (AOCS, 2013) was followed, the changes are listed in **Table 3.1**.

**Table 3.1.** Information on the nine working solutions.

| Standards | Preparing methods |
|---|---|
| Calibration I (PP-3-MCPD, 55 µg/mL) | Pipette 550µL of the stock solution (Solutions: Standard, PP-3-MCPD) into a 10 mL volumetric flask and fill up to the mark with the solvent. |
| Calibration II (PP-3-MCPD, 5.5µg/mL) | Pipette 1 mL of the Calibration I solution into a 10 mL volumetric flask and fill up to the mark with the solvent |
| Calibration III (PP-2-MCPD, 55 µg/mL) | Pipette 550µL of the stock solution (Solutions: Standard, PP-2-MCPD) into a 10 mL volumetric flask and fill up to the mark with the solvent. |
| Calibration IV (PP-2-MCPD, 5.5 µg/mL) | Pipette 1 mL of the Calibration III solution into a 10 mL volumetric flask and fill up to the mark with the solvent |
| Calibration V (Gly-P, 100 µg/mL) | Pipette 1 mL of the stock solution (Solutions: Standard, Gly-P) into a 10 mL volumetric flask and fill up to the mark with the solvent |
| Calibration VI (Gly-P, 10 µg/mL) | Pipette 1 mL of the Calibration V solution into a 10 mL volumetric flask and fill up to the mark with the solvent |
| Internal standard I (PP-3-MCPD-d5, 40 µg/mL) | Pipette 400 µL of the stock solution (Solutions: Standard, PP-3-MCPD-d5) into a 10 mL volumetric flask and fill up to the mark with the solvent. |
| Internal standard II (PP-2-MCPD-d5, 40 µg/mL) | Pipette 400 µL of the stock solution (Solutions: Standard, PP-2-MCPD-d5) into a 10 mL volumetric flask and fill up to the mark with the solvent. |
| Internal standard III (Gly-P-d5, 40 µg/mL) | Pipette 400 µL of the stock solution (Solutions: Standard, Gly-P-d5) into a 10 mL volumetric flask and fill up to the mark with the solvent |

PP-3-MCPD, 1,2-bis-palmitoyl-3-chloropropanediol; PP-2-MCPD, 1,3-dipalmitoyl-2-chloropropanediol; Gly-P, glycidyl palmitate.

### *3.2.3. Sample preparation*

The general principle of this method is the conversion of glycidyl esters into 3-monobrompropanediol esters with sodium bromide and diluted sulphuric acid up front, which is then followed by an acid catalysed hydrolysis of all esters (16h, 40°C, with sulphuric acid in methanol) in order to release the bound contaminants (2-MCPD, 3-MCPD and glycidol). Finally, the free contaminants are derivatised with phenylboronic acid prior to gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis.

The extraction method of the 2-, 3-MCPD esters and GEs, which was used in this experiment, followed basically the AOCS official method Cd29a_13 (AOCS, 2013) with some slight modifications. Firstly, GC-MS/MS was used for the measurement instead of GC-MS, for additional selectivity. Secondly, an extra internal standard PP-2-MCPD-d5 were applied, so that 2-MCPD could be quantified on its own internal standard, which improves the accuracy of 2-MCPD results. The third modification concerns the reconstitution of the dry residue in the final step in iso-octane instead of n-heptane. Fourthly, before transferring the supernatant to the GC vial, 50 µL 1,2,3,4-TCN solution was added to each sample as a syringe standard. This syringe standard was used to monitor the GC injection reproducibility and sensitivity drift of the MS/MS instrument over time.

### *3.2.4. GC-MS/MS*

The instrumental measurement was performed according to the AOCS official method Cd29a_13 (AOCS, 2013), but with a modification of using GC-MS/MS instead of GC-MS. GC-MS/MS analyses were carried out using a Varian CP-3800 GC and a Varian CP-8400

autosampler, combined with Varian 1200L Quadrupole MS/MS system from Varian (USA), equipped with an DB-35ms fused-silica column (30m x 0.25 mm ID x 0.25µm film thickness), purchased from Agilent (USA).

Gas chromatographic settings: The injector was set at 250°C on pulsed splitless mode with a pulse pressure of 30.0 psi and a pulse duration of 1.20 min (Varian type 1079 EFC). 2 µL samples were injected and carried by 1 mL/min He. The transfer line temperature was set at 300°C. The GC oven temperature program started at 100˚C for 1 min, ramped to 160°C at 20°C/min, hold for 1 min at 160°C, ramped to 180°C at 4°C/min, from 180°C to 340°C at 30°C/min, hold for 4.70 min at 340°C. The total run duration is 20.03 min.



Mass spectrometer settings: The ionisation was set at 70 eV. The monitored mass transitions are described in **Table 3.2**.

**Table 3.2.** The optimised MS/MS settings for the PBA derivative of each compound.

| Compound | Qualifier ion transition | Quantifier ion transition | Collision energy (eV) |
|---|---|---|---|
| 2-MCPD | 196>91, 196>134 | 196.3>104.3 | 16 |
| 2-MCPD-d5 | 201>93.3, 201>136.3 | 201.3>104.3 | 25 |
| 3-MCPD | 196>91 | 196.4>147.3 | 8 |
| 3-MCPD-d5 | 201>93, 201>106 | 201.4>150.3 | 8 |
| 3-MBPD | 240>91, 240>105 | 240.0>147.0 | 12 |
| 3-MBPD-d5 | 245>93 | 245.0>150.0 | 10 |

MCPD, monochloropropanediol; MBPD, monobrompropanediol, PBA, phenylboronic acid.

All oil samples were analysed in duplicate. Data used in this study were the average value of the two replicates of each oil sample.

### *3.2.5. Collision Energy Optimisation*

The most abundant daughter ions were identified by infusion of standard solutions of the PBA derivative of the target analytes. The transitions are mentioned in **Table 3.2**. Afterwards, the optimal collision energies were determined, and these values are mentioned in **Table 3.2**. These mass transitions correspond to the PBA derivatives of the unbound native target compounds and their internal standards.

**Quality Assurance and Quality Control:** The chemical analysis of the samples is performed according to the AOCS official method Cd29a_13 (AOCS, 2013). This method is implemented in our laboratory and experiments demonstrated that the method (with slight modifications as mentioned earlier) meets the method performance requirements as specified in Cd29a_13. Quality is controlled by analysis of blank samples, the use of deuterated internal standards, duplicate analysis of every individual sample and spiking experiments.

### *3.2.6. Statistical Analysis*

One-way analysis of variance (ANOVA) was performed to assess the differences in concentrations of the compounds between types of oils. Subsequently, Tamhane's T2 post hoc tests were applied for pairwise comparisons. $p < 0.05$ was used throughout the study. The statistical analyses resulting in the figures were performed using MATLAB (R2015b, The MathWorks Inc., Natick, MA, USA). The ANOVA and post-hoc tests were carried out in XLstat (Addinsoft, New York, NY, USA).

## 3.3. Results and discussion

### *3.3.1. The QA of the measurements*

Seven compounds (3-MCPD-d5, 3-MCPD, 2-MCPD-d5, 2-MCPD, Glycidol-d5, Glycidol, 1,2,3,4-TCN) were, after sample preparation, analysed by GC-MS/MS. The Total Ion Current (TIC) chromatograms of a standard solution, an EVOO sample and a ROO sample, which were selected randomly from the large sample set, are presented in **Figure 3.1**. 1,2,3,4-TCN is added to every sample prior to injection as an internal standard to monitor the injector and detector performance throughout the sample sequence.

The internal standards (e.g. 3-MCPD-d5), eluted prior to the native compounds (e.g. 3-MCPD). This is in agreement with previous reports (Abd Razak et al., 2012). From **Figure 3.1**, it is clear that traces of 2-MCPD, 3-MCPD and glycidol could be detected in ROO, whereas they were below the limit of quantification in EVOO.

To illustrate assay accuracy, the standard curves were established using the optimised parameters in **Table 3.2**. The results in **Figure 3.2** show the relationship between the ratio of the amount of 3-MCPD to the amount of 3-MCPD-d5 and the ratio of corresponding peak areas. In accordance with the AOCS method Cd 29a-13, 100 ± 10 mg of blank oil (containing no 2-MCPD/3-MCPD esters and GEs) were added to the calibration samples to improve the robustness of the method, since the oil matrix helps to retain the analytes during the evaporation step. The calibration curves of 3-MCPD were determined in triplicate between and after the whole sample series. The values of a and b of those three curves are quite similar only with small differences and all the values of $R^2$ are more than 0.999. According to the AOCS method criteria, the values of b and $R^2$ of the three calibration curves meet the requirements, which are $|b| < 0.02$, $R^2 > 0.99$. It demonstrates the stability of the measurement system, as well as a good repeatability and linearity of this method. The calibration curves were used to calculate the concentrations of the target compounds in the oil samples in the remainder of the study.

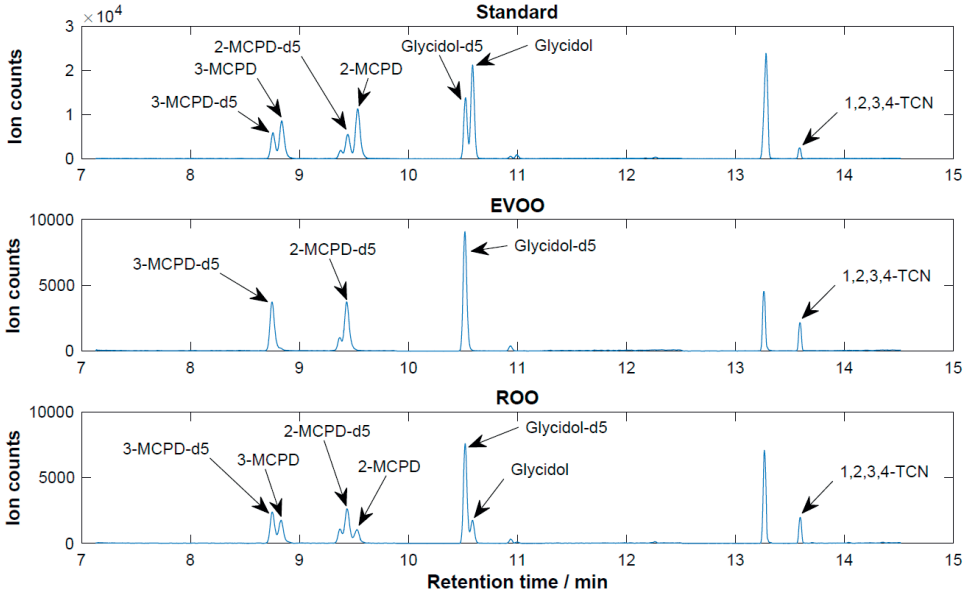


**Figure 3.1.** Chromatograms of the PBA derivatives of the target compounds in a standard solution, an EVOO sample and a ROO sample. EVOO, extra virgin olive oil; PBA, phenylboronic acid; ROO, refined olive oil.

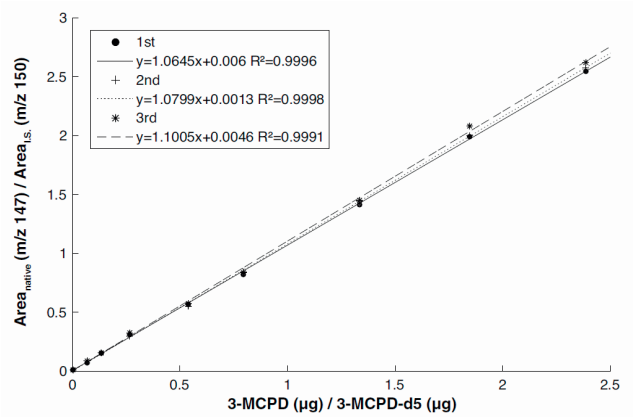


**Figure 3.2.** Three calibration curves of 3-MCPD. X-axis is ratio of the amount of standard (3-MCPD) to the amount of internal standard (3-MCPD-d5). Y-axis is the ratio of corresponding peak areas. MCPD, monochloropropanediol.

### *3.3.2. MCPD esters and GEs contents in relation to vegetable oil type*

The 94 samples, including 30 EVOO, 18 ROO, 16 POO, 8 C-VEGE, 12 R-VEGE and 10 blends, were subjected to GC-MS/MS analysis. The results of the measurements of the 3-MCPD esters, 2-MCPD esters and GEs in the five pure types of oils are presented in box plots (**Figure 3.3**).

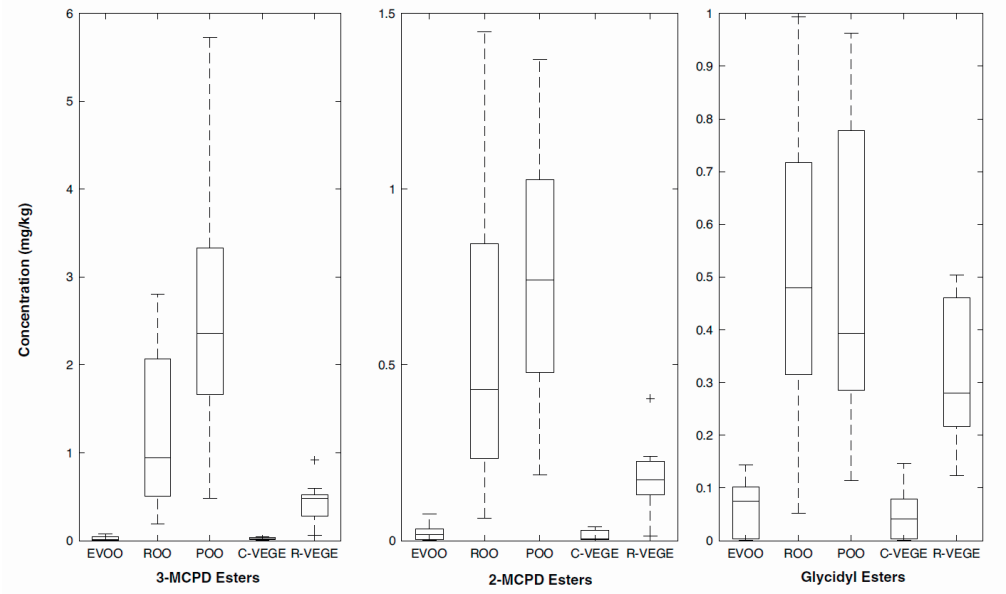


**Figure 3.3.** Box plots of the measurements of the 3-MCPD esters, 2-MCPD esters and glycidyl esters in the five types of oils. The central mark represents the median value, the edges of the line are the minimum and maximum values, the edges of the box are first quartile and third quartile values for the five types of oil. MCPD, monochloropropanediol.

As shown in **Figure 3.3**, the concentrations of the 2- and 3-MCPD esters and the GEs vary in the ranges 0-6 mg/kg, 0-1.5 mg/kg and 0-1 mg/kg, respectively. The concentrations of 3-MCPD esters in samples are at least twice the concentrations of 2-MCPD esters. This may be due to the two positions (1- and 3-) of glycerol available to be chlorinated and resulting in 3-MCPD esters, compared with only one position (2-) resulting in 2-MCPD ester formation. There seems hardly any correlation between MCPD esters and GEs concentrations in the oil samples. It is generally believed that GEs formation to be independent from MCPD ester formation (EFSA, 2016).

**Table 3.3.** Mean concentrations of the 3-MCPD esters, 2-MCPD esters and GEs in the five types of oils and statistical comparisons (ANOVA and Tamhane's T2 post hoc tests, $p < 0.05$).

| | 3-MCPD esters (mg/kg) | 2-MCPD esters (mg/kg) | GEs (mg/kg) |
|---|---|---|---|
| POO | 2.896 [a] | 1.031 [a] | 0.694 [a] |
| ROO | 1.210 [a] | 0.558 [a] | 0.516 [a] |
| R-VEGE | 0.427 [b] | 0.176 [b] | 0.426 [ab] |
| EVOO | 0.026 [c] | 0.021 [c] | 0.059 [b] |
| C-VEGE | 0.022 [c] | 0.015 [c] | 0.049 [b] |
| *p* value | < 0.0001 | < 0.0001 | 0.012 |

Different superscript letters (a, b, c) in a column indicate significant differences from each other. C-VEGE, cold pressed vegetable oil; EVOO, extra virgin olive oil; GEs, glycidyl esters; MCPD, monochloropropanediol; POO, pomace olive oil; ROO, refined olive oil; R-VEGE, refined vegetable oil.

The mean concentrations of the three compounds and significant differences between oil types are presented in **Table 3.3**. Clearly, the concentrations of the three compounds in the cold-pressed oils (EVOO, C-VEGE) are significantly lower than in POO and ROO. R-VEGE shows values between these two groups. The differences between the olive oil grades and the vegetable oil grades are discussed in the following paragraphs.

*3.3.2.1. Comparison of cold pressed olive oil and lower grades of olive oils*

The concentrations of the three compounds in all individual oil samples are presented in **Figure 3.4**. The data have been sorted according to the measured concentration of 3-MCPD esters. The 3-MCPD concentrations measured in the ROO and POO samples are considerable and significantly higher than in EVOO (**Table 3.3**). Experiments with EVOO carried out by Matthäus and Pudel (2013) revealed that in EVOO approx. 1 mg/kg 3-MCPD esters could be generated after 2 hours of high temperature heating. On the other hand, Ozdikicierler et al. (2016) detected no 3-MCPD esters and GEs in POO during steam distillation. These discrepancies with our current results are likely to be due to the fact that in our study samples of commercial origin were used and industrial processes had been applied to those samples. Moreover, it may be due to the degradation of the esters after a long time of heating (Ermacora & Hrncirik, 2014).



According to previous studies, several factors significantly promote the formation of 3-MCPD esters, such as temperature, heating time, pH value, moisture content, pressure and oil types (Hamlet et al., 2015; Ozdikicierler et al., 2016). High temperature is the main factor that can cause 3-MCPD esters and GEs formation (Abd Razak et al., 2012; Hrncirik & van Duijn, 2011). Previous studies also indicated that high temperature is employed in both ROO (Li et al., 2016) and POO (Moral & Mendez, 2006). Addition of a large amount of water in the degumming process and the use of high temperatures in the deodorisation process could also attribute to the higher formation of glycidol in refined oil (Wang et al., 2017). Furthermore, the results of the current study reveal that the contents of 3-MCPD esters and GEs in POO are higher than in most of the ROO samples. Because the refining process takes place in both ROO and POO, the differences originate most probably from the phase before refining. There are three possibly reasons for the high concentrations, 1) an additional high temperature drying treatment happened before the refining process (Moral & Mendez, 2006; Ozdikicierler et al., 2016), 2) the extra exposed time of olive pomace to water, which results in the increased chance of monoacylglycerols and diacylglycerols (DAG) formation which are precursors of bound 3-MCPD and glycidyl esters (Shahidi, 2005), 3) presence of more precursors in the oil (because of its lower quality) prior to refining.

*3.3.2.2. Comparison of cold pressed and refined vegetable oils*

In order to be able to compare the results of the olive oils, a small set of other cold pressed and refined vegetable oils were analysed as well (C-VEGE and R-VEGE). C-VEGE

consisted of seven rapeseed oils and one sunflower oil, the latter of which is number four in **Figure 3.4**. R-VEGE comprised three types of oils: the first three samples are rapeseed oils, number 4-6 and 12 are the peanut oils, number 7 to 11 are the sunflower oils.

It can be seen from **Figure 3.4** that the concentrations of C-VEGE are considerably lower than those of the R-VEGE samples, which is in agreement with the olive oil samples. The most likely explanation is, in line with the olive oils, the high temperature treatment during refining process (Abd Razak et al., 2012; Hrncirik & van Duijn, 2011). It also appears that the concentrations of those three compounds in the refined peanut oil samples have the tendency to be higher than in the other two types of seed oils (particularly the MCPD esters in sample 12 and GEs in sample 6), although the number of peanut samples was limited. The results may be due to the peanut ripeness level result in high DAG levels in peanut oils which are the precursors of the esters (Akhtar et al., 2014; Ayres, 1983).

*3.3.2.3. Comparison of olive oils and other vegetable oils*

Both EVOO and C-VEGE are cold pressed oils, which means that during the whole processing, the extraction temperatures are controlled not exceeding 27 °C for EVOO (Boselli et al., 2009), 75-80°C for rapeseed oil (Cvengros, 1995) and 38–40°C for sunflower oil (Bendini et al., 2011). As shown in **Figure 3.4**, there is no large difference between EVOO and C-VEGE: the value ranges of 3-MCPD esters, 2-MCPD esters and GEs are 0-0.08 mg/kg, 0-0.08 mg/kg and 0-0.15 mg/kg, respectively.

In general, the values of the 3-MCPD esters in ROO and POO are larger than in the other refined vegetable oils. Similar results were found in previous studies, which has already mentioned above, indicating that olive oil and peanut oil generate higher levels of 3-MCPD esters than seed oils (rapeseed oil, sunflower oils) due to elevated DAG levels (Franke et al., 2009; Hamlet et al., 2015; Ramli et al., 2015; Zelinkova et al., 2006).

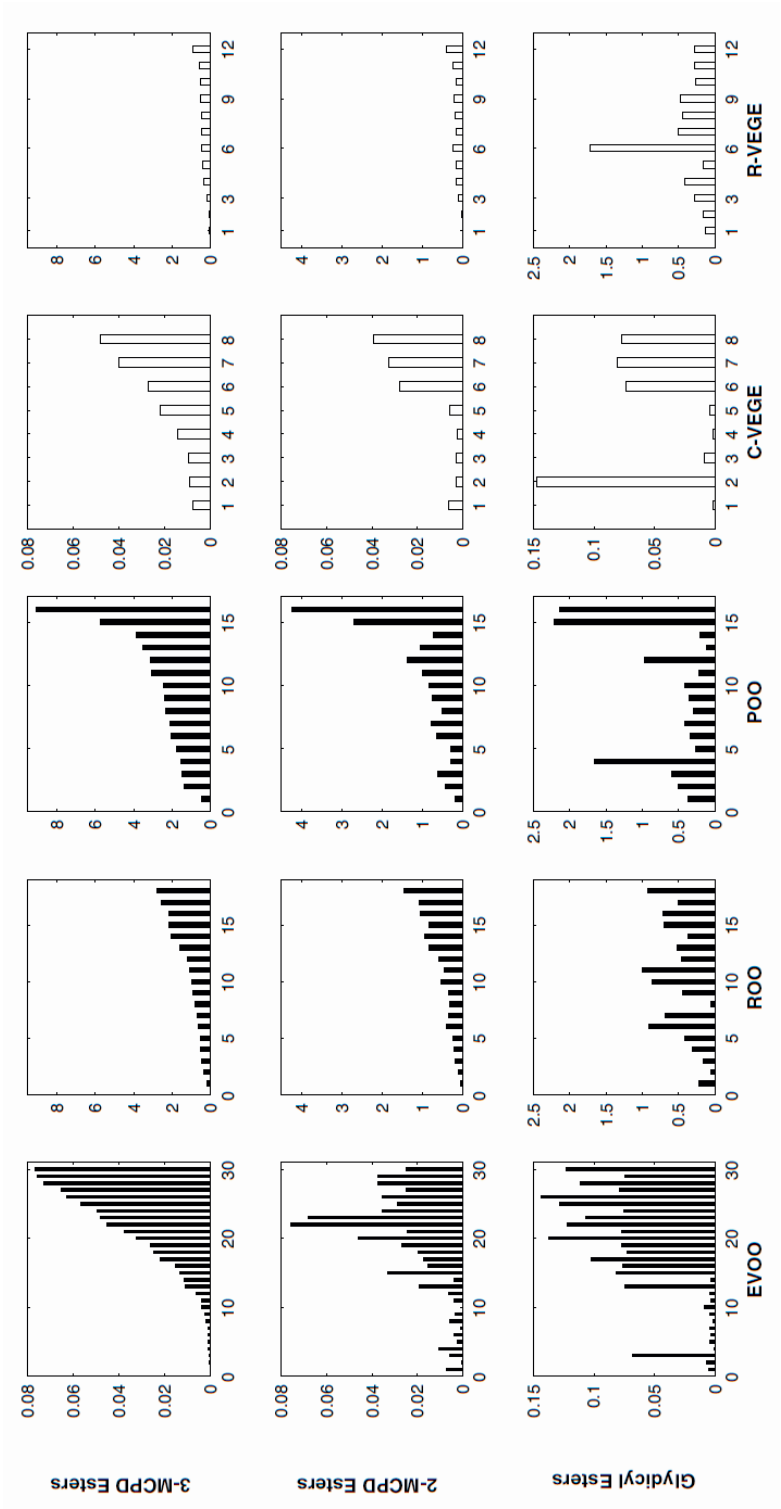


**Figure 3.4.** The concentrations of 3-MCPD esters, 2-MCPD esters and GEs in the individual samples of the five types of oils (mg/kg). Samples are sorted according to an increasing 3-MCPD esters content. GEs, glycidyl esters; MCPD, monochloropropanediol.

### *3.3.3. Authentication considerations*

In order to evaluate the values of the three compounds for authentication practices, all data are presented together in **Figure 3.5a**. This is a three dimension scatter plot based on the measurement data of the 3-MCPD esters, 2-MCPD esters and GEs. In the plot, one outlier sample showing the highest concentrations for all three compounds is lacking for legibility reasons. The EVOO samples, which are located in the origin point, are completely separated from ROO and POO in the three dimensions, see **Figure 3.5c** and **Figure 3.5d**. The plot also shows that 2-MCPD and 3-MCPD concentrations are fairly correlated for ROO but not so much for POO, which shows a more scattered pattern.

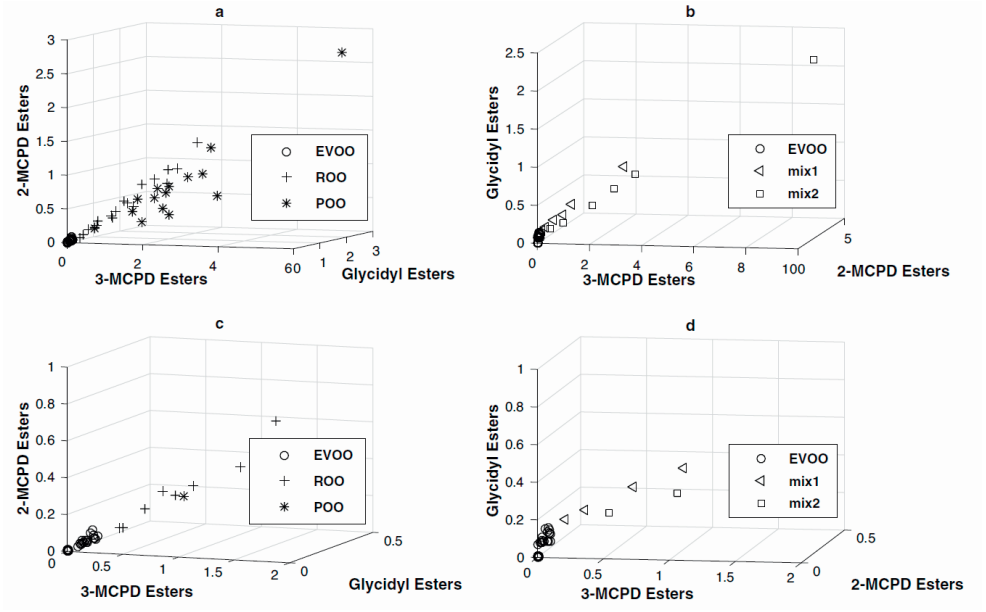


**Figure 3.5.** Three dimension scatter plots of the three compounds in (a) EVOO, ROO and POO; (b) the EVOO samples and mixtures of EVOO/ROO (mix1) and EVOO/POO (mix2); (c) zoomed in section of plot a; (d) zoomed in section of plot b (mix 1 refers to samples comprised 10%, 20%, 30%, 40% and 50% of ROO in EVOO. mix 2 refers to samples comprised 10%, 20%, 30%, 40% and 50% of POO in EVOO). EVOO, extra virgin olive oil; MCPD, monochloropropanediol; POO, pomace olive oil; ROO, refined olive oil.

In order to examine potential matrix effects when examining mixtures, mixtures of EVOO and ROO or POO samples were analysed. These samples, with increasing lower grade oil concentrations show a gradual, linear change in MCPD esters and GEs concentrations (**Figure 3.5b**: mix1 and mix2): $R^2 = 0.98$ for ROO and $R^2 = 0.97$ for POO. Obviously, when mixing EVOO with lower grade olive oils, the concentrations of MCPD esters and GEs is fully determined by the fractions of the two types of oil.

In view of fraud detection, the 95% upper bounds of the EVOO sample set were subsequently calculated in order to set upper limits for real EVOO. This resulted in the

following values: 0.036 mg 3-MPCD/kg oil, 0.028 mg 2-MCPD/kg oil and 0.078 mg GE/kg oil. It is obvious that all 100% ROO or POO would be easily discriminated from the real EVOO, they are all exceeding these values considerably. However, the smarter fraudster will mix oils. The most difficult scenario would be the admixture of an EVOO with relatively low levels of the three compounds for the EVOO population with an ROO or POO with also low levels of the three compounds for their populations. Therefore, for this worst case scenario mixtures of EVOO at the 95% lower bound level and ROO or POO also at their 95% lower bound level were considered. The concentrations in EVOO at 95% lower bound are 0.016 mg 3-MPCD/kg oil, 0.014 mg 2-MCPD/kg oil and 0.041 mg GE/kg oil; for ROO 0.804 mg 3-MPCD/kg oil, 0.365 mg 2-MCPD/kg oil and 0.369 mg GE/kg oil; for POO 1.804 mg 3-MPCD/kg oil, 0.479 mg 2-MCPD/kg oil and 0.328 mg GE/kg oil. For calculation of the 'virtual' concentrations in the worst case scenario mixtures, a linear relationship was considered based on the results of the mixture analysis above. An example for the calculation of the 3-MCPD concentration in a mixture of EVOO and POO is shown below.



$$[3\text{-MCPD (mix)}] = \frac{Mass\ (EVOO)}{Mass\ (EVOO + POO)} \times [3\text{-MCPD (EVOO)}] + \frac{Mass\ (POO)}{Mass\ (EVOO + POO)} \times [3\text{-MCPD (POO)}]$$

The resulting values for the mixtures were compared to the set upper limits, i.e. 0.036 mg 3-MPCD/kg oil, 0.028 mg 2-MCPD/kg oil and 0.078 mg GE/kg oil (see above) in order to determine the lowest levels at which ROO or POO mixed into EVOO could be detected. ROO and POO admixtures to EVOO would be detectable at 2% w/w levels based on 3-MCPD esters, at 5% based on 2-MCPD esters, as well as at 13% (ROO) and 14% (POO) based on GEs.

These results are very promising. MCPD esters seem a very sensitive marker group for detection of ROO or POO in EVOO. The methodology is robust, an internationally accepted method is applied, and the marker itself is robust from a fraud perspective too. It is hard to remove these compounds from ROO or POO. Certainly, a wider set of olive oils, including soft deodorised oils, as well as other vegetable oils will be required for further confirmation. Furthermore, it has to be kept in mind that the manual sample preparation method is fairly labour intensive, although nowadays fully automated sample preparation robots, coupled to GC-MS(/MS) are available for quick and routinely analysis of oil samples allowing a wide application of such oil authenticity testing approach (Jacq et al., 2008; Parkinson et al., 2004).

### *3.3.4. Safety considerations*

As already mentioned MCPD esters have toxic effects, but what about the concentrations measured in the oils? Considering the TDI for 3-MCPD esters (0.8 µg/kg bw per day) (EFSA, 2016), the intake for an adult person (60 kg) would amount 48 µg 3-MCPD esters per day. For the mean olive oil concentrations (Table 1), this intake would be theoretically reached by consumption of olive oil only with an intake of 1845.6 g EVOO/day, 39.6 g ROO/day (ca. 3 table spoons) or 16.8 g POO/day (ca. 1 table spoon). One can imagine that these ROO or POO volumes can be met in practice. For a full evaluation larger sets and more

kinds of oils would need to be examined and compared to consumption data to understand the potential relevance of the 3-MCPD ester contamination in this kind of oils. In addition, 3-MCPD esters may be ingested through other foods, adding up to the total exposure. Nevertheless, it is obvious that the refined oils in the current study may contribute to the daily intake of 3-MCPD esters for users of these oils and most likely to the intake of 2-MCPD esters and GEs as well.

### 3.4. Conclusions

The present study was designed to evaluate the value of MCPD esters and GEs for authentication of the premium processing grades of olive oils and other vegetable oils. Cold-pressed oils showed significantly lower levels of MCPD esters and GEs than their refined counterparts. Calculations revealed that 3-MCPD esters, 2-MCPD esters and GEs would allow detection of adulteration of EVOO with 2%, 5% and 13-14% ROO or POO (95% confidence) based on the current set. Therefore, this approach appears very promising and sensitive to detection of EVOO fraud with lower processing grade oils.

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# Chapter 4

## Rapid, robust distinction of extra virgin olive oil from other olive oil grades using PTR-QiToF-MS fingerprints and one class classification models

This chapter has been submitted for publication as: Yan J., Alewijn M., & van Ruth S. M., Rapid, robust distinction of extra virgin olive oil from other olive oil grades using PTR-QiToF-MS fingerprints and one class classification models.

**Abstract**

To develop a simple, rapid but robust analytical and chemometric procedure to distinguish premium quality extra virgin olive oil (EVOO) from the lower grades of olive oils, proton-transfer-reaction quadrupole ion guide time-of-flight mass spectrometry (PTR-QiToF-MS) was applied. The volatile organic compounds (VOCs) of 390 edible oils were analysed: EVOO (255 samples), refined olive oil (57 samples), pomace olive oil (18 samples) and some other edible oils that could serve as potential adulterants (60 samples). A robust one-class classification approach was applied and four different algorithms were compared. The models' performance was estimated by a training set, additional test sets and validation sets. Principal component analysis indicated a good visual separation between EVOO and non-EVOO samples, and pinpointed the distinct VOCs related to the EVOO identity. In the one class classification evaluation, the k-nearest neighbours model presented best results: the training set and the validation set showed 100% (140 out of 140) and 96% (24 out of 25) correct prediction for the EVOO, the additional test set and the validation set present 95% (104 out of 110) and 96% (24 out of 25) for non-EVOO groups, respectively. For this most successful model, formic acid (m/z 47.012), dimethyl sulphide (m/z 63.026) and hexenal (m/z 99.081) are key compounds for the distinction of EVOO from the other oils. Application of the newly developed and validated method showed that 10% of the 90 retail olive oil samples were highly suspicious. This work indicates that PTR-QiToF-MS combined with the one class classification approach allows rapid and robust discrimination between EVOO and non-EVOO.

**Keywords:** authentication; extra virgin olive oil; food fraud; one-class classification; volatile organic compounds.

### 4.1. Introduction

Analytical fingerprinting combined with multivariate classification is an approach that allows non-targeted discrimination between groups of products and is applied for more complex food authentication questions. This also makes it more difficult for fraudsters to circumvent. Most studies result in chemical or physical profiles which are combined with multivariate classification methods to establish a class discrimination based on product characteristics. Usually a large or smaller database of samples is generated and a model developed (Acierno et al., 2016; Taiti et al., 2015), which can be used to predict class membership of future samples based on their fingerprints. Although many methods have been published, robust validation of these methods are less common (Alewijn et al., 2016).

Two main categories of classification models can be distinguished: multi-class and one-class classification (OCC). Multi-class classification is mostly applied when at least two classes can be meaningfully defined, whereas OCC is a better choice for broad anomaly testing. This means that if the authentication issue is aimed at the characterisation of a single interest category (a positive/target object) and the nature of "the negative class" is diverse and not really one group, then OCC is the best choice. Furthermore, OCC is used in cases when the number of negative class samples are few as well (Oliveri & Downey, 2012; Zhang et al., 2015). Several OCC methods, such as one-class k-nearest neighbours (OCkNN) (Khan & Madden, 2014; Munroe & Madden, 2005), one-class support vector machine (OCSVM) (Krawczyk et al., 2014; Manevitz & Yousef, 2002), principal component analysis residue-based one-class classification (PCAROCC) (Gemperline, 2006), soft independent modelling of class analogies (SIMCA) (Branden & Hubert, 2005; Xu et al., 2011) and OCSVM can be applied for authenticity classification of a single class versus all others.

Extra virgin olive oil (EVOO), extracted by a traditional cold pressing method, is a well-known target for fraudsters. It is more expensive than other vegetable oils, not only because of its nutritional components but also because of its perceived premium organoleptic qualities. Although various kinds of olive oil adulteration exists, unauthorised blending of EVOO with refined olive oil (ROO), pomace olive oil (POO) or lower priced vegetable oils (VEG) has become a big issue in Mediterranean countries (Aparicio & Harwood, 2013), such as Italy, Spain and Greece, the major olive oil producers. Various control methods or detection methods are applied to limit the impact of premium olive oil fraud. However, educated fraudsters will try to circumvent tests all the time. Therefore, the exploration of innovative and reliable analytical methods is helpful to protect the market from EVOO fraud.

Many chemical analytical methodologies have been reported to verify olive oil authenticity based on volatile and non-volatile compounds. Besides the large number of analytical methods based on non-volatile compounds of olive oil (Christopoulou et al., 2004; Yan et al., 2018), it is considered as a promising strategy to assess the volatile organic compounds (VOCs) for high quality olive oil certification (Angerosa et al., 2004). Previous

studies have established that enzymatic reactions are mainly responsible for the formation of the aroma components of olive oil. Furthermore, the aroma also relies on the maturity of the olive fruit, storage time of the olives and extraction technological conditions, such as malaxation time and temperature (Angerosa et al., 1999; Salas et al., 1999). It has also been reported that main VOCs that contribute to a fragrant and delicate flavour are removed during the oil refining process (Morales et al., 1999). Thus, it seems promising to distinguish different processing grades of olive oils by their VOCs.

Proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS), as a high resolution and high sensitivity analytical tool, has been used for real-time analysis of VOCs. It is established on the implementation of chemical ionisation by proton transfer from hydronium ions (Fabris et al., 2010; Taiti et al., 2015). Published studies on PTR-ToF-MS concerned olive fruits (Masi et al., 2015), coffee (Yener et al., 2014), honey (Schuhfried et al., 2016), peppers (Taiti et al., 2015), ham (del Pulgar et al., 2011), milk (Fabris et al., 2010; Liu et al., 2018), chocolate (Acierno et al., 2016) and so on. Moreover, the application of PTR-ToF-MS in olive oil authentication (Sabbatini et al., 2018) and some attempts to link spectral data from PTR-ToF-MS to sensory characteristics (Marone et al., 2017; Taiti & Marone, 2017) were recently carried out. Usually a limited number of samples were considered in these studies. PTR-ToF-MS has not been used so far to distinguish between olive oils of different processing grades in a robust way. Moreover, PTR-ToF-MS coupled with a quadrupole ion guide (PTR-QiToF-MS), as an improved instrument in the market, has been applied in some topics, but not yet for EVOO. Since large quantities of mass data are generated by the PTR-QiToF-MS technique, which adds to the difficulty to evade this kind of tests, chemometric tools are needed to process and eventually interpret the information.

In order to develop a simple, rapid but robust analytical and chemometric procedure, PTR-QiToF-MS fingerprints are used to distinguish EVOO from its lower grades counterparts. This study is sub-divided into three parts: 1) broad discrimination between EVOO and lower grade olive oils, as well as other botanical vegetable oils suitable for EVOO adulteration by VOC patterns; 2) evaluation of four OCC models based on different algorithms and selection of the most solid and robust OCC model; 3) elucidation of the important discriminating VOCs.

## 4.2. Materials and methods

### *4.2.1. Materials*

A total of 390 edible vegetable oils of different varieties, harvested and processed in 2016, were gathered from producers, traders and retailers across Europe in 2016 and 2017. They included a training set (140 EVOO), an additional test set (110 non-EVOO), a validation set (25 EVOO and 25 non-EVOO) and a market survey set (90 EVOO labelled samples).

**Table 4.1.** Sample types and numbers of sample sets used in the study. The training set was used to build one class classification models; the additional test set (T) was measured at the same time as the training set and was used for testing and choosing the final model; the validation set (V) was measured three months after the training set and was used to test performance of the models; the market survey set (M) were commercial retail samples.

| Sample set | Type [a] | Number |
|---|---|---|
| Training set | EVOO | 140 |
| Additional test set | T-ROO | 45 |
| | T-POO | 15 |
| | T-VEG | 50 |
| Validation set | V-EVOO | 25 |
| | V-ROO | 12 |
| | V-POO | 3 |
| | V-VEG | 10 |
| Market survey set | M-EVOO | 90 |
| Total | | 390 |

[a] EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil; VEG, other botanical vegetable oils.



The training set comprised 140 authentic EVOO samples (from reputable sources and additionally verified by fatty acid fingerprints combined with chemometrics, ultraviolet-visible spectra analysis (IOC, 2017)) and 2/3-monochloropropanediol and glycidyl esters analysis (Yan et al., 2018). The 140 samples included 50 EVOO originating from Italy, 33 from Spain, 16 from the EU (not specified), 12 from Greece, 10 from Portugal, 7 from Australia, 4 from the USA, 3 from Turkey, 2 from Uruguay, 1 from Peru, 1 from Morocco and 1 from Tunisia. For internal cross validation samples, 30% of the samples were randomly selected from the 140 authentic EVOO. The additional test set (non-EVOO samples, measured at the same time as the training set) consisted of 45 ROO, 15 POO and 50 other botanical VEG. The latter included 18 rapeseed, 13 sunflower, 7 peanut, 3 walnut, 3 hazelnut, 2 avocado, 2 sesame and 2 grapeseed oils. The validation set was measured completely independent of the training and additional test set, i.e. three months after the analysis of the training set comprised 25 EVOO, 12 ROO, 3 POO and 10 VEG (3 rapeseed, 2 walnut, 1 sunflower, 1 avocado, 1 almond, 1 linseed and 1 sesame). The market survey set included 90 commercial retail EVOO labelled samples, which were produced in Italy (32), Spain (27), EU (18), Greece (8), Tunisia (3) and Portugal (2).

**Table 4.1** summarises the information of all the analysed samples. Prior to analysis, all the samples were stored in capped bottles and were kept in the dark at room temperature until analysis.

### *4.2.2. PTR-QiToF-MS analysis*

For each oil sample, 5 mL of oil was transferred into a 250 mL flask which was capped with a silicone septum. The closed flask was immersed in a water bath at 30 ºC for 30 min to

let the headspace air and the sample equilibrate before analysis. Two replicates of each sample were measured with the same method on different days.

The headspace measurements of all oil samples were performed by using a PTR-QiToF-MS instrument (Ionicon Analytik GmbH, Innsbruck, Austria). The instrumental conditions for the proton transfer reaction were as follow: drift voltage 999 V, drift temperature 61 ± 1 °C, drift pressure 3.803 mbar affording an E/N value of 133.7 ± 1 Townsend (1 Td = $10^{-17}$ $cm^2$ $V^{-1}$ $s^{-1}$).

The laboratory air was measured for the first 10 s as a blank before each sample, after that, the flask was delivered directly to the inlet of the PTR-QiToF-MS system. The headspace VOCs were transferred to the PTR-QiToF-MS via a heated (60 ± 0.5 °C) peek inlet tube at an air flow rate of 61 ± 2 mL/min for 1 min. Each sample was measured for 30 s at an acquisition rate of 1 spectrum per second. The random sequence was used to avoid possible systematic memory effects. Results were stored in the system automatically.

### *4.2.3. VOCs data pre-processing*

All the raw data obtained from the PTR-QiToF-MS machine were integrated by PTRwid software (Holzinger, 2015). The unified mass list with the ion count per second (cps) of each sample were provided after the autonomous mass scale calibration, as described by Holzinger (2015). The average of the thirty sample scans and the average of the ten blank scans were calculated separately. The VOCs concentrations in ppbv (parts per billion by volume) were calculated from cps according to equation (1), which is shown below (Cappellin et al., 2012b), using a constant reaction rate coefficient (k = 2 × $10^{-9}$$cm^3$/s), $[H_3O^+]_{measured}$ indicates the measured concentration (cps) of isotopic of protonated water, at m/z 21.022. Subsequently, the unit of $cm^{-3}$ was converted to ppbv, on the basis of ideal gas equation (2).

$$[VOC] = \frac{1}{kt} \times \frac{[VOC{\cdot}H^+]_{measured}}{[H_3O^+]_{measured} \times 487} \times \frac{\sqrt{(m/z)_{H_3O^+}}}{\sqrt{(m/z)_{VOC{\cdot}H^+}}} \quad (1)$$

$$PV = nRT \quad (2)$$

After unit conversion, the blank corrected results of each sample were calculated by subtracting the average of each sample's 10 blank cycles. The replicates of each sample were checked by using autocorrelation (Box & Jenkins, 1976) and the sample was removed when the correlation value was below 0.9. Averages were calculated based on the data of two replicate measurements.

Ions not related to the sample, such as $N_2^+$, $NO^+$, $O_2^+$ and water clusters [$H_2O^+$, $H_3^{[18]}O^+$, $(H_2O)_2{\cdot}H^+$, $H_2O{\cdot}H_2^{[18]}O{\cdot}H^+$, $(H_2O)_3{\cdot}H^+$] signals at m/z 28.005, 29.997, 31.989, 18.010, 21.022, 37.028, 39.032 and 55.039, respectively, were removed. After the data pre-processing, 295 mass peaks in the range from m/z 18.033 to m/z 207.204 remained. The data matrix of the

training set consisted of 295 (mass peaks) × 140 (samples) data points, the data matrix of the additional test set of 295 (mass peaks) × 110 (samples) points, the data matrix of the validation set of 295 (mass peaks) × 50 (samples) points and the data matrix of the market survey of 295 (mass peaks) × 90 (samples) points, which were all subjected to further statistical analysis.

#### *4.2.4. Statistical analysis*

In this study, the model evaluation sticks to the steps in **Figure 4.1**. The full mass spectral data matrices were subjected to PCA for a preliminary inspection. PCA was carried out by Pirouette 4.5 (Infometrix Inc., Woodville, WA, USA). Then OCC was applied to distinguish between EVOO and three groups of non-EVOO samples comparing several algorithms, i.e. OCkNN, OCSVM, PCAROCC and SIMCA. All those calculations were performed by scripts developed in R 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria). The method on how to predict the authenticity of a sample based on each algorithm can be found in our previous work (Yan et al., 2019). Then, the percentages of samples predicted as EVOO in each sample set were calculated. The 1-10 consecutive factors and four data pre-processing methods (mean-centring, $\log^{10}$ scaling, auto-scaling and row-wise normalisation) were applied in conjunction with those four algorithms (OCkNN, OCSVM, PCAROCC and SIMCA) to build various models. Any model was built with the training set (140 EVOO) with application of 25 times repeated leave-30%-out random cross validation. The best model was assigned to be the model performing best for each algorithm with the best setting of number of factors and pre-processing methods. Afterwards, the additional test set was subjected to the corresponding classification models for testing and the best model was selected in terms of minimum false EVOO assignments. Subsequently, the model with best performance was verified based on the results of validation set.

Afterwards, the VOCs contributing to the separation with the best model were examined using a receiver operating characteristic (ROC, or area under the ROC curve, AUC) curve based approach. A ROC curve is a graphical plot illustrating the performance of a binary classifier by varying its discrimination threshold parameter (Yenigun et al., 2017), moreover, the AUC value was commonly applied to represent expected performance of the classification. The higher the value, the better the performance of the discrimination. This value was applied to solve a binary comparison problem previously, which shows similarities with the one-class classification method (EVOO versus all other oils) in the current study. In order to specify the possible adulterants to EVOO, three sub-groups of adulterants (ROO, POO and VEG) were used to compare with EVOO individually to test the performance of this binary comparison technique.

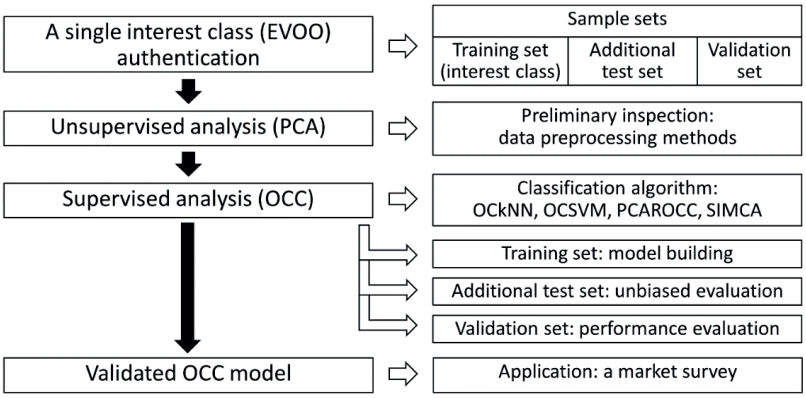


**Figure 4.1.** Roadmap for the evaluation of one class classification methods. EVOO, extra virgin olive oil; OCC, one-class classification; OCkNN, one-class k-nearest neighbours; OCSVM, one-class support vector machine; PCA, principal component analysis; PCAROCC, principal component analysis residue-based one-class classification; SIMCA, soft independent modelling of class analogies.

In order to find important VOCs for discrimination, the ROC curves were created using leave-one-mass-out spectral data for binary classifier (EVOO versus ROO, EVOO versus POO, EVOO versus VEG). Afterwards, the AUC values of each curve were calculated. All those calculations were performed by scripts developed in R 3.3.3. Since there are 295 VOCs, 295 AUC values were collected for each binary classification. Therefore, the important VOCs were verified based on the following rule: the lower the AUC value, the worse the discrimination performance and the more the important the removed VOC.

Subsequently, the significant difference of concentrations of those key VOCs in different processing grades of olive oils and other vegetable oils was assessed using analysis of variance (ANOVA, 95% confidence level, $p < 0.05$) and pairwise comparison was performed with least significant difference tests (LSD). ANOVA was performed by SPSS statistic software version 23 (IBM, Chicago, IL, USA).

## 4.3. Results and discussion

### *4.3.1. PTR-QiToF-MS profiles of VOCs*

The 390 oil samples were subjected to PTR-QiToF-MS analysis and 295 masses in the range of m/z 18.033 to 207.204 were acquired for each sample. The $\log^{10}$ transformed mass spectra for four groups from the training set and the additional test set, i.e. EVOO (n = 140), T-ROO (n = 45), T-POO (n = 15), T-VEG (50), are presented in **Figure 4.2**.

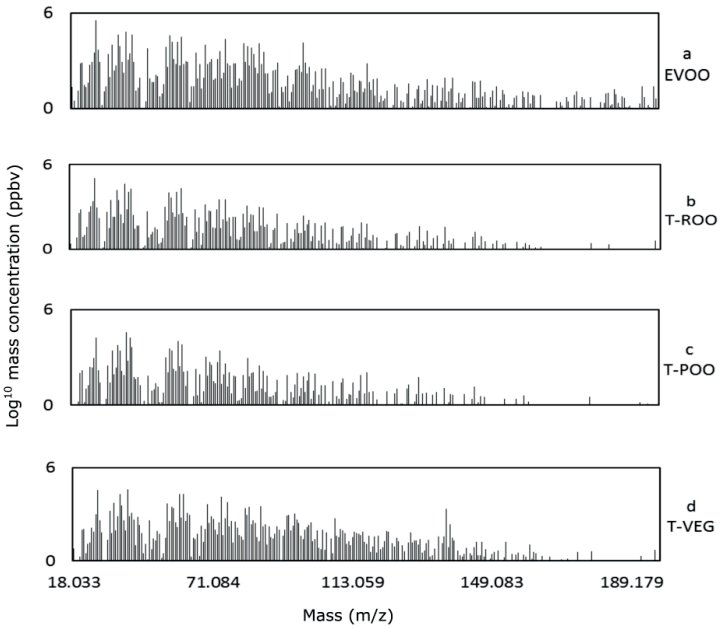


**Figure 4.2.** $Log^{10}$ transformed average spectral profiles of VOCs of four sample groups (a EVOO, b T-ROO, c T-POO and d T-VEG). VOCs, volatile organic compounds. For sample group codes, see Table 4.1.

According to the PTR-QiToF-MS spectral profiles, the mass intensities of the first half range from m/z 18.033 to m/z 113.059 are higher than those in the second half mass range in each sample group, which is ranging from m/z 113.096 to m/z 207.204. The total sums of masses ranging from 18.033 to 113.059 in EVOO, T-ROO, T-POO and T-VEG are 780 ppmv, 276 ppmv, 127 ppmv and 217 ppmv, respectively. This indicates that the concentration of VOCs in EVOO in general is considerably higher than for the other three groups. Meanwhile, the total sums of masses ranging from m/z 113.096 to m/z 207.204 are 2 ppmv, 0.4 ppmv, 0.4 ppmv and 3 ppmv, respectively. These results suggest that the distinct differences between the groups are promising for further investigations of the ability to separate the EVOO from the non-EVOO samples.

### *4.3.2. Unsupervised statistical comparison of VOCs: PCA*

In order to explore the data and to obtain a preliminary indication of the differences between the training set (EVOO) and the additional test set (T-ROO, T-POO and T-VEG), all 295 mass spectra obtained from PTR-QiToF-MS were subjected to PCA.

Four pre-processing methods (mean-centring, $log^{10}$ scaling, auto-scaling and row-wise normalisation) and combinations thereof were applied to perform the PCA analysis. After comparison of those methods, mean-centring and $log^{10}$ scaling is the best combination of the pre-processing methods. The PCA scores of the four oil groups show that the first 10 principal

components (PCs) explained 91% of the total variance. The first three dimensions of scores plot and loadings plot are presented in **Figure 4.3**.

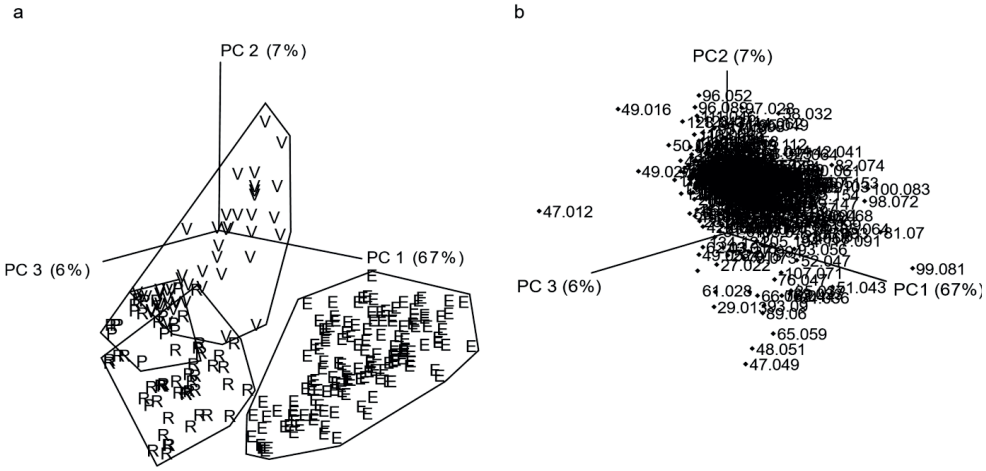


**Figure 4.3.** Scores plot (a) and loadings plot (b) of the first three dimensions of the PCA (data pre-processing = mean-centring and $\log^{10}$ scaling) of the mass spectral data of the olive oil training set (E refers to EVOO) and additional test set (R refers to T-ROO; P refers to T-POO; V refers to T-VEG). For sample group codes, see Table 4.1.

As can be seen in **Figure 4.3a**, a distinct separation between EVOO and the other three groups appears: EVOO is located in the bottom right corner, while the other three groups are located in the top and bottom left sides, with some overlap among the other three groups. T-ROO samples are located closer to the EVOO sample group than the other two groups, which indicates greater similarity. T-VEG is stretching along PC2, with those located at the lower end of the plot showing overlap with T-ROO and T-POO, and they are primarily sunflower oils.

Furthermore, the loadings plot shows the importance of each variable to the corresponding samples in scores plot. In **Figure 4.3b**, the loadings plot shows that all masses are scattered in the plot and make a contribution to the discrimination. And it is obvious that masses m/z 47.012, 47.049, 48.051, 49.016, 49.027, 51.043, 65.059, 66.062, 75.044, 81.07, 85.064, 98.072, 99.081, 100.083 contribute to the EVOO discrimination. Mass m/z 47.012 is the one associated with high concentration in the non-EVOO sample group. All other masses show higher concentrations in the EVOO sample group.

The separation of the EVOO and other samples observed in this exploratory multivariate statistical analysis indicates the presence of underlying features which allows distinction of EVOO. Further dedicated classification and validation statistics help to develop a solid method which can be applied in practice and which will be presented in the following section.

### *4.3.3. Supervised statistical comparison of VOCs: OCC*

#### *4.3.3.1. Classification models based on four algorithms*

To evaluate the ability to discriminate EVOO from lower grade counterparts and other vegetable oils, four algorithms (SIMCA, PCAROCC, OCkNN and OCSVM) were applied to estimate OCC models for EVOO authentication. These models were constructed on the basis of the training set (EVOO) using various data pre-processing methods and considering various model parameters. The best performing model for each algorithm is presented in **Table 4.2**. Their prediction ability is further detailed by their classification rates for the additional test set (T-ROO, T-POO and T-VEG). Their performances are specified by the results of the independent validation set (V-EVOO, V-ROO, V-POO and V-VEG).

**Table 4.2.** Percentages of samples predicted as EVOO in the four OCC models [a].

| Classification algorithm | | OCkNN | OCSVM | PCAROCC | SIMCA |
|---|---|---|---|---|---|
| Parameters [b] | | 2 | 0.002 | 4 | 3 |
| Mean-centring [c] | | √ | √ | √ | √ |
| $Log^{10}$ scaling | | √ | √ | √ | √ |
| Auto-scaling | | × | × | √ | √ |
| EVOO [d] (n = 140) (%) | | 100 | 100 | 99 | 99 |
| Additional test set (%) | T-ROO (n = 45) | 9 | 9 | 9 | 4 |
| | T-POO (n = 15) | 0 | 0 | 0 | 0 |
| | T-VEG (n = 50) | 4 | 6 | 2 | 10 |
| Validation set (%) | V-EVOO (n = 25) | 96 | 92 | 88 | 84 |
| | V-ROO (n = 12) | 8 | 8 | 8 | 0 |
| | V-POO (n = 3) | 0 | 0 | 0 | 0 |
| | V-VEG (n = 10) | 0 | 0 | 0 | 10 |

[a] The percentage values are the average values from 25 times repeated leave-30%-out cross validation partial models; [b] The best number of factors for SIMCA and PCAROCC or k nearest neighbours for OCkNN or kernel coefficient for OCSVM; [c] mark "√" / "×" indicates that this data pre-processing method was or was not used in the model; [d] cross validation for the EVOO model is leave-30%-out. For sample group codes see Table 4.1. For classification algorithm codes see Figure 4.1.

All four types of models assigned the EVOO samples well to the EVOO class (99-100%), which is not surprisingly considering the approach. Only one EVOO sample was misclassified using the SIMCA and PCAROCC algorithms. Thus, OCkNN and OCSVM models presented slightly better classification ability. In terms of the comparison of OCkNN and OCSVM, the additional test set resulted in ≥ 94% correct predictions. More specifically, both algorithms resulted in 4 ROO misclassified samples. Furthermore, application of the OCkNN and OCSVM models resulted also in 2 and 3 VEG incorrectly classified samples, respectively. All together the OCkNN revealed a fairly good and the best prediction ability of the four models.

Subsequently the validation sets were measured three months after the training set. SIMCA and PCAROCC models show < 90% correct EVOO prediction rates. On the contrary, OCkNN and OCSVM present > 90% correct EVOO prediction rates. Regarding the non-EVOO prediction in the validation set, the rates of successful prediction are all > 90%. Moreover, the incorrectly predicted V-EVOO sample of the OCkNN model was also incorrectly predicted by

the other models. Therefore, both OCkNN and OCSVM models performed better than the others when considering the validation results.

When we take all results into account, the OCkNN model demonstrates best prediction performance, the training set and the validation set showed 100% (140 out of 140) and 96% (24 out of 25) correct prediction for the EVOO and the additional test set and the validation set present 95% (104 out of 110) and 96% (24 out of 25) for non-EVOO groups, respectively.

*4.3.3.2. OCkNN model*

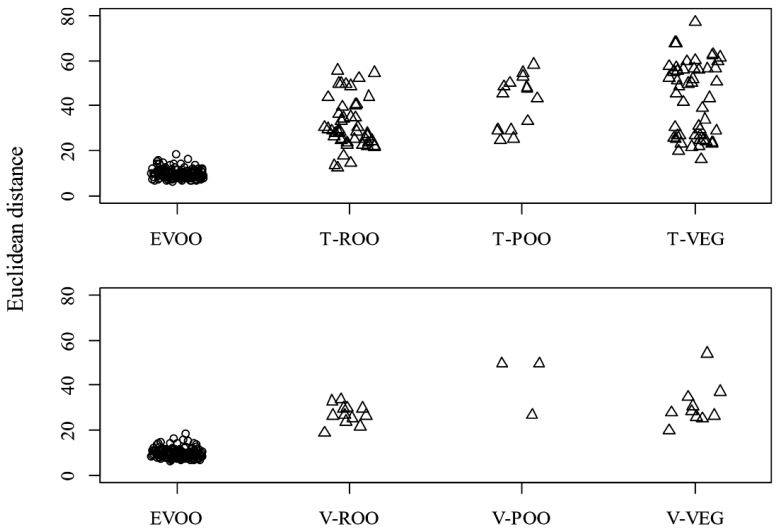


**Figure 4.4.** Stripplots of a) training set (EVOO) (circle marks) versus additional test sets (T-ROO, T-POO and T-VEG) (triangle marks) and b) training set (EVOO) (circle marks) versus and validation sets (V-ROO, V-POO and V-VEG) based on Euclidean distance. For sample group codes, see Table 4.1.

Given the prediction performance, OCkNN is examined in greater detail below. OCkNN relies on the Euclidean distance from the evaluated object to its k nearest neighbours, the stripplots of which are presented in **Figure 4.4** for EVOO (training set) versus additional test set samples (T-ROO, T-POO and T-VEG) and external validation samples (V-ROO, V-POO and V-VEG).

A clear difference between EVOO and other oil samples is visible with the Euclidean values of the EVOO group being much lower than those of the other sample sets. It is evident that the EVOO group shows a narrow distribution in comparison to the others. The T-VEG group shows the largest range of values (16.09-77.35), which is likely to be due to the variety of botanical origins of the vegetable oil samples in this group.

More precisely, a threshold value of the OCkNN model for EVOO authentication was set at 18.50 based on the values of all EVOO samples. Test samples with scores below the

threshold value are classified as EVOO, while samples with scores higher than the threshold value are classified as non-EVOO. **Figure 4.4** shows same results as **Table 4.2**, although T-POO, V-POO and V-VEG are completely separated from the EVOO population, the distributions of EVOO with T-ROO, T-VEG and V-ROO have small overlapping zone.

Considering all, the results show solid and robust performance of the OCkNN model of the VOC data.

### *4.3.4. Distinct VOCs*

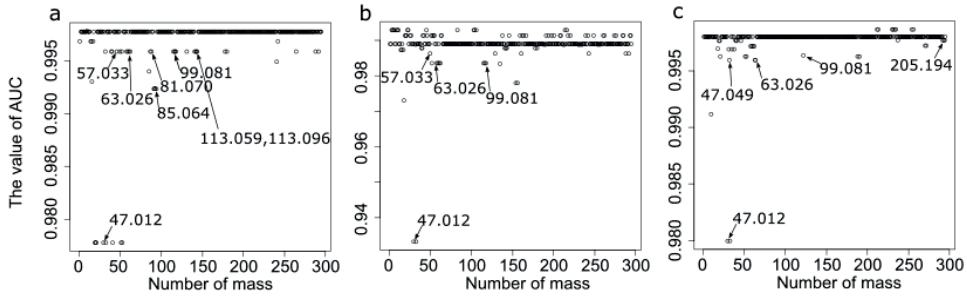


**Figure 4.5.** The AUC values of 295 VOCs for a) EVOO versus ROO, b) EVOO versus POO, c) EVOO versus VEG. AUC, the area under the ROC curve; ROC, receiver operating characteristic. For sample group codes, see Table 4.1.

The important VOCs, that contributed to the separation between EVOO and all other oils in OCkNN model, were examined by their AUC values. As shown in **Figure 4.5**, eight (m/z 47.012, 57.033, 63.026, 81.070, 85.064, 99.081, 113.059 and 113.096), four (m/z 47.012, 57.033, 63.026 and 99.081) and five (m/z 47.012, 47.049, 63.026, 99.081 and 205.194) compounds contribute to the EVOO/ROO, EVOO/POO and EVOO/VEG separation, respectively. The compounds were tentatively identified based on their masses and likely chemical formulas (Fabris et al., 2010) as well as based on information from literature (**Table 4.3**). All ten tentatively identified masses show significantly differences (ANOVA, $p < 0.05$) across the four groups. Furthermore, post-hoc tests show individual differences between the groups tested. Seven out of those 10 masses showing significant differences between groups reveal a significant difference in VOCs concentrations between the EVOO group and all other oil groups. To illustrate the characteristic of VOCs in different oil groups, all the 51 tentatively identified masses information were established in supplementary material.

Three VOCs appear key to the three separations m/z 47.012, 63.026 and 99.081. VOC m/z 47.012 is most likely formic acid. According to previous studies (Paradiso et al., 2018), formic acid is derived as a secondary product from oxidative pathways or from decomposition of 2,4-(E-E)-decadienal. Most remarkable is that its concentration is lower for EVOO than for the lower grade olive oils. Dimethyl sulphide (m/z 63.026) is a sulphur compound that is associated with the unpleasant odour of “beetroot” and “organic” (Angerosa et al., 2004;

Vezzaro et al., 2011). It was found in a higher concentration for EVOO than for ROO and POO. This is due to the refining process which removes most of the odour, including dimethyl sulphide. (E)-3-hexenal/(Z)-3-hexenal (m/z 99.081) contribute to the "green" aroma of products and is present at higher concentrations in EVOO compared to the other oils. This finding is consistent with the results of Angerosa et al. (2004). This group found that the aroma of high quality olive oil generally attributed to a "green" sensation, such as cut grass, leaf, apple or other fruits like aroma. All three VOCs deserve special attention as they are being important VOCs for the distinction of EVOO from lower grade oils.

#### *4.3.5. Application of the model in practice: a market survey*

Considering the successful validation of the methodology, the combined VOC – OCkNN model approach was applied in practice. A survey of 90 retail M-EVOO samples was conducted with the newly developed and validated approach. The 90 samples were analysed by PTR-QiToF-MS and their identity predicted by the OCkNN model presented in the previous sections. Nine of the 90 retail EVOO samples were classified as non-EVOO (10%). Considering the performance of the model during validation, these samples are highly suspicious. Future confirmatory/additional analyses will provide insights in the cause and type of adulterations.

### 4.4. Conclusions

This new approach using the spectral fingerprints obtained with rapid and non-destructive PTR-QiToF-MS in combination with robust OCC is a promising screening methodology for the distinction of EVOO from other olive oil grades as well as other vegetable oils that are potential adulterants. The robust OCC model has the ability to discriminate EVOO samples from lower grades counterparts as well as from other vegetable oils with $\geq$ 90% correct prediction ability. Key compounds for distinction are formic acid (m/z 47.012), dimethyl sulphide (m/z 63.026) and hexenal (m/z 99.081). In practice 10% of the 90 retail EVOO samples appeared not to be beyond doubt.

**Table 4.3.** Tentatively identified mass peaks of training set (EVOO) and additional test set (T-ROO, T-POO, T-VEG) as well as average concentrations, standard deviations (SD), statistical comparisons (one-way ANOVA and post-hoc LSD tests, $p < 0.05$) and aroma attribution.

| Measured protonated mass (m/z) | Protonated chemical formula | Tentative identification | References * | Average ± SD (ppbv) | | | | *p*-value | Attributes/Notes | References ** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | EVOO (n = 140) | T-ROO (n = 45) | T-POO (n = 15) | T-VEG (n = 50) | | | |
| 47.012 | $CH_3O_2^+$ | Formic acid | [8] | (3±3) ×10$^{3\ c}$ | (12±11) ×10$^{3\ b}$ | (17±16) ×10$^{3\ a}$ | (1±2) ×10$^{3\ c}$ | <0.001 | - | - |
| 47.049 | $C_2H_7O^+$ | Ethanol | [1, 3, 5, 9] | (45±55) ×10$^{3\ a}$ | (18±25) ×10$^{3\ b}$ | (4±8) ×10$^{3\ bc}$ | (0.4±0.6) ×10$^{3\ c}$ | <0.001 | floral, green, alcoholic, ripe apple and grassy | [2], [10] |
| 57.033 | $C_3H_5O^+$ | 2-Propenal | [6, 10] | (39±33) ×10$^{3\ a}$ | (11±12) ×10$^{3\ b}$ | (4±5) ×10$^{3\ b}$ | (3±5) ×10$^{3\ b}$ | <0.001 | - | - |
| 63.026 | $C_2H_7OS^+$ | Dimethyl sulphide | [4] | 921±1930 $^{a}$ | 45±54 $^{b}$ | 16±11 $^{ab}$ | 1262±3569 $^{a}$ | 0.019 | organic, wet earth, beetroot | [2], [10] |
| 81.070 | $C_6H_9^+$ | Cyclohexadiene (Terpene fragment) | [4, 12] | (12±12) ×10$^{3\ a}$ | (0.3±0.3) ×10$^{3\ b}$ | (0.07±0.07) ×10$^{3\ b}$ | (3±15) ×10$^{3\ b}$ | <0.001 | - | - |
| 85.064 | $C_5H_9O^+$ | (E)-2-pentenal/(Z)-2-Pentenal /(E)-2-methyl-2-butenal/1-penten-3-one | [1, 3, 5] | (7±6) ×10$^{3\ a}$ | (0.3±0.2) ×10$^{3\ b}$ | (0.1±0.1) ×10$^{3\ b}$ | (0.4±0.8) ×10$^{3\ b}$ | <0.001 | green, apple, floral, pleasant | [2] |
| 99.081 | $C_6H_{11}O^+$ | (E)-3-hexenal/(Z)-3-hexenal /(E)-2-hexenal/(Z)-2-hexenal/2-methyl-4-pentenal | [1, 3-5, 7] | (13±11) ×10$^{3\ a}$ | (0.2±0.2) ×10$^{3\ b}$ | (0.07±0.1) ×10$^{3\ b}$ | (0.1±0.2) ×10$^{3\ b}$ | <0.001 | artichoke, green, floral, green leaves, grassy, apple-like | [2], [10] |
| 113.059 | $C_6H_9O_2^+$ | Dimethyl-furanone/methyl-cyclopentanedione/ cyclotene/sorbic acid | [6, 11] | 171±308 $^{a}$ | 10±7 $^{b}$ | 5±4 $^{b}$ | 36±55 $^{ab}$ | <0.001 | - | - |
| 113.096 | $C_7H_{13}O^+$ | Heptenal/(E) -2-heptenal | [4, 7] | 131±125 $^{a}$ | 30±22 $^{b}$ | 26±30 $^{b}$ | 76±120 $^{b}$ | <0.001 | correlated with the rancidity perception | [2] |
| 205.194 | $C_{15}H_{25}^+$ | β-Caryophyllene/copaene | [1, 3] | 23±22 $^{a}$ | 4±7 $^{b}$ | 0.4±0.7 $^{b}$ | 4±17 $^{b}$ | <0.001 | - | - |

* References for tentative identification compounds, ** References for aroma attribution of each compound: [1] Angerosa et al. (1998); [2] Angerosa et al. (2004); [3] Cappellin et al. (2012a); [4] Iraqi et al. (2005); [5] Luna et al. (2006); [6] Masi et al. (2015); [7] Morales et al. (2005); [8] Paradiso et al. (2018); [9] Vezzaro et al. (2011); [10] Vichi et al. (2003); [11] Yener et al. (2014); [12] Yener et al. (2016). Different superscript letters (a, b, c) in a row indicate significant differences from each other. For sample group codes see Table 4.1.

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# Chapter 5

## Handheld near-infrared spectroscopy for distinction of extra virgin olive oil from other olive oil grades substantiated by compositional data

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**Abstract**

Miniaturisation of analytical technology has paved the way for in-situ screening of foods. In the current study, the spectral features of olive oils were examined by handheld near-infrared spectroscopy to explore the technology's capabilities to distinguish extra virgin olive oil (EVOO) from lower grade oils. 80 EVOO, 40 refined olive oil and 10 pomace olive oil (POO) samples were analysed for their spectral and compositional features. The latter included analysis of the fatty acids (FAs), the pigments chlorophylls and carotenoids, chromatic coordinates and moisture contents. The 1350-1570 nm wavelength range appeared most suitable for distinction of the oils. One-class classification models with three different classifiers were subsequently estimated using this range, and their quantitative performance was assessed from probabilistic data. Soft independent modelling of class analogies models appeared to predict the identity of the oils best and with a high success rate. Compared to the other oils, POO comprised a significantly higher and lower proportion of polyunsaturated and monounsaturated FAs, respectively. Higher contents of chlorophylls, carotenoids and moisture were noted for EVOO. The relevant spectral information for distinction of the oils correlated strongly with the degree of unsaturation of the oils as well as their levels of chlorophylls, carotenoids and moisture.

**Keywords:** Food fraud; in-situ; one-class classification; pigments; portable.

### 5.1. Introduction

The European Commission (2013) and the International Olive Council (IOC) (2016) released regulations and standards which define various olive oil grades. Three main common commercial grades are: extra virgin olive oil (EVOO), refined olive oil (ROO) and pomace olive oil (POO). EVOO, which is extracted from good quality olive fruits by mechanical or other physical treatments, is considered the best quality olive oil. All grades have to meet particular compositional requirements, but for EVOO the strictest apply. Due to the premium quality, the price of EVOO is higher than those of ROO and POO. As a result, EVOO is also the olive oil most vulnerable to adulteration (van Ruth et al., 2018). Lower grade olive oils, such as ROO (Karbasian et al., 2015) and POO (Yang & Irudayaraj, 2001), are considered perfect adulterants given their similar gross compositions. Hence, blending of EVOO with these oils is relatively harder to be detected than the presence of oils of other botanical origins (rapeseed oil, peanut oil, (high oleic) sunflower oil, etc.) in EVOO. However, EVOO, ROO and POO differ in their minor compound contents, since numerous compounds such as free fatty acids (FAs), volatile compounds, metals, pigments, moisture, etc. are eliminated from olive oil during (mild) refining treatments (Antonopoulos et al., 2006; Bendini et al., 2009). Furthermore, some compounds are formed during the (mild) refining process and these are sufficiently persistent to appear in the final product. These compounds are stigmastadienes (IOC, 2017a), fatty acid alkyl esters (Perez-Camino et al., 2008), monochloropropanediol (MCPD) esters and glycidyl esters (GEs) (Yan et al., 2018). Their presence in olive oil has been proven markers of the (mild) refining process and allows distinction of retail ROO and POO from EVOO. These methods are generally suitable as confirmatory methods but labour-intensive at the same time.



Simple, rapid and in-situ analyses for food quality control have the great advantage of delivering analytical results immediately at the sampling location, rather than days later in a laboratory where a small selection of samples have been analysed. It allows very early intervention, in case anomalies are detected. It can also be used for screening purposes to efficiently select samples to be sent to the laboratory for confirmatory analysis. With the miniaturisation of analytical devices over the last decade, these measurement techniques have received more and more attention. For instance near-infrared spectroscopy (NIR), which is a rapid, non-destructive, cost-efficient and easy-to-use technique, has led to a boost in the number of in-situ applications in diverse academic and industrial fields (Casale & Simonetti, 2014; Porep et al., 2015; Vanstone et al., 2018). Apart from in-line applications, more and more small, portable and handheld devices have become available.

Handheld NIR has been applied in lipid analysis for quantitation of vegetable oil in diesel (Paiva et al., 2015), for quantitation of trans fat in edible oils (Birkel & Rodriguez-Saona, 2011), for purity determination of copaiba oils (Moreira et al., 2018) and for detection of lard adulteration in palm oil (Basri et al., 2017). Furthermore, specific olive (oil) applications concern the on-line evaluation of the oil content of olives during olive processing (Giovenzana

et al., 2018) and the measurement of olive oil acidity (Grossi et al., 2019). However, the application of handheld NIR for distinction of EVOO from lower grade counterparts has not been done before. Considering those all, this kind of technology may also be promising as a rapid screening technique for distinction of olive oil grades.

In this study, we explored rapid and non-destructive handheld NIR technology for its capabilities to distinguish EVOO from other olive oil grades. One-class classification models for sorting the olive oil grades were estimated using three types of classifiers and various data pre-treatments. Model performance was compared by evaluation of the probability distributions based on kernel density estimates (KDE). To examine the underlying causes for the spectral differences of the oils, compositional analyses were conducted and the resulting data correlated with the spectral data. The analyses involved the FAs, chlorophylls, carotenoids and moisture contents as well as chromatic coordinates of all samples.

## 5.2. Materials and methods

### *5.2.1. Materials*

A set of 130 commercial olive oil samples was selected from a pool of 400 oil samples from European sources in 2016-2017 and comprised 80 EVOO, 40 ROO and 10 POO samples. All samples complied with EU olive oil grade legislative requirements in regard to FA compositional requirements and spectroscopic tests (extinction coefficient K232, K268 and ΔK values) (IOC, 2017b). They were also evaluated for their MCPD esters and GEs contents (Yan et al., 2018) prior to the study. All samples were stored in capped bottles and were kept in the dark in a basement (18 °C) for six months before analysis.

### *5.2.2. Handheld NIR analysis*

A handheld NIR Pro 1700ES spectrometer (JDSU, Milpitas, CA, USA), palm-sized (45 × 42 mm), weighing 60 g and with a working wavelength range of 908-1676 nm (Correia et al., 2018), was applied. The spectrometer employs a linear variable filter as the light dispersing element which results in high signal-to-noise ratio (Correia et al., 2018). The absorption spectra of the olive oil samples were acquired at room temperature (21 ± 1 °C). The transmittance mode was applied with a measurement time of 0.25-0.50 s, an integration time of 11 ms and 100 scan counts. Signal intensity was recorded every 6.2 nm for the wavelength range of 908-1676 nm, resulting in a spectrum of 125 points. Four mL of olive oil was transferred into a disposable plastic cuvette, then the cuvette was placed in the sample chamber of the device and analysed. Samples were measured in random order to avoid possible systematic memory effects and measurements of each sample were carried out in duplicate on day one. On day two, all samples were measured again in duplicate in random order. Hence, in total four replicates of each oil sample were collected, and the average spectral intensities of each sample were used for further

data analysis. The handheld NIR data matrix consisted eventually of 130 samples × 125 wavelengths.

### *5.2.3. Fatty acid analysis*

The sample preparation and gas chromatographic (GC) analysis was conducted according to the official methods (International Organization for Standardization, 2015, 2017). For the GC analysis, an Agilent HP7890A GC (Agilent Technologies, Inc., Wilmington, DE, USA) was equipped with a 100 m × 0.25 mm × 0.2 µm film thickness fused silica capillary column (Varian, Palo Alto, CA) coupled to a flame ionisation detector. The following working conditions were applied: initial column temperature, 120 °C; final column temperature, 240 °C; heating ramp, 4.0 °C $min^{-1}$; hold time, 7 min 240 °C; run time, 37 min; carrier gas, hydrogen; constant flow, 1.0 mL $min^{-1}$; injection, 1 µL; split, 1 : 100; injector temperature, 250 °C; detector temperature, 250 °C. Duplicate measurements of all samples were conducted. The average was calculated and used for further analysis. In total, 15 FAs were measured and the sum of (un)saturated FAs (saturated FA, SFA; monounsaturated FA, MUFA; polyunsaturated FA, PUFA) were calculated. The iodine values (IV, g 100 $g^{-1}$) and saponification numbers (SN, mg KOH $g^{-1}$) of each sample were calculated from their FA compositions as described by Kalayasiri et al. (1996). The FA data matrix consisted of 130 samples × 20 variables.

### *5.2.4. Chlorophylls and carotenoids analysis*

The total chlorophylls and carotenoids contents of all oil samples were measured by ultraviolet-visible (UV-VIS) absorption spectroscopy using a Cary 100 UV-VIS spectrophotometer (Varian Inc., Palo Alto, CA, USA). The measurement was carried out according to the method described by Minguezmosquera et al. (1991). All samples were measured in duplicate.

### *5.2.5. Chromatic coordinates CIELAB analysis*

Chromatic coordinates L*, a* and b* (referred to as CIELAB) were measured using the mobile application Colour Grab 3.6.1 (Loomatix Ltd., München, Germany) at room temperature. This system expresses colour as three values: L* for the lightness from black (0) to white (100), a* from green (-) to red (+) and b* from blue (-) to yellow (+). The analysis was replicated six times for each sample. The averaged values of L*, a* and b* of each sample were used for further data analysis.

### *5.2.6. Moisture content analysis*

Approximately 5 g of test sample was weighed for analysis. The moisture content was determined according to ISO standard 662 (International Organization for Standardization, 1998), method B.

### *5.2.7. Statistical analysis*

#### *5.2.7.1. Univariate analysis*

Non-parametric Kruskal-Wallis tests were applied for group comparisons due to non-normality of the various data. The latter was determined with the Shapiro-Wilk method. Subsequent pairwise comparisons were carried out using Mann-Whitney U-tests. These data analyses were performed using SPSS statistic 23 software (IBM, Chicago, IL, USA). A significance level of $p < 0.05$ was used throughout the study.

Area under the spectral curve (AUC) is calculated according to equation (1):

$$\text{AUC} = \sum_{n=2}^{125} \frac{(Abs_n + Abs_{n-1})}{2} \times (Wav_n - Wav_{n-1}) \quad , \quad (1)$$

where Abs is the absorbance of the wavelength, Wav is the value of the wavelength and n is the number of the wavelength.

#### *5.2.7.2. Exploring the spectral data: unsupervised analysis*

Principal component analysis (PCA) was carried out to explore the spectral data set using several pre-processing methods, such as 1st derivative, 2nd derivative (Paiva et al., 2015; Panford & Deman, 1990), standard normal variate (SNV), multiplicative scatter correction (MSC) (Amigo et al., 2015), mean-centring, auto-scaling and $\log^{10}$ scaling. The best pre-processing combination was selected to obtain the best separated PCA distribution. Analyses were carried out using Pirouette 4.5 software (Infometrix, WA, USA).

#### *5.2.7.3. Classification of olive oils by their spectral data: Supervised analysis*

For the development of one-class classification models, the spectral data of the 80 EVOO samples were divided into a training set of 50 samples and an external validation set of 30 samples. The training set was used to develop one-class classification models and was subjected to 25 times repeated leave-30%-out random cross-validation for a first impression of the performance of the models. The performance of the models was also evaluated by independent samples: by the external validation EVOO set of 30 samples and the non-EVOO challenge set which consisted of 40 ROO and 10 POO samples.

Three classifiers, namely one-class k-nearest neighbours (OCkNN) (Khan & Madden, 2014; Munroe & Madden, 2005), one-class support vector machine (OCSVM) (Manevitz & Yousef, 2002) and soft independent modelling of class analogies (SIMCA) (Branden & Hubert, 2005), were applied for classification of the samples. Specifically, OCkNN considered the Euclidean distance from the evaluated object to its k nearest neighbours, OCSVM determined the distance from the evaluated object to the boundary enclosing the target class (model), SIMCA calculated the distance from the evaluated object to the model centre. Subsequently,

the distance value of each sample was compared with the threshold value (95% confidence intervals of all the training samples' values). When the sample value was below the threshold value, this sample was considered to be within normal range and thus predicted as target sample (EVOO). Consecutive factors 1-10 and five data pre-processing methods (i.e. 1st derivative, 2nd derivative, SNV, MSC, mean-centring, auto-scaling and $\log^{10}$ scaling) were applied in conjunction with the mentioned three classifiers to estimate various models. The model performing best for each algorithm with the best setting of number of factors and pre-processing methods was selected for validation. The performance of the models was evaluated using the external EVOO validation set, the ROO and POO challenge sets. The model resulting in fewest false assignments was considered the best. KDE was applied to generate a non-parametric distribution for class probability based on the distance data of each model. All calculations were performed by scripts developed in R 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

Prediction performance was assessed using the following indicators: sensitivity (the proportion of actual positive cases that were correctly identified), specificity (the proportion of actual negative cases that were correctly identified) and accuracy (the proportion of the total number of predictions that were correct). These indicators are defined according to equations (2-4):

$$\text{Sensitivity} = \frac{TP}{TP + FN} \quad , \quad (2)$$

$$\text{Specificity} = \frac{TN}{TN + FP} \quad , \quad (3)$$

$$\text{Accuracy} = \frac{TP + TN}{TP + TN + FP + FN} \quad , \quad (4)$$

where TP, TN, FP and FN denote the true positive, the true negative, the false positive and the false negative number of samples, respectively.

*5.2.7.4. Correlation analysis*

The correlations between the spectral data and all other parameters were assessed by computing Pearson correlation coefficients (r). Paired samples t-tests were applied to assess significance of the correlation coefficients ($p < 0.05$). All calculations were performed by scripts developed in R 3.3.3.

## 5.3. Results and discussion

### *5.3.1. Handheld NIR analysis*

#### *5.3.1.1. Spectral profiles*

All olive oil samples were subjected to handheld NIR analysis. The average absorbance spectra of the three olive oil grades are presented in **Figure 5.1**. The spectral profiles exhibit four characteristic bands rich in molecular information (Zone A-D). In **Table 5.1,** formerly reported molecular characteristics are assigned to these major spectral bands and wavelengths.

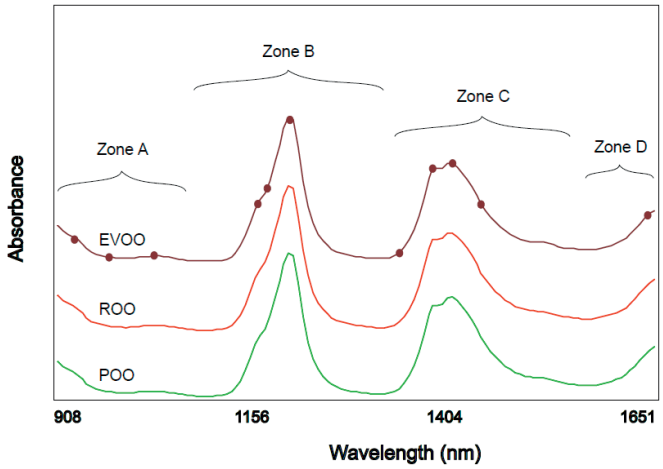


**Figure 5.1.** Average absorbance spectra of extra virgin olive oils (EVOO), refined olive oils (ROO) and pomace olive oils (POO) acquired by handheld near-infrared spectroscopy. Brackets identify the four main spectral bands (Zones A, B, C and D) and dots the special wavelengths to which is referred in Table 5.1 (930 nm, 982 nm, 1038 nm, 1162 nm, 1181 nm, 1212 nm, 1348 nm, 1391 nm, 1416 nm, 1453 nm and 1664 nm).

For Zone A (908-1090 nm), wavelengths 930 nm and 1038 nm have been reported to relate to the vibration of methylene groups (Fernandez-Cabanas et al., 2011; Picouet et al., 2018; 2001), which is characteristic for fats in general. Wavelength 982 nm is related to the second overtone of O-H stretching, and this is associated with the water content of oil samples (Fernandez-Cabanas et al., 2011). **Table 5.1** shows that the absorption intensity for the three wavelengths varied for the grades, with EVOO differing significantly from POO, but not from ROO (Mann-Whitney U-tests, $p < 0.05$).

In Zone B (1090-1330 nm), multiple molecular groups (methylene group, methyl group and cis double bond) are associated with this spectral band. In addition, the fourth overtone of the C-O stretching is expected to be reflected in the spectral range around 1162 nm as well (Workman, 2008). The band centred at 1212 nm corresponds to the second overtone of C-H stretching (Yang & Irudayaraj, 2001). Furthermore, the bands near 1181 nm have been associated with the second overtone of the fundamental C-H absorption of pure FAs containing

cis double bonds, such as oleic acid (Hourant et al., 2000). It is known that oils rich in unsaturated FAs present high absorption in the vicinity of 1164 nm (Hourant et al., 2000). The absorbance intensity at wavelength 1181 nm differed significantly between POO (0.364 ± 0.013), on the one hand, and EVOO (0.353 ± 0.003) and ROO (0.352 ± 0.002) on the other hand (Mann-Whitney U-tests, $p < 0.05$). Results may imply that POO comprises a higher unsaturated FAs fraction than the other grades.

**Table 5.1.** Average absorbance intensities and standard deviations of the four spectral bands and special wavelengths for three olive oil grades and tentative assignment of related molecular structures.

| Zone | Band location Wavelength (nm) | Molecule | Functional group | Vibration | Absorbance intensity | | | Reference * |
|---|---|---|---|---|---|---|---|---|
| | | | | | EVOO (n = 80) | ROO (n = 40) | POO (n = 10) | |
| A | 908-930 | $-CH_2-$ | C-H | 3rd overtone | | | | [1] |
| | 930 | $-CH_2-$ | C-H | 3rd overtone | 0.095 ± 0.004 $^b$ | 0.095 ± 0.002 $^b$ | 0.107 ± 0.014 $^a$ | [2] |
| | 982 | $H_2O$ | O-H | 2nd overtone | 0.017 ± 0.004 $^b$ | 0.017 ± 0.002 $^b$ | 0.030 ± 0.014 $^a$ | [3] |
| | 1038 | $-CH_2-$ | C-H | Combination | 0.024 ± 0.004 $^b$ | 0.024 ± 0.002 $^b$ | 0.036 ± 0.014 $^a$ | [2] |
| B | 1090-1180 | $-CH_2-$ | C-H | 2nd overtone | | | | [4] |
| | 1100-1200 | $-CH_3$ | C-H | 2nd overtone | | | | [1,4] |
| | 1150-1260 | -CH=CH- | C-H | 2nd overtone | | | | [4] |
| | 1162 | -C=O | C-O | 4th overtone | 0.230 ± 0.003 $^b$ | 0.229 ± 0.002 $^b$ | 0.242 ± 0.013 $^a$ | [1,4] |
| | 1181 | -CH=CH- | C-H | 2nd overtone | 0.353 ± 0.003 $^b$ | 0.352 ± 0.002 $^b$ | 0.364 ± 0.013 $^a$ | [4] |
| | 1212 | $-CH_2-$ | C-H | 2nd overtone | 0.620 ± 0.004 $^b$ | 0.621 ± 0.002 $^{ab}$ | 0.628 ± 0.012 $^a$ | [2] |
| C | 1350-1430 | $-CH_2-$ | C-H | Combination | | | | [1,4] |
| | 1360-1420 | $-CH_3$ | C-H | Combination | | | | [4] |
| | 1390-1450 | $H_2O$ | O-H | 1st overtone | | | | [4] |
| | 1390-1450 | $-CH_2-$ | C-H | 1st overtone | | | | [4] |
| | 1350 | -C=O | C-O | 3rd overtone | 0.038 ± 0.003 $^b$ | 0.036 ± 0.002 $^b$ | 0.047 ± 0.011 $^a$ | [1] |
| | 1391 | $-CH_2-$ | C-H | Combination | 0.404 ± 0.003 $^a$ | 0.399 ± 0.003 $^b$ | 0.409 ± 0.011 $^a$ | [2,4] |
| | 1416 | $-CH_2-$ | C-H | 1st overtone | 0.430 ± 0.004 $^b$ | 0.426 ± 0.004 $^c$ | 0.445 ± 0.012 $^a$ | [2,4] |
| | 1453 | $H_20$ | O-H | 1st overtone | 0.252 ±0.004 $^b$ | 0.252 ± 0.004 $^b$ | 0.271 ± 0.012 $^a$ | [3] |
| D | 1650-1670 | $-CH_2-$ | C-H | 1st overtone | | | | [4] |
| | 1664 | -CH=CH- | C-H | 1st overtone | 0.187 ± 0.004 $^b$ | 0.186 ± 0.004 $^b$ | 0.194 ± 0.008 $^a$ | [5] |
| AUC | | | | | 101 ± 2 $^b$ | 100 ± 2 $^b$ | 107 ± 4 $^a$ | |

* [1] Paiva et al. (2015); [2] Yang and Irudayaraj (2001); [3] Fernandez-Cabanas et al. (2011); [4] Hourant et al. (2000); [5] Christy et al. (2004). Different superscript letters (a, b, c) in a row indicate significant differences from each other (Kruskal-Wallis tests and Mann-Whitney U-tests, $p < 0.05$). EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil; AUC, area under the spectral curve.

For Zone C (1350-1570 nm), the band from 1350 nm to 1450 nm is resulting from a combination of C-H stretching and deformation of the concerned molecule (methylene group and methyl group) (Hourant et al., 2000; 2015). The band ranging from 1390 nm to 1450 nm corresponds to the first overtone of methylene group and water (Hourant et al., 2000). The wavelengths 1391 nm and 1416 nm relate to the combination of C-H stretching and deformation and the first overtone of C-H stretching vibration, respectively (2000; Yang & Irudayaraj, 2001). The absorption intensity at both wavelengths is significantly higher for EVOO than for ROO (Mann-Whitney U-tests, $p < 0.05$).

For Zone D (a narrow spectral band from 1650 nm to 1676 nm), the band most likely relates to the first overtone of C-H stretching. The wavelength 1664 nm corresponds to the first overtone of cis double bonds (Christy et al., 2004; Sato et al., 1991). Remarkably, the absorbance of POO at this wavelength (0.194 ± 0.008) is significantly higher than the absorbance of EVOO (0.187 ± 0.004) and ROO (0.186 ± 0.004) (Mann-Whitney U-tests, $p < 0.05$).

Furthermore, **Table 5.1** shows that the AUC of EVOO (101 ± 2) is significantly lower than that of POO (107 ± 4) (Mann-Whitney U-tests, $p < 0.05$). This indicates that EVOO has a lower absorption intensity than POO, which is in agreement with previous research (Yang & Irudayaraj, 2001). On the other hand, the AUC value of EVOO (101 ± 2) does not differ significantly from that of ROO (100 ± 2) which indicates the similarity of the two grades.

Summarising the above, the absorbance of the olive oil grades at various spectral bands showed considerable overlap. Since it appears difficult to distinguish the three olive oil grades simultaneously by a single wavelength absorbance, we proceeded with a multivariate approach.

*5.3.1.2. Exploring the spectral data*

In order to explore the data, the handheld NIR spectral data were subjected to PCA (**Figure 5.2**). The combination of 2$^{nd}$ derivative and auto-scaling appeared the most suitable pre-processing method since it resulted in a good separation between EVOO and the other two olive oil grades.

A distinct separation between EVOO and the lower grade olive oils appears along factor 1 in **Figure 5.2a**. The EVOO samples cluster in the right hand side quadrants, whereas ROO and POO are located at the left. This indicates that the separation is mainly driven by the variables loading on factor 1. Therefore, the loadings of the wavelengths on factor 1 are shown in **Figure 5.2c**. The spectral data in the range of 1348-1571 nm in Zone C present high positive and negative loadings. The high positive loadings indicate an association with EVOO, whereas a high negative loading is associated with ROO and POO. This is fairly promising for further distinction of the oils; therefore, this wavelength range was selected for further data processing. In **Figure 5.2b**, the absorbance in the selected wavelength range show sufficient differences between the samples of the three olive oil grades. Previous studies reported that this band is mainly dominated by the vibration of -$CH_2$- and $H_2O$ (Hourant et al., 2000; Paiva et al., 2015; Yang & Irudayaraj, 2001). Moreover, the absorption band of the first overtone of $H_2O$ highly perturbs the absorption at these wavelengths (Hourant et al., 2000). This may imply that the moisture contents of the olive oils contribute to the differences in absorbance in this range. On the other hand, the wavelength 982 nm (loading factor 1 = 0.12), 1181 nm (0.14), 1350 nm (0.16), 1391 nm (-0.17) and 1453 nm (0.16) in **Figure 5.2c** present the highest absolute loading values. **Table 5.1** shows that 982 nm and 1453 nm correspond to the overtone of water and 1181 nm is associated with the second overtone of the fundamental C-H absorption of FAs containing cis double bonds (Hourant et al., 2000). Furthermore wavelength 1391 nm corresponds to a combination of C-H vibrations that characterise oils. These results reveal that moisture content and abundance of double bonds in the FAs may contribute to the distinction of the olive oil grades.

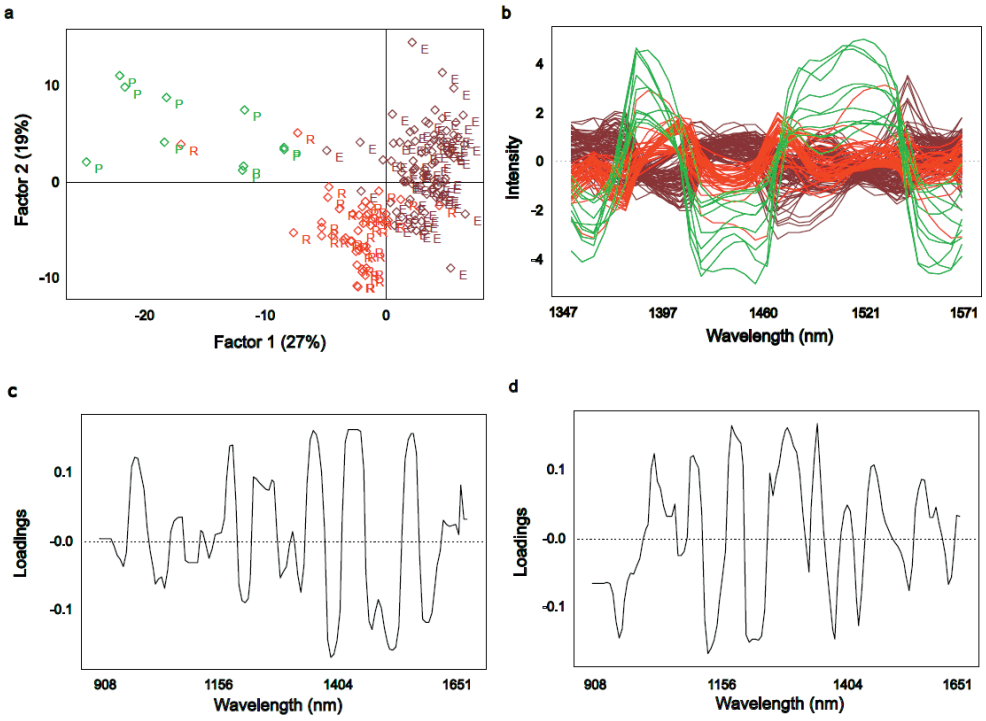


**Figure 5.2.** Results of principal component analysis of the handheld near-infrared spectral data of three olive oil grades (data pre-processing: 2nd derivative and auto-scaling): a) scores plot; b) absorbance in the range of 1347-1571 nm; c) factor 1 loadings of wavelengths; d) factor 2 loadings of wavelengths. E in grey, extra virgin olive oil; R in red, refined olive oil; P in green, pomace olive oil.

ROO samples mainly grouped in the lower quadrants and POO samples in the upper quadrants. The separate groupings of the grades are mainly associated with the 1100-1317 nm wavelength range in Zone B (**Figure 5.2**d). The wavelengths 1038 nm (loading factor 2 = 0.13), 1162 nm (-0.15), 1212 nm (0.15) and 1416 nm (-0.15) showed highest absolute loadings and they are more associated with ROO and POO.

Summarising the results of the exploratory analyses, wavelength range Zone C shows largest differences between EVOO and lower grade olive oils, whereas Zone B reflects differences between ROO and POO.

*5.3.1.3. Classification of olive oils by their spectral data*

One-class classification models were estimated using the absorbance data of the wavelength range of 1348-1571 nm and three classifiers (OCkNN, OCSVM, SIMCA) in order to distinguish between EVOO and other olive oil grades. The performance of each model in terms of their sensitivity, specificity and accuracy values is presented in **Table 5.2** for the internal validation, external validation, ROO and POO challenge sets, respectively. For OCkNN and OCSVM 98% correct classification rates were noted for EVOO (sensitivity score) for

internal and external validation, which means only one EVOO was misclassified in both cases. SIMCA resulted in a 100% sensitivity score, i.e. all individual EVOO samples were correctly identified as authentic EVOO. Specificity of the models followed the same ranking of the classifiers. Consequently, also the accuracy of SIMCA (100%) was higher than those of the other two models. The application of the challenge ROO and POO sets using SIMCA showed that POO samples were efficiently discriminated from EVOO (specificity of 100%) and 35 out of 40 ROO samples were classified correctly (specificity of 88%). OCkNN and SIMCA showed better performance than OCSVM, with SIMCA performing slightly better than OCkNN.

**Table 5.2.** One-class classification models and their internal/external/challenge sets validation results.

| | | Sensitivity | Specificity | Accuracy |
|---|---|---|---|---|
| EVOO vs InVal set | OCkNN [a] | 98% | 98% | 98% |
| | OCSVM [b] | 98% | 98% | 98% |
| | SIMCA [c] | 100% | 100% | 100% |
| EVOO vs ExVal set | OCkNN | 98% | 100% | 99% |
| | OCSVM | 98% | 100% | 99% |
| | SIMCA | 100% | 100% | 100% |
| EVOO vs ROO challenge set | OCkNN | 98% | 90% | 94% |
| | OCSVM | 98% | 85% | 92% |
| | SIMCA | 100% | 88% | 94% |
| EVOO vs POO challenge set | OCkNN | 98% | 100% | 98% |
| | OCSVM | 98% | 100% | 98% |
| | SIMCA | 100% | 100% | 100% |

[a] OCKNN refers to one-class k-nearest neighbours, the best model was estimated with 2nd derivative pre-processing and 3 factors; [b] OCSVM refers to one-class support vector machine, the best model was estimated with 2nd derivative and auto-scaling pre-processing and 2 factors; [c] SIMCA refers to soft independent modelling of class analogies, the best model was estimated with 2nd derivative pre-processing and 2 factors. EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil; InVal, internal validation; ExVal, external validation.

KDE was used to estimate the probability density function of the spectral data. Probability distributions for each olive oil grade and classifier applied are presented in **Figure 5.3**. Samples were classified based on a threshold value predicted from the training and validation sets. Samples with a calculated distance smaller than the threshold value were classified in the positive group (authentic EVOO), whereas samples with a calculated distance larger than the threshold value were classified in the negative group (non-authentic EVOO). The threshold values are 0.0024 for OCkNN, 1.5 for OCSVM and 1.0 for SIMCA. In **Figure 5.3**, the modelling set presented normal distributions for all three models. However, ROO and POO in combination with OCSVM and SIMCA show non-normal distributions. For ROO, a small tail exists at the right hand side, i.e. three ROO samples showed larger distance values than others. Furthermore, the distribution of POO shows two peaks, which may be due to the limited size of this sample group. As expected, distributions for the training and cross-validation sets are similar, as well as the distributions of the training and external EVOO validation sets. Regarding distributions of the EVOO and the two challenge sets, a full separation would be the most desirable. EVOO and ROO distributions show some overlap, whereas EVOO and POO overlap hardly. These results are in alignment with the accuracy scores in **Table 5.2**.

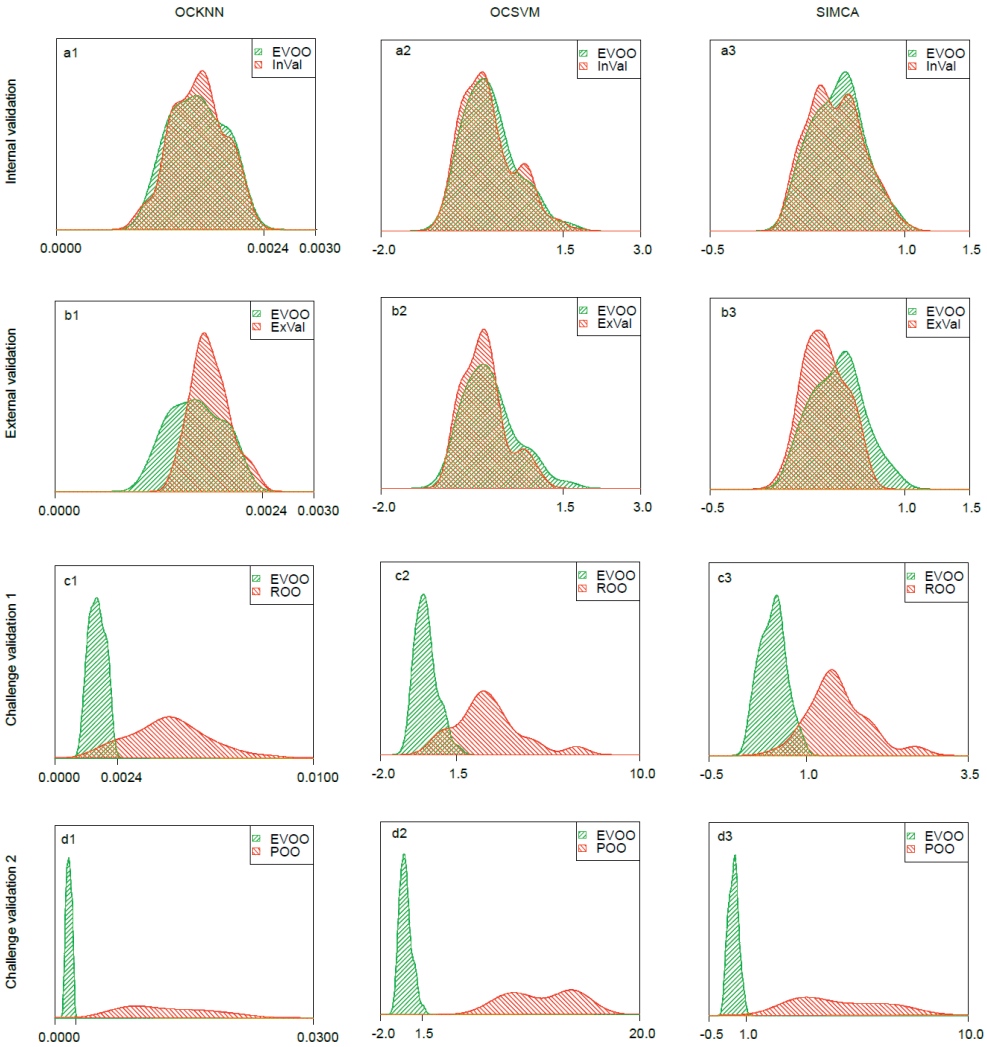


**Figure 5.3.** Comparison of kernel density estimate based probability distributions for classification models of three classifiers. Distributions are shown for the extra virgin olive oil training set (EVOO in green) versus (a) the internal EVOO validation set, (b) the external EVOO validation set, (c) the refined olive oil (ROO) challenge set and (d) the pomace olive oil (POO) challenge set (all in red). InVal, internal validation; ExVal, external validation. OCkNN, one-class k-nearest neighbours; OCSVM, one-class support vector machines; SIMCA, soft independent modelling of class analogies.

### *5.3.2. Compositional analysis*

#### *5.3.2.1. Fatty acids*

The relative abundance of 15 FAs, the sum of the relative abundance of (un)saturated FAs (SFA, MUFA and PUFA), IV and SN are listed in **Table 5.3**. The three olive oil grades

present considerable overlap in regard to the range of relative abundance of the FAs. However, the EVOO samples differed significantly from ROO and POO samples for 8 out of 15 and 5 out of 15 FAs, respectively (Mann-Whitney U-tests, $p < 0.05$). The three most abundant FAs in the olive oils are C16:0, C18:1*n*9 and C18:2*n*6; they account for over 85 % of total FAs. The average concentration of C16:0 of EVOO (12.5 ± 1.7 %) was higher than that of ROO (11.5 ± 0.8 %) and POO (11.7 ± 0.5 %). EVOO (71.5 ± 3.6 %) showed significantly higher concentrations of C18:1*n*9 than POO (69.6 ± 1.1 %) (Mann-Whitney U-tests, $p < 0.05$), but did not differ significantly from ROO (72.1 ± 1.6 %). On the other hand, the concentration of C18:2*n*6 in EVOO (7.8 ± 1.7 %) was significantly lower than in POO (9.8 ± 0.9 %) (Mann-Whitney U-tests, $p < 0.05$). These results are in alignment with previous studies (Ambrosewicz et al., 2012; Chiavaro et al., 2008). Moreover, the sum of unsaturated FAs of EVOO, both MUFA and PUFA, differed significantly from POO (Mann-Whitney U-tests, $p < 0.05$), which is also in agreement with one of the previous studies (Chiavaro et al., 2008).

**Table 5.3.** Average value (Mean), standard deviations (SD), minimum (Min), maximum (Max) and significance of differences (*p* value) of fatty acids (C16:0, etc., g 100 $g^{-1}$), iodine values (IV, g 100 $g^{-1}$) and saponification numbers (SN, mg KOH $g^{-1}$) determined in three olive oil grades.

| Parameter | EVOO (n = 80) | | | ROO (n = 40) | | | POO (n = 10) | | | *p* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Min | Max | Mean ± SD | Min | Max | Mean ± SD | Min | Max | |
| C16:0 | 12.5 ± 1.7$^{a}$ | 8.5 | 17.5 | 11.5 ± 0.8$^{b}$ | 10.3 | 13.7 | 11.7 ± 0.5$^{ab}$ | 10.8 | 12.3 | <0.05 |
| C17:0 | 0.1 ± 0.0 | 0.0 | 0.2 | 0.1 ± 0.0 | 0.1 | 0.1 | 0.1 ± 0.0 | 0.1 | 0.1 | 0.90 |
| C18:0 | 2.8 ± 0.6$^{b}$ | 1.8 | 4.3 | 3.4 ± 0.5$^{a}$ | 1.9 | 4.1 | 2.8 ± 0.2$^{b}$ | 2.3 | 3.1 | <0.05 |
| C20:0 | 0.5 ± 0.1$^{a}$ | 0.4 | 0.7 | 0.4 ± 0.0$^{b}$ | 0.3 | 0.5 | 0.5 ± 0.0$^{a}$ | 0.5 | 0.5 | <0.05 |
| C22:0 | 0.1 ± 0.0$^{b}$ | 0.1 | 0.2 | 0.1 ± 0.0$^{c}$ | 0.1 | 0.2 | 0.2 ± 0.0$^{a}$ | 0.2 | 0.2 | <0.05 |
| C24:0 | 0.1 ± 0.0$^{b}$ | 0.0 | 0.2 | 0.1 ± 0.0$^{b}$ | 0.0 | 0.1 | 0.1 ± 0.0$^{a}$ | 0.1 | 0.1 | <0.05 |
| SFA | 16.0 ± 1.6 | 13.0 | 20.9 | 15.6 ± 0.5 | 14.6 | 16.4 | 15.4 ± 0.5 | 14.7 | 16.0 | 0.46 |
| C16:1*n*9 | 0.1 ± 0.0 | 0.1 | 0.2 | 0.1 ± 0.0 | 0.1 | 0.2 | 0.1 ± 0.0 | 0.1 | 0.1 | 0.27 |
| C16:1*n*7 | 0.9 ± 0.3 | 0.4 | 1.9 | 0.9 ± 0.2 | 0.4 | 1.7 | 0.8 ± 0.1 | 0.8 | 0.9 | 0.75 |
| C17:1*n*7 | 0.1 ± 0.1 | 0.1 | 0.3 | 0.1 ± 0.0 | 0.1 | 0.2 | 0.1 ± 0.0 | 0.1 | 0.2 | 0.88 |
| C18:1*n*9 | 71.5 ± 3.6$^{a}$ | 60.0 | 76.0 | 72.1 ± 1.6$^{a}$ | 67.4 | 74.4 | 69.9 ± 1.1$^{b}$ | 68.1 | 72.1 | <0.05 |
| C18:1*n*7 | 2.1 ± 0.5$^{b}$ | 1.1 | 4.1 | 2.3 ± 0.4$^{a}$ | 1.4 | 3.0 | 2.4 ± 0.2$^{a}$ | 2.0 | 2.6 | <0.05 |
| C20:1*n*9 | 0.3 ± 0.1$^{a}$ | 0.2 | 0.5 | 0.3 ± 0.0$^{b}$ | 0.3 | 0.4 | 0.4 ± 0.0$^{a}$ | 0.3 | 0.4 | <0.05 |
| MUFA | 75.1 ± 2.9$^{a}$ | 65.5 | 78.8 | 75.8 ± 1.3$^{a}$ | 71.7 | 77.5 | 73.5 ± 1.0$^{b}$ | 72.2 | 75.9 | <0.05 |
| C18:2*n*6 | 7.8 ± 1.7$^{b}$ | 5.6 | 13.0 | 7.6 ± 1.1$^{b}$ | 6.0 | 11.2 | 9.8 ± 0.9$^{a}$ | 7.8 | 10.8 | <0.05 |
| C18:3*n*3 | 0.8 ± 0.1$^{a}$ | 0.6 | 1.0 | 0.6 ± 0.0$^{b}$ | 0.5 | 0.7 | 0.6 ± 0.1$^{a}$ | 0.5 | 0.7 | <0.05 |
| C22:6*n*3 | 0.0 ± 0.0$^{a}$ | 0.0 | 0.1 | 0.0 ± 0.0$^{b}$ | 0.0 | 0.0 | 0.0 ± 0.0$^{ab}$ | 0.0 | 0.0 | <0.05 |
| PUFA | 8.5 ± 1.7$^{b}$ | 6.3 | 13.56 | 8.3 ± 1.1$^{b}$ | 6.6 | 11.9 | 10.5 ± 1.0$^{a}$ | 8.4 | 11.5 | <0.05 |
| IV | 83.7 ± 1.3$^{b}$ | 80.2 | 86.8 | 83.8 ± 1.1$^{b}$ | 81.8 | 86.6 | 85.7 ± 1.01$^{a}$ | 84.0 | 87.2 | <0.05 |
| SN | 200.2 ± 0.6$^{a}$ | 198.4 | 201.7 | 200.0 ± 0.5$^{a}$ | 199.1 | 201.1 | 199.4 ± 0.37$^{b}$ | 198.6 | 199.9 | <0.05 |

Different superscript letters (a, b, c) in a row indicate significant differences from each other (Kruskal-Wallis tests and Mann-Whitney U-tests, $p < 0.05$). SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil.

A higher IV corresponds to a higher degree of unsaturation (Kalayasiri et al., 1996). In **Table 5.3**, IV of EVOO (83.7 ± 1.3 g 100 $g^{-1}$) was significantly lower than that of POO (85.7 ± 1.0 g 100 $g^{-1}$) (Mann-Whitney U-tests, $p < 0.05$), but was similar to the IV of ROO (83.8 ± 1.1 g 100 $g^{-1}$). This result confirms the assumptions in section 3.1.1. that POO presents a higher level of unsaturation than the other two olive oil grades. A higher SN implies a short carbon chain length (Pham et al., 2013). The SN value of EVOO (200.2 ± 0.6 mg KOH $g^{-1}$) was significantly higher than the SN value of POO (199.4 ± 0.4 mg KOH $g^{-1}$) (Mann-Whitney U-

tests, $p < 0.05$). Although the absolute difference is small, its significance implies a consistent slightly smaller FA chain length in EVOO in comparison to POO.

*5.3.2.2. Chlorophylls, carotenoids, chromatic coordinates CIELAB and moisture*

All samples were subjected to total chlorophylls analysis, total carotenoids analysis, chromatic coordinates CIELAB determinations and moisture content measurements. Average values are listed in **Table 5.4**.

The chlorophylls and carotenoids are considered the two main pigment groups in olive oil. They determine the characteristics and commercial quality of olive oil, especially EVOO (Marquez, 2003). **Table 5.4** shows that the concentration of total chlorophylls in EVOO varied from 0.2 to 6.3 mg $kg^{-1}$ and total carotenoids from 0.4 to 2.9 mg $kg^{-1}$, respectively. The large variation in concentrations within the EVOO group is due to the fact that pigments in EVOO vary with fruit ripeness and oil freshness (Marquez, 2003; Minguezmosquera et al., 1991). Nevertheless, the chlorophylls content in EVOO ($3.6 \pm 1.6$ mg $kg^{-1}$) was significantly higher than in ROO ($0.5 \pm 0.3$ mg $kg^{-1}$) and POO ($0.8 \pm 0.4$ mg $kg^{-1}$) (Mann-Whitney U-tests, $p < 0.05$). Similarly, the carotenoids content of EVOO ($1.7 \pm 0.5$ mg $kg^{-1}$) was significantly higher than those of ROO ($0.3 \pm 0.2$ mg $kg^{-1}$) and POO ($0.6 \pm 0.2$ mg $kg^{-1}$) (Mann-Whitney U-tests, $p < 0.05$). This is primarily due to removal of a large fraction of the pigments in the latter oils during the refining process (Antonopoulos et al., 2006).



The chromatic coordinates L*, a* and b* were determined to investigate the colour of the olive oils. The L* value of the EVOO group ($81.3 \pm 5.9$) was significantly lower than that of ROO ($91.3 \pm 3.0$) and POO ($91.7 \pm 3.2$) groups (Mann-Whitney U-tests, $p < 0.05$). This indicates that EVOO is darker in colour than the other grades. The mean values of a* for the EVOO ($-2.1 \pm 1.2$) and POO ($-1.9 \pm 1.6$) groups were significantly lower than the value for the ROO group ($1.2 \pm 1.2$) (Mann-Whitney U-tests, $p < 0.05$). This means that EVOO and POO possessed a stronger green colour than ROO, while ROO exhibited also some red colour. Furthermore, the b* value for the EVOO group ($58.1 \pm 12.4$) differed significantly from that of the ROO ($14.4 \pm 6.3$) and POO ($24.9 \pm 6.5$) groups (Mann-Whitney U-tests, $p < 0.05$). These results indicate that EVOO exhibited the strongest yellow colour intensity of the three olive oil grades. Regarding this aspect, EVOO was followed by POO and ROO, respectively. It is interesting that with this simple smartphone application one is able to roughly distinguish EVOO from the other olive oil grades by their colours.

The moisture contents of EVOO ranged from 0.1 to 0.2 g 100 $g^{-1}$, which shows a small overlap with ROO (0.0-0.1 g 100 $g^{-1}$) and POO (0.0-0.1 g 100 $g^{-1}$). The difference between the olive oil grades was, however, significant (Mann-Whitney U-tests, $p < 0.05$). Multiple filtering treatments of the refining process eliminate water in olive oil (Bakhouche et al., 2014). Therefore, also moisture content limits are higher for EVOO ($\leq 0.2$ g 100 $g^{-1}$) than for lower grade olive oils ($\leq 0.1$ g 100 $g^{-1}$) in IOC standards (IOC, 2016).

**Table 5.4**. Average values (Mean), standard deviations (SD), minimum (Min), maximum (Max) and significance of differences ($p$ value) of chlorophylls (mg $kg^{-1}$), carotenoids (mg $kg^{-1}$), chromatic coordinates CIELAB (L*, a* and b*) and moisture content (g 100 $g^{-1}$) determined in three olive oil grades.

| Parameter | EVOO ($n$ = 80) | | | ROO ($n$ = 40) | | | POO ($n$ = 10) | | | $p$ value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Min | Max | Mean ± SD | Min | Max | Mean ± SD | Min | Max | |
| Chlorophylls | 3.6 ± 1.6 $^{a}$ | 0.2 | 6.3 | 0.5 ± 0.3 $^{b}$ | 0.0 | 1.6 | 0.8 ± 0.4 $^{b}$ | 0.3 | 0.2 | <0.05 |
| Carotenoids | 1.7 ± 0.5 $^{a}$ | 0.4 | 2.9 | 0.6 ± 0.2 $^{b}$ | 0.1 | 1.2 | 0.6 ± 0.2 $^{b}$ | 0.4 | 0.8 | <0.05 |
| L* | 81.3 ± 5.9 $^{b}$ | 65.8 | 91.5 | 91.7 ± 3.2 $^{a}$ | 82.9 | 96.8 | 91.7 ± 3.2 $^{a}$ | 83.6 | 95.0 | <0.05 |
| a* | -2.1 ± 1.2 $^{b}$ | -5.2 | 0.3 | 1.2 ± 1.2 $^{a}$ | -0.8 | 3.1 | -1.9 ± 1.6 $^{b}$ | -4.4 | 0.4 | <0.05 |
| b* | 58.1 ± 12.4 $^{a}$ | 14.1 | 77.7 | 14.4 ± 6.3 $^{b}$ | 6.3 | 36.5 | 24.9 ± 6.5 $^{b}$ | 17.4 | 36.9 | <0.05 |
| Moisture | 0.1 ± 0.0 $^{a}$ | 0.1 | 0.2 | 0.1 ± 0.0 $^{b}$ | 0.0 | 0.1 | 0.1 ± 0.0 $^{b}$ | 0.0 | 0.1 | <0.05 |

Different superscript letters (a, b) in a row indicate significant differences from each other (Kruskal-Wallis tests and Mann-Whitney U-tests, $p < 0.05$). EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil.

### *5.3.3. Correlation of spectral and compositional data*

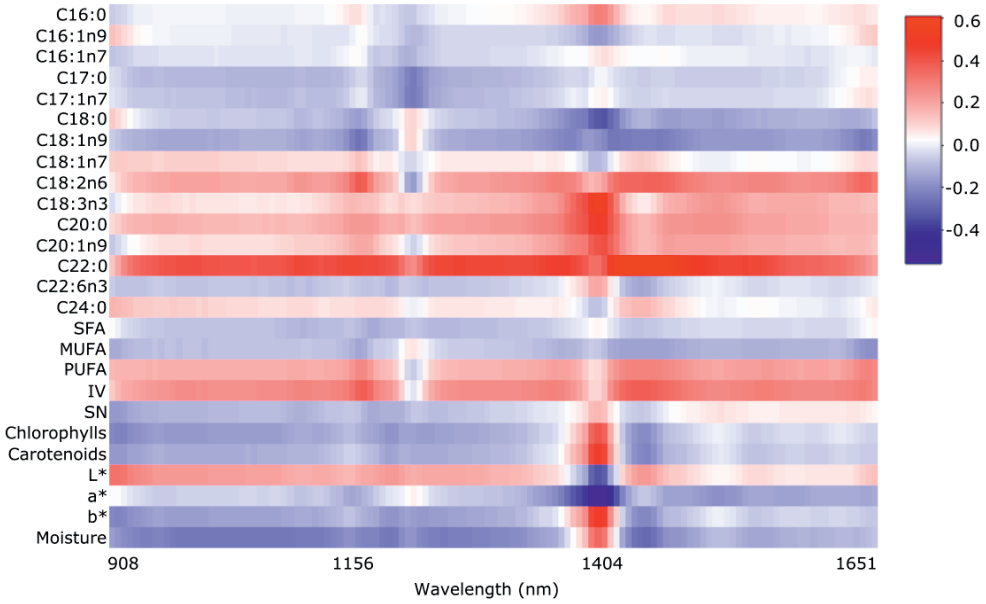


**Figure 5.4.** Pearson correlation coefficients for spectral data (horizontal axis) versus compositional data (vertical axis). Positive and negative coefficients are coloured red and blue, respectively. Larger absolute values of the coefficient (r) are presented as darker colours; coefficients < -0.17 and > + 0.17 indicate significant correlations (Paired samples t-test, $p < 0.05$).

The correlation coefficients (r) of the spectral and compositional data were calculated and are presented in **Figure 5.4**. Initially, the spectral data and FA data were correlated. The entire spectrum and the compositional characteristics C18:2*n*6, C22:0, PUFA and IV correlated significantly ($r > 0.17$, $p < 0.05$). Three of these characteristics, C18:2*n*6, PUFA and IV, have a relationship with the degree of unsaturation. The higher their values, the higher the absorption. Many correlations between compositional characteristics and the wavelength range selected for the classification models (1348-1571 nm) were observed. For instance, for C18:3*n*3, C20:0,

C20:1*n*9, C22:0, PUFA and IV, significant positive correlations were noted ($r > 0.17$, $p < 0.05$), whereas C18:0 and C18:1*n*9 were significantly negatively correlated with absorbance in this wavelength range ($r < -0.17$, $p < 0.05$). These results align with previous studies which also reported the impact of FAs on the absorption in the NIR range (Christy et al., 2004). Absorption behaviour of NIR spectra can be particularly explained by the degree of unsaturation of the fats and oils (Hourant et al., 2000; Sato, 2002).

The other compositional features were also correlated with the spectral data. L* and moisture content present a statistically significant correlation ($r > 0.17$, $p < 0.05$) with the spectral region of 908-1350 nm (Zone A and Zone B). Lightness of the oil and its moisture content seem to be related to the absorption intensity in this spectral range. Furthermore, wavelength 1391 nm in the selected wavelength range, which corresponds to the combination of C-H stretching and deformation, showed significant correlation ($p < 0.05$) with chlorophylls ($r = 0.36$), carotenoids ($r = 0.43$), L* ($r = -0.30$), a* ($r = -0.52$), b* ($r = 0.44$) and moisture content ($r = 0.31$). Wavelength 1453 nm, which results from the first overtone of O-H, is significantly correlated with chlorophylls ($r = -0.17$), carotenoids ($r = -0.19$), L* ($r = 0.21$), b* ($r = -0.19$) and moisture content ($r = -0.27$) ($p < 0.05$). These correlations are interesting because 1391 nm and 1453 nm contributed highly to the EVOO distinction. These results indicate that chlorophylls, carotenoids and moisture contents might played an important role in the absorption intensity of the oils at wavelength 1391 nm and 1453 nm in addition to the unsaturation degree of the oils. Pigments apparently also affected the chromatic coordinates CIELAB of the oils. Moreover, moisture content presents a significant correlation ($p < 0.05$) with two spectral bands in the selected wavelength range, i.e. in the range of 1391-1404 nm and 1435-1460 nm. This is in agreement with previous studies, in which the range of 1390-1453 nm was assigned to the first overtone of $H_2O$ (Fernandez-Cabanas et al., 2011; Hourant et al., 2000).



### 5.4. Conclusions and considerations

Based on the 130 commercial olive oil samples used in this study, the handheld NIR appeared promising for distinction of pure olive oil grades. This new methodology is especially of interest because of its potential in situ use and application by non-scientists. The degree of unsaturation of the FAs as well as chlorophylls, carotenoids and moisture contents appear to be related to the spectral differences between the olive oil grades. This study is a good first step towards a useful practical application. The latter will require, however, expansion of the olive oil database to cover more compositional variation in practice. It is also imperative to look into blends of grades in future studies, since most fraud in practice are admixtures rather than full replacements. Another point for future consideration is, like for any authentication method, the robustness of the methodology against attempts of fraudsters to outfox the scientists.

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# Chapter 6

## A sound approach: Exploring a rapid and non-destructive ultrasonic pulse echo system for vegetable oils characterisation

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**Abstract**

A rapid and non-destructive ultrasonic pulse echo system was developed for vegetable oils characterisation. To understand the differences in the ultrasonic properties of the oils, physical traits, such as their viscosity and density, were related to the ultrasonic data. In turn, these physical traits were correlated with the fatty acid (FA) compositions of the oils. Eighty oil samples, including 30 extra virgin olive oil (EVOO), 15 refined olive oil (ROO), 15 pomace olive oil (POO), 10 rapeseed oil, 5 sunflower oil and 5 peanut oil samples, were analysed for their sound properties, viscosities, densities and FA compositions. It was observed that the ultrasonic velocity of EVOO decreased linearly with increase in temperature, the temperature coefficient of ultrasonic velocity in EVOO was -2.92 $m \cdot s^{-1} \cdot °C^{-1}$. The ultrasonic velocity of EVOO (1453 ± 2 m/s) differed significantly from those of POO and the oils of other botanical origin, but not from the velocity of ROO. Ultrasonic velocity was strongly positively correlated with the density and negatively correlated with the viscosity of the oils. The higher density and lower viscosity of the oils were in turn related to a higher unsaturation degree of the oils. Hence, oils with a higher proportion of unsaturated fat present higher densities and lower viscosities, which resulted in higher ultrasonic velocity values. Ultrasonic measurements allow rapid, non-destructive analysis. This first application for characterisation of these oils is promising.

**Keywords:** Density; fatty acids; rheology; ultrasonic velocity.

### 6.1. Introduction

Extra virgin olive oil (EVOO) extracted from fresh olive fruits using traditional cold pressing methods is the highest quality olive oil available commercially. Because of its high nutritional value, premium organoleptic quality and high price, it turns out to be an attractive target for food fraud. EVOO adulteration is considered a common fraudulent practice in the olive oil industry and these adulterations include the addition of lower grade olive oils (refined olive oil (ROO) and pomace olive oil (POO)) and other vegetable oils (rapeseed oil (RSO), sunflower oil (SFO) and peanut oil (PNO)) (De Oliveira & Catharino, 2015).

In order to ensure food safety and assess EVOO characterisation, the European Commission (2013) and IOC (2016) released official methods on the characteristics of olive oil and on the relevant methods of analysis. Moreover, a large number of potential techniques for EVOO characterisation have been reported (Bajoub et al., 2018), which can be divided into chromatography (Yan et al., 2018), mass spectrometry (Marone et al., 2017), vibrational spectroscopy (Wang et al., 2016), nuclear magnetic resonance (Fragaki et al., 2005), DNA-based techniques (Pasqualone et al., 2016) and other techniques (Chiavaro et al., 2008). Rapid, inexpensive and non-invasive analytical approaches for EVOO characterisation are continually being sought (Persuric et al., 2018; Squeo et al., 2019), whereas the sound properties, which could be interesting markers for rapid and non-destructive analysis, have hardly received any attention to date. Sound (0-20 kHz) measurements have been applied as an effective approach for salt profiling and identification (van Ruth et al., 2019). Analyses using the ultrasound (> 20 kHz) range could also be promising for food characterisation. Ultrasonic spectroscopy is a development of the pulse-echo technique which uses broadband (0.5-10 MHz) ultrasound and analyses the spectra of the echo pulses (Brown, 1973). It is a promising technique for rapid and non-destructive analyses. The ultrasonic waves generated from an ultrasonic transducer, propagate through the oil samples. Thus, measurement of the characteristics of these ultrasonic waves may then show differences which relate to properties of vegetable oils.



Measurement of the ultrasonic velocity (speed of sound) is the basis of most ultrasonic techniques used to evaluate the properties of foods (McClements, 1997). Furthermore, ultrasonic velocity has been correlated to the rheological properties of vegetable oils (Gladwell et al., 1985) and the density of oils (Sankarappa et al., 2005). Measurements of ultrasonic velocity have been employed to characterise edible oils (Alouache et al., 2015; Alouache et al., 2018), such as for solid fat content analysis (Singh et al., 2004), recycled edible oils evaluation (Ali & Ahmad, 2018) and frying oil degradation assessments (Benedito et al., 2007; Benedito et al., 2002). Moreover, researchers have previously reported a potential correlation between ultrasonic velocities of olive oil and their chemical composition (Kumari et al., 2017; McClements & Povey, 1988), which has been proposed as an alternative method for compositional analyses. Therefore, ultrasonic measurements could be an alternative and promising approach for rapid and non-destructive assessment of the integrity of oils.

Considering the vulnerability of olive oil to fraud, olive oil would be an interesting target to explore this type of approach.

In this study, a pulse-echo ultrasonic system was employed to explore the characterisation of the vegetable oils and to examine the underlying causes for the ultrasonic velocity differences between the oils. The detailed objectives of this study are 1) to evaluate a developed ultrasonic velocimetry measurement system for the vegetable oils characterisation; 2) to investigate the correlation between ultrasonic measurements and the viscosity and density of the oils; 3) to relate the viscosity and density of the oils to their FA compositions.

## 6.2. Materials and methods

### *6.2.1. Samples*

Eighty vegetable oil samples were collected, and their authenticity was confirmed by official methods, including FA compositional fingerprinting and spectrophotometric tests measuring extinction coefficients (K232, K268 and ΔK) (IOC, 2017). They were also evaluated for their monochloropropanediol esters contents (Yan et al., 2018). They included olive oil samples: 30 EVOO, 15 ROO and 15 POO samples, as well as oils of other botanical origins which are often found as adulterants: 10 RSO, 5 SFO and 5 PNO samples. Prior to analysis, samples were stored in capped bottles, which were kept in the dark at room temperature until analysis.

### *6.2.2. Ultrasonic velocity analysis*

The ultrasonic measurements were carried out using a purpose-built pulse-echo system. This pulse-echo system, shown schematically in **Figure 6.1a**, is composed of a sample platform (**Figure 6.1b**), an immersion transducer (diameter 12.54 mm, Panametrics-NDT, Olympus NDT U.K. Ltd., Rotherham, South Yorkshire, UK) with a central frequency of 5 MHz, a computer controlled pulse generator/receiver (Panametrics-NDT Model 5800, Olympus NDT U.K. Ltd., Rotherham, South Yorkshire, UK), an oscilloscope (Tektronix TDS 210, Tektronix UK Ltd., Bracknell, UK) and a computer (Dell, Texas, US).

As can be seen in **Figure 6.1b**, a special sample platform which is fixed on a stainless-steel base plate was custom fabricated and consisted of a transparent plastic tube (2), a solid stainless-steel cylinder (3) and a micrometre (4) (fixed on one side of a vertical stainless-steel bracket). A good seal between the plastic cylinder and the solid stainless-steel cylinder is managed by rubber rings to avoid leakage, and the generated volume is used as a sample cell (63 mm internal diameter). The transducer (1) is placed in a holder fixed to the micrometre. The distance between the transducer surface and the bottom of the sample cell can be measured with the micrometre (0.01 mm), with an integrated spring in the micrometre maintaining a

constant position force. The sample platform was placed into a temperature-controlled water bath to maintain the sample temperature (23.5 °C ± 0.1 °C). When recording a measurement, the face of the transducer had to be placed below the oil surface as shown in **Figure 6.1b**. Any air bubbles trapped in the oil on the transducer face were manually removed prior to testing by swiping over the surface of the transducer with finger.

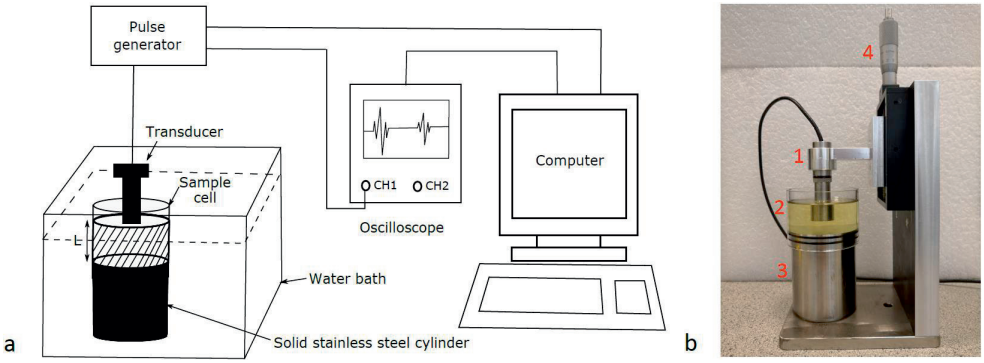


**Figure 6.1** a Schematic diagram of ultrasonic pulse-echo system. L, length of the half-way wave path in oil samples. b Sample platform of the pulse-echo system. (1) transducer; (2) transparent plastic cylinder; (3) solid stainless-steel cylinder; (4) micrometre.

The principle of the pulse echo system is similar to that reported by other researchers (Alouache et al., 2016; Awad et al., 2012) but presents some differences. The computer controlled pulse generator/receiver is used to produce/generate monopolar electrical pulses, which are converted to ultrasonic pulses by the transducer. The pulse generator/receiver was operated in pulse echo mode with a pulse repetition frequency of 1 kHz, 25 μJ pulse energy with 20 dB of input attenuation and 20 dB of receiver amplification. The generated ultrasonic pulse propagates through the vegetable oils from the transducer until it reflects from the stainless-steel cylinder face (solid reflector), back to the same transducer, which acts as a receiver and converts the returned ultrasonic pulses into electrical signals that are displayed on the oscilloscope. Subsequently, the ultrasonic signal from each sample was transferred to the computer via a general-purpose interface bus interface with the oscilloscope. The signal acquisition was repeated with different separations between the transducer face and the stainless-steel base using a MATLAB (R2015b, The MathWorks Inc., Natick, MA, USA) program.

The ultrasonic velocity can be calculated by analysing the times of the echoes received at the oscilloscope. As shown in **Figure 6.1a**, the distance between the transducer and the bottom of the sample cell (L) is equal to half the distance propagated by the ultrasonic pulse. Six measurements were carried out under six consecutive distances (10, 12, 14, 16, 18 and 20 mm) for each sample. The amount of the oils used under each measurement distance are 31.17,

37.41, 43.64, 49.88, 56.11 and 62.34 mL, respectively. The ultrasonic velocity (V) can be thus calculated by two measurements under two consecutive distances using equation (1):

$$V = \frac{2(L_2 - L_1)}{T_2 - T_1} \quad , \quad (1)$$

where $L_1$ is the lower distance of one-way echo path (m); $L_2$ is the consecutive next distance of one-way echo path (m), which is larger than $L_1$; $T_1$ is the time of receiving the first reflected signal (s) under the distance of $L_1$; $T_2$ is the time of receiving the first reflected signal (s) under the distance of $L_2$; V is the propagation velocity of the ultrasound in samples (m/s).

Since six distances were measured for each sample, five velocity values were calculated. The final velocity of each sample was the average of the five velocity values. This device was calibrated by measuring the ultrasonic velocity of water (1499 ± 3 m/s at 26 °C) (Engineering ToolBox, 2004).

### *6.2.3. Viscosity analysis*

The apparent viscosity of the oils was measured using a Haake Roto Visco 1 rotational viscometer (Thermo Fisher Scientific, GmbH, Karlsruhe, Germany), equipped with a Z41 concentric cylinder and Z43 cup. Each sample (10 mL) was loaded into the gap between the cylinder and the cup, before being allowed to equilibrate at 20 °C for 1 min before analysis. In the first step, the sample was subjected to a shear rate ramp from 0 to 200 $s^{-1}$ over 2 min, then held at 200 $s^{-1}$ for 2 min before returning shear rate from 200 to 0 $s^{-1}$ over 2 min. The test was carried out at 20.0 ± 0.1 °C, and the temperature was controlled by a digital recirculating water bath. The average viscosity was taken at a shear rate of 200 $s^{-1}$. The equation of the viscosity (see Equation (2)) was used to describe the rheological properties of the samples:

$$\eta = \frac{\tau}{\gamma} \quad , \quad (2)$$

where $\eta$ is the viscosity (Pa.s), $\tau$ is the shear stress (Pa), $\gamma$ is the shear rate ($s^{-1}$). Duplicate measurements of all samples were conducted.

### *6.2.4. Density analysis*

The density values were calculated using a gravimetric method. The flask and the 25 mL glass pipette were weighed together ($m_1$) by electronic lab balance (± 0.001 g), then the 25 mL ($v$) oil sample was pipetted into the flask by the glass pipette. Subsequently, the flask, the glass pipette and the oil were weighed together ($m_2$). The density of the oil was obtained by using equation (3):

$$\rho = \frac{m_2 - m_1}{v} \times 1000 \quad , \quad (3)$$

where $\rho$ is the density of the sample (kg/m$^3$), $m_1$ is the total weight of the empty flask and the glass pipette (g), $m_2$ is the total weight of the oil sample, the flask and the glass pipette (g), and $v$ is the accurate volume of the sample (mL). The tests were carried out at room temperature (20.0 ± 0.1 °C). Duplicate measurements were conducted for all samples.

### *6.2.5. Measurement of fatty acids*

#### *6.2.5.1. Fatty acid methyl esters preparation*

The 100 μL of the internal standard solution (50 mg tritridecanoin (C13:0) and 50 mg methyl undecanoate (C11:0) dissolved by 10 mL pentane) was pipetted into a gas chromatography (GC) vial and the solution was evaporated to dry under a stream of nitrogen. Then, one droplet of each oil sample (approximately 10-20 mg) was transferred into the prepared GC vial and the vial was closed with a magnetic cap. Then, the samples were placed in the autosampler (Gerstell MPS, GmbH, Germany). Subsequently, an automatic boron trifluoride transmethylation procedure was conducted for the sample preparation as reported in ISO 12966-2 (2017).

#### *6.2.5.2. Fatty acid methyl ester analysis*

The gas chromatographic analysis of fatty acids (FAs) was carried out according to ISO 12966-4 (2015). The Agilent HP7890A GC (Agilent Technologies, Inc., Wilmington, DE, USA) was equipped with a 100 m × 0.25 mm × 0.2 μm film thickness fused silica capillary column (Varian, Palo Alto, CA) coupled to a flame ionisation detector. Working condition: initial column temperature, 120 °C; final column temperature, 240 °C; heating ramp, 4.0 °C/min; hold time, 7 min at 240 °C; run time, 37 min; carrier, hydrogen; constant flow, 1.0 mL/min; injection, 1 μL; split, 1:100; injector temperature, 250 °C; detector temperature, 250°C.



Individual fatty acid methyl esters (FAMEs) were identified by comparison with external standards. The internal standard C13:0 was employed for the quantification of individual FAMEs. In addition, the performance of transesterification was evaluated by internal standards C13:0 and C11:0. Blanks were performed prior to analysing each batch of samples for the stability test of the machine.

Iodine values (IV, g I/100g) and Saponification numbers (SN, mg KOH/g) of 80 vegetable oils were calculated from their FA compositions as described by Kalayasiri et al. (1996). Duplicate measurements of all samples were conducted.

### *6.2.6. Statistical analysis*

Means and standard deviations were calculated for each sample and oil group. Non-parametric Kruskal-Wallis tests were applied for group comparisons due to non-normality nature of the data. The pairwise comparisons were carried out by Mann-Whitney U-tests ($p <$

0.05). All of the above data analysis methods were performed by SPSS statistic 23 (IBM, Chicago, IL, USA).

The correlations between the ultrasonic velocity, viscosity and density, as well as between these physical characteristics and the FAs compositional data were assessed by computing Pearson correlation coefficients (r). Paired samples t-tests were applied to assess significance of the correlation coefficients. All calculations were performed by scripts developed in R 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria).

Partial least squares (PLS) regressions with leave-one-out cross validation were carried out, using Pirouette 4.5 (Infometrix, USA) to create predictive models based on FAs data. The FAs dataset was pre-processed in three ways, including auto-scaling, mean-centring and range-scaling. The performance of models was evaluated by coefficient of determination for cross-validation ($r^2$) and standard error of cross-validation (SEV), since SEV is the best single estimate of the prediction capability of these kinds of models (Fernandez-Cabanas et al., 2011).

## 6.3. Results and discussion

### *6.3.1. Pulse echo system - ultrasonic velocity measurements*

#### *6.3.1.1. Development of the measurement system*

In terms of the sample platform, oil was placed in a sample cell of 63 mm internal diameter with adjustable height from 0 to 50 mm, which illustrate the maximum amount of oil required for the measurement is less than 150 mL. The amount of the oils used under each measurement distance are 31.17 mL (10 mm), 37.41 mL (12 mm), 43.64 mL (14 mm), 49.88 mL (16 mm), 56.11 mL (18 mm) and 62.34 mL (20 mm). Moreover, the measurement time for each sample at a certain distance from generating signal to receiving and recording the signal is less than 30 s, and this could be reduced with automation of the transducer positioning.

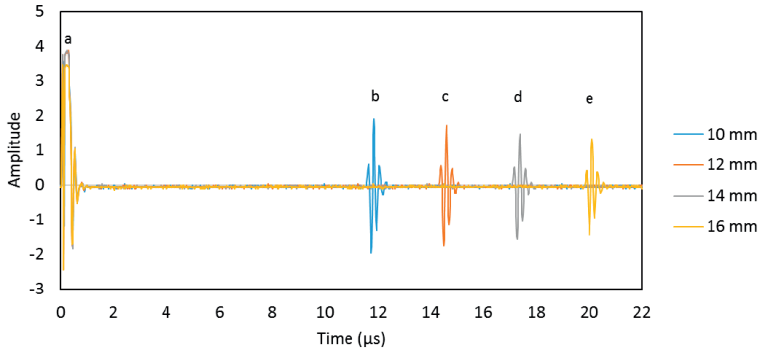


**Figure 6.2.** Plot of the first propagation signal of extra virgin olive oil analysed at four distances. a refers to the transmitter pulse; b, c, d, e refer to the first propagation signal at the distance of 10, 12, 14, 16 mm, respectively.

**Figure 6.2** shows the variation in ultrasonic propagation delay of EVOO under four different distances (10 mm, 12 mm, 14 mm and 16 mm). The first signal (a) in **Figure 6.2** is the transmitter pulse without propagating through the oil sample and is electronic cross-talk between the pulser and the amplifier. Thus, the first propagation signal is the second one (b), the time delay of receiving the first propagation signal (the time when the signal reaches its peak) increases when the distance increases, i.e., from 11.862 μs (b) to 14.614 μs (c), 17.364 μs (d) and 20.116 μs (e). Because of the difficulty of picking out the peak of the first signal, the velocity of ultrasound cannot be calculated using the time and distance directly, but applying the relative time between two distances is an alternative way for velocity calculation. In this case, the mean value of velocity of EVOO is 1453.84 ± 0.47 m/s.

*6.3.1.2. Initial evaluation of the measurement system*

Previous studies reported that the ultrasonic properties may vary when the measurement temperature is changed (Benedito et al., 2002; Sankarappa et al., 2005). The variation of the ultrasonic velocity with temperature in EVOO was measured over a temperature range from 18 to 36 °C, using an ultrasonic frequency of 5 MHz. The signal was recorded at 2 °C intervals and the temperature was controlled by a water bath. As shown in **Figure 6.3**, the velocity was observed to be decreasing with the increase of temperature in the applied temperature range, which results in a velocity decrease from 1470 to 1416 m/s. Sankarappa et al. (2005) explained that velocity changes with temperature are attributed to changes in intermolecular distance with temperature.



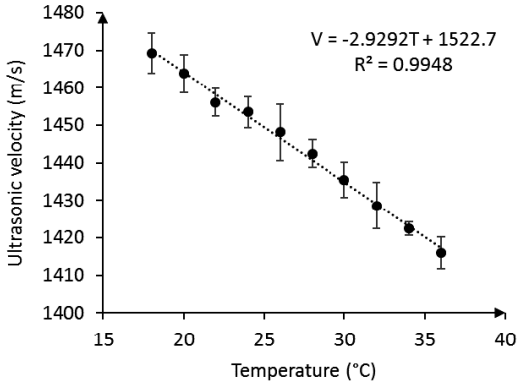


**Figure 6.3.** Variation of ultrasonic velocity with temperature for extra virgin olive oil at the ultrasonic frequency of 5 MHz.

**Figure 6.3** reveals that the correlation coefficient is more than 0.99, therefore, ultrasonic velocity is linearly related to the temperature of the olive oil over the temperature range studied. This is in agreement with other observations made in olive oil (Benedito et al., 2002) and other oils (sunflower oil and refined groundnut oil) (Sankarappa et al., 2005). However, a previous study also reported that velocity decreased nonlinearly with increasing temperature in coconut

oil, castor oil and unrefined Kardi (safflower) oil (Sankarappa et al., 2005). Moreover, the temperature coefficient for ultrasonic velocity in EVOO is -2.93 $m \cdot s^{-1} \cdot °C^{1}$. This slope is close to the value reported by Benedito et al. (2002) for olive oil (ranges from -3.37 to -3.54 $m \cdot s^{-1} \cdot °C^{-1}$) and McClements and Povey (1988) (-3.28 $m \cdot s^{-1} \cdot °C^{-1}$). This illustrates that the assay accuracy of this pulse echo system is high. Since the ultrasonic measurements are temperature dependent, all 80 samples were measured at a constant temperature (23.5 °C ± 0.1°C) in the subsequent study to obtain repetitive and reliable results.

### *6.3.2. Ultrasonic velocity of the different oils*

The 80 vegetable oils, including 30 EVOO, 15 ROO, 15 POO, 10 RSO, 5 SFO and 5 PNO, were subjected to ultrasonic velocity measurements using the developed pulse echo system, and the results are presented in **Table 6.1**. As can be seen in **Table 6.1**, the ultrasonic velocities of the six categories are in the range of 1450 to 1462 m/s and significant differences between groups were observed (Kruskal-Wallis test, $p < 0.05$). Specifically, a significant difference exists between EVOO (1453 ± 2 m/s) and the other vegetable oils (RSO (1460 ± 1 m/s), SFO (1461 ± 1 m/s) and PNO (1458 ± 1 m/s)), which also can be noticed in **Table 6.1**. Furthermore, a significant difference in mean values between EVOO (1453 ± 2 m/s) and POO (1456 ± 1 m/s) was established. This result may be explained by the fact that POO is extracted from olive residue with the help of chemicals and/or high temperatures, which results in a lower quality oil compared with EVOO, with different characteristics (Gunstone, 2011). However, there is no significant difference between EVOO (1453 ± 2 m/s) and ROO (1455 ± 1 m/s). This may due to the fact that EVOO and ROO are primarily a mixture of triacylglycerols with a similar pattern, with only 0.5-1.5% FAs, mono- and diacylglycerols and non-glyceridic constituents (Gunstone, 2011). Thus, differences in composition are relatively small.

Three vegetable oils (RSO, SFO and PNO) present higher velocity values compared with the olive oils with average values of 1460 ± 1 m/s, 1461 ± 1 m/s and 1458 ± 1 m/s, respectively. Meanwhile, the velocities of SFO and RSO are higher than the velocity of PNO, this trend is in accordance with earlier observations reported by Coupland and McClements (1997).

In the following sections we explore underlying causes for the differences established.

### *6.3.3. Viscosity and density of the different oils*

All samples were subjected to viscosity and density measurements, the results of which are presented in **Table 6.1**, along with the significant differences between oil groups. The viscosity of EVOO (83.3 ± 1.2 mPa.s) is significantly higher than that of RSO (73.1 ± 3.9 mPa.s) and SFO (67.3 ± 2.9 mPa.s) and is significantly lower than that of PNO (86.7 ± 1.5 mPa.s). Furthermore, the viscosity of EVOO (83.3 ± 1.2 mPa.s) is significantly lower than ROO (86.3 ± 0.8 mPa.s) and POO (86.4 ± 1.4 mPa.s). This may be due to the high temperature during the

refining process, which leads to the formation of long-chain FAs (polymers), which results in the increase of viscosity (Kreps et al., 2017).

**Table 6.1.** Averages and standard deviations of ultrasonic velocity, viscosity and density of 80 oil samples, including 30 extra virgin olive oil samples (EVOO), 15 refined olive oils (ROO), 15 pomace oils (POO), 10 rapeseed oils (RSO), 5 sunflower oils (SFO) and 5 peanut oils (PNO).

| Type | Ultrasonic velocity (m/s) | Viscosity (mPa.s) | Density (kg/m$^3$) |
|---|---|---|---|
| EVOO (n = 30) | 1455 ± 2 | 83.6 ± 0.4 | 913 ± 0 |
| | 1456 ± 1 | 82.9 ± 0.3 | 911 ± 0 |
| | 1455 ± 0 | 83.4 ± 0.2 | 912 ± 0 |
| | 1454 ± 2 | 85.5 ± 0.1 | 911 ± 1 |
| | 1454 ± 2 | 82.5 ± 0.2 | 912 ± 0 |
| | 1455 ± 3 | 82.8 ± 0.1 | 909 ± 0 |
| | 1452 ± 1 | 83.4 ± 0.2 | 911 ± 0 |
| | 1456 ± 3 | 84.2 ± 0.2 | 909 ± 0 |
| | 1453 ± 2 | 84.5 ± 0.3 | 912 ± 1 |
| | 1452 ± 0 | 81.4 ± 0.2 | 911 ± 1 |
| | 1455 ± 0 | 84.1 ± 0.2 | 912 ± 0 |
| | 1453 ± 2 | 82.1 ± 0.4 | 913 ± 1 |
| | 1453 ± 3 | 83.8 ± 0.2 | 913 ± 1 |
| | 1454 ± 1 | 83.0 ± 0.3 | 914 ± 1 |
| | 1456 ± 3 | 86.4 ± 0.4 | 913 ± 1 |
| | 1454 ± 1 | 82.8 ± 0.2 | 909 ± 1 |
| | 1453 ± 2 | 83.5 ± 0.3 | 911 ± 0 |
| | 1452 ± 1 | 85.5 ± 0.2 | 911 ± 1 |
| | 1452 ± 1 | 82.7 ± 0.4 | 913 ± 0 |
| | 1452 ± 2 | 84.3 ± 0.2 | 909 ± 0 |
| | 1452 ± 2 | 83.3 ± 0.4 | 912 ± 1 |
| | 1450 ± 1 | 82.0 ± 0.2 | 912 ± 0 |
| | 1455 ± 3 | 81.9 ± 0.3 | 911 ± 0 |
| | 1453 ± 4 | 83.6 ± 0.4 | 916 ± 1 |
| | 1453 ± 2 | 84.3 ± 0.2 | 912 ± 1 |
| | 1452 ± 2 | 83.0 ± 0.3 | 913 ± 0 |
| | 1454 ± 1 | 82.6 ± 0.2 | 911 ± 0 |
| | 1452 ± 0 | 82.2 ± 0.4 | 914 ± 1 |
| | 1448 ± 1 | 82.4 ± 0.3 | 914 ± 0 |
| | 1454 ± 3 | 83.3 ± 0.2 | 912 ± 0 |
| Mean ± SD* | 1453 ± 2 $^{c}$ | 83.3 ± 1.2 $^{b}$ | 912 ± 2 $^{d}$ |
| ROO (n = 15) | 1455 ± 2 | 87.1 ± 0.2 | 913 ± 0 |
| | 1453 ± 2 | 86.6 ± 0.2 | 912 ± 1 |
| | 1454 ± 1 | 87.0 ± 0.3 | 913 ± 1 |
| | 1454 ± 4 | 86.4 ± 0.4 | 913 ± 0 |
| | 1455 ± 2 | 84.7 ± 0.4 | 913 ± 1 |
| | 1456 ± 0 | 85.6 ± 0.3 | 914 ± 1 |
| | 1457 ± 3 | 85.5 ± 0.2 | 913 ± 1 |
| | 1454 ± 3 | 86.1 ± 0.3 | 913 ± 0 |
| | 1454 ± 2 | 88.0 ± 0.3 | 913 ± 0 |
| | 1455 ± 1 | 86.0 ± 0.1 | 912 ± 0 |
| | 1456 ± 4 | 86.0 ± 0.2 | 913 ± 0 |
| | 1455 ± 3 | 86.9 ± 0.1 | 913 ± 0 |
| | 1454 ± 2 | 86.5 ± 0.2 | 913 ± 1 |
| | 1456 ± 4 | 85.8 ± 0.2 | 910 ± 0 |
| | 1453 ± 2 | 85.7 ± 0.2 | 912 ± 0 |
| Mean ± SD | 1455 ± 1 $^{bc}$ | 86.3 ± 0.8 $^{a}$ | 913 ± 1 $^{cd}$ |
| POO (n = 15) | 1457 ± 1 | 88.0 ± 0.2 | 911 ± 0 |
| | 1456 ± 1 | 85.8 ± 0.2 | 912 ± 1 |
| | 1457 ± 2 | 85.8 ± 0.3 | 911 ± 1 |
| | 1456 ± 3 | 86.1 ± 0.3 | 915 ± 0 |
| | 1456 ± 2 | 90.4 ± 0.3 | 914 ± 1 |
| | 1455 ± 2 | 87.4 ± 0.3 | 913 ± 0 |
| | 1457 ± 8 | 87.6 ± 0.3 | 916 ± 0 |
| | 1455 ± 3 | 84.7 ± 0.3 | 915 ± 1 |
| | 1457 ± 1 | 85.5 ± 0.3 | 916 ± 0 |
| | 1457 ± 2 | 86.2 ± 0.3 | 917 ± 1 |
| | 1455 ± 1 | 86.2 ± 0.3 | 917 ± 1 |
| | 1454 ± 1 | 85.7 ± 0.2 | 914 ± 0 |
| | 1456 ± 2 | 86.7 ± 0.2 | 916 ± 0 |
| | 1458 ± 2 | 85.7 ± 0.2 | 914 ± 1 |
| | 1457 ± 2 | 84.9 ± 0.1 | 918 ± 1 |
| Mean ± SD | 1456 ± 1 $^{b}$ | 86.4 ± 1.4 $^{a}$ | 915 ± 2 $^{bc}$ |
| RSO (n = 10) | 1461 ± 3 | 72.1 ± 0.2 | 920 ± 1 |
| | 1461 ± 1 | 72.2 ± 0.3 | 921 ± 4 |
| | 1460 ± 2 | 73.4 ± 0.2 | 922 ± 3 |
| | 1459 ± 2 | 70.6 ± 0.1 | 915 ± 1 |
| | 1460 ± 3 | 71.9 ± 0.2 | 919 ± 1 |
| | 1462 ± 2 | 65.1 ± 0.3 | 920 ± 1 |
| | 1462 ± 3 | 75.5 ± 0.2 | 917 ± 1 |
| | 1461 ± 3 | 74.9 ± 0.2 | 918 ± 1 |
| | 1459 ± 2 | 74.8 ± 0.2 | 917 ± 1 |
| | 1459 ± 3 | 80.2 ± 0.3 | 915 ± 0 |
| Mean ± SD | 1460 ± 1 $^{a}$ | 73.1 ± 3.9 $^{c}$ | 919 ± 3 $^{a}$ |
| SFO (n = 5) | 1461 ± 1 | 64.8 ± 0.1 | 921 ± 1 |
| | 1460 ± 2 | 67.1 ± 0.2 | 922 ± 0 |
| | 1461 ± 5 | 65.8 ± 0.2 | 921 ± 1 |
| | 1462 ± 4 | 66.5 ± 0.2 | 921 ± 1 |
| | 1460 ± 4 | 72.2 ± 0.2 | 920 ± 0 |
| Mean ± SD | 1461 ± 1 $^{a}$ | 67.3 ± 2.9 $^{d}$ | 921 ± 1 $^{a}$ |
| PNO (n = 5) | 1458 ± 5 | 87.6 ± 0.2 | 916 ± 1 |
| | 1457 ± 3 | 87.5 ± 0.2 | 916 ± 0 |
| | 1458 ± 2 | 86.8 ± 0.2 | 916 ± 0 |
| | 1457 ± 4 | 84.2 ± 0.2 | 915 ± 0 |
| | 1458 ± 5 | 87.6 ± 0.3 | 915 ± 0 |
| Mean ± SD | 1458 ± 1 $^{b}$ | 86.7 ± 1.5 $^{a}$ | 915 ± 1 $^{b}$ |

Different letters (a, b, c) indicate significant differences between oil groups (Kruskal-Wallis and Mann-Whitney U tests, $p < 0.05$).

As shown in **Table 6.1**, the three olive oils (EVOO, ROO, POO), with viscosities ranging from 81-88 mPa.s, are more viscous than RSO (73.1 ± 3.9 mPa.s) and SFO (67.3 ± 2.9

mPa.s). This may be due to the fact that the majority of bonds in olive oil are single bonds with a "zig-zag" configuration, which cause higher viscosities than other bonds (Schaschke et al., 2006). However, the viscosity of PNO (86.7 ± 1.5 mPa.s) does not significantly differ from ROO (86.3 ± 0.8 mPa.s) and POO (86.4 ± 1.4 mPa.s), which is possibly due to the broad similarity in composition.

The density values of EVOO (912 ± 2 kg/m$^3$) differ significantly from those of the other three vegetable oils (RSO, SFO and PNO; **Table 6.1**). These differences are mainly due to differences in chemical composition (Kalogianni et al., 2011). Furthermore, a significant difference between density values of EVOO (912 ± 2 kg/m$^3$) and POO (915 ± 2 kg/m$^3$) is observed, but no significant difference between EVOO (912 ± 2 kg/m$^3$) and ROO (913 ± 1 kg/m$^3$). It is evident that the density of EVOO is lowest for all olive oils. And this is in agreement with the previous study reported that unrefined oils have lower densities than their refined counterparts (Sankarappa et al., 2005).

### *6.3.4. Correlation of the ultrasonic velocity, viscosity and density of the oils*

In order to explore the correlation of the ultrasonic velocity, viscosity and density in vegetable oils, the relationship between velocity data and the viscosity and density data are shown in **Figure 6.4a** and **6.4b**, respectively. The EVOO is located in the lower right hand corner of the velocity/viscosity plot (**Figure 6.4a**) and fully separated from the oils of other botanical origins (RSO, SFO and PNO). EVOO presents somewhat lower viscosity and velocity values than the lower grade olive oils (ROO and POO). Subsequently, the correlation coefficient (r) was calculated for the ultrasonic velocity and viscosity. A significant negative correlation between the velocity and the viscosity (r = -0.64, $p < 0.05$) was observed, which is in agreement with previous research (Alouache et al., 2016; Alouache et al., 2015).

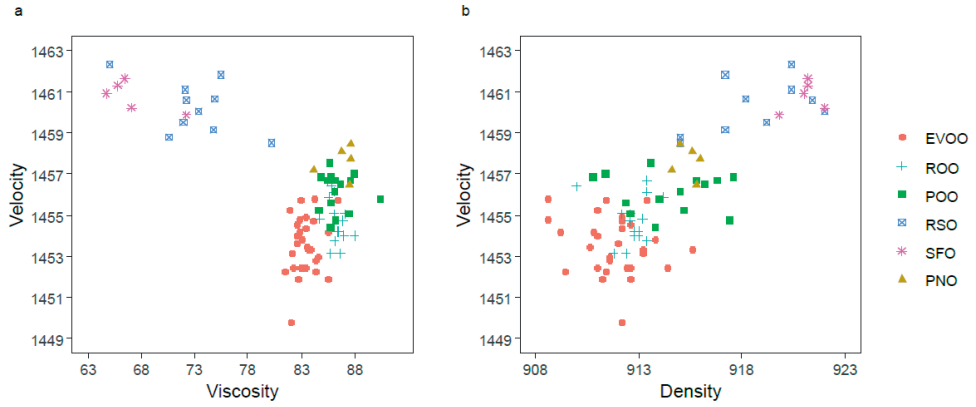


**Figure 6.4.** Scatter plots of ultrasonic velocity versus a) viscosity and b) density data of 30 extra virgin olive oils (EVOO), 30 other olive oil grade samples (15 refined olive oils (ROO) and 15 pomace oils (POO)) and 20 oils of other botanical origins (10 rapeseed oils (RSO), 5 sunflower oils (SFO) and 5 peanut oils (PNO)).

Comparing the velocity and density values, it appears that the EVOO is located in the lower left side corner of the corresponding plot (**Figure 6.4b**). EVOO can be discriminated from RSO, SFO and PNO using velocity and density values, but EVOO has a slight overlap with POO and presents considerable overlap with ROO. A significant positive correlation ($r = 0.75$, $p < 0.05$) between the velocity and density was observed.

The results above reveal that the intermolecular structure may be responsible for the velocity differences. Further exploration of correlation of ultrasonic velocity, viscosity, density and intermolecular composition would be useful.

### *6.3.5. Fatty acid composition of the oils*

The 80 oils were analysed for their FA compositions, the results of which are listed in **Table 6.2**. The raw data of the FAME analysis are shown in supplementary material (Table S2). All 17 FAs showed significant differences ($p < 0.05$) between the six oil categories, as well as for the sum of (un)saturated FAs (SFA, MUFA and PUFA). The three most abundant FAs in the six oil categories are C16:0, C18:1n9 and C18:2n6, which account for more than 85% of total FAs.

**Table 6.2.** Averages and standard deviations of relative fatty acid composition (FA), iodine values (IV) and saponification numbers (SN) of six types of edible oils.

| Chemical properties (g/100g) | EVOO (n = 30) | ROO (n = 15) | POO (n = 15) | RSO (n = 10) | SFO (n = 5) | PNO (n = 5) | *p* value |
|---|---|---|---|---|---|---|---|
| C14:0 | 0.05 ± 0.00 $^{e}$ | 0.02 ± 0.00 $^{e}$ | 0.02 ± 0.00 $^{d}$ | 0.05 ± 0.00 $^{b}$ | 0.08 ± 0.05 $^{a}$ | 0.03 ± 0.00 $^{c}$ | <0.05 |
| C15:0 | 0.05 ± 0.00 $^{d}$ | 0.05 ± 0.00 $^{d}$ | 0.00 ± 0.00 $^{e}$ | 0.02 ± 0.00 $^{a}$ | 0.02 ± 0.00 $^{b}$ | 0.05 ± 0.00 $^{c}$ | <0.05 |
| C16:0 | 12.64 ± 1.58 $^{a}$ | 11.97 ± 1.02 $^{ab}$ | 11.60 ± 0.46 $^{b}$ | 4.51 ± 0.36 $^{d}$ | 6.96 ± 0.71 $^{c}$ | 8.48 ± 1.29 $^{c}$ | <0.05 |
| C17:0 | 0.08 ± 0.04 $^{a}$ | 0.08 ± 0.02 $^{a}$ | 0.08 ± 0.05 $^{a}$ | 0.07 ± 0.05 $^{a}$ | 0.04 ± 0.05 $^{b}$ | 0.08 ± 0.05 $^{a}$ | <0.05 |
| C18:0 | 2.63 ± 0.43 $^{b}$ | 3.20 ± 0.56 $^{a}$ | 2.86 ± 0.21 $^{b}$ | 1.56 ± 0.06 $^{c}$ | 3.26 ± 0.05 $^{a}$ | 2.61 ± 0.55 $^{b}$ | <0.05 |
| C20:0 | 0.50 ± 0.04 $^{b}$ | 0.43 ± 0.04 $^{d}$ | 0.47 ± 0.02 $^{c}$ | 0.18 ± 0.04 $^{f}$ | 0.27 ± 0.06 $^{e}$ | 1.56 ± 0.20 $^{a}$ | <0.05 |
| C22:0 | 0.15 ± 0.02 $^{e}$ | 0.14 ± 0.03 $^{e}$ | 0.20 ± 0.05 $^{d}$ | 0.36 ± 0.05 $^{c}$ | 0.76 ± 0.06 $^{b}$ | 3.12 ± 0.33 $^{a}$ | <0.05 |
| C24:0 | 0.05 ± 0.03 $^{e}$ | 0.04 ± 0.03 $^{e}$ | 0.09 ± 0.00 $^{d}$ | 0.12 ± 0.05 $^{c}$ | 0.26 ± 0.045 $^{a}$ | 2.07 ± 0.17 $^{b}$ | <0.05 |
| SFA | 16.07 ± 1.49 $^{ab}$ | 15.88 ± 0.70 $^{ab}$ | 15.34 ± 0.40 $^{b}$ | 6.94 ± 0.39 $^{d}$ | 11.64 ± 0.60 $^{c}$ | 17.96 ± 1.94 $^{a}$ | <0.05 |
| C16:1n9 | 0.13 ± 0.09 $^{a}$ | 0.12 ± 0.05 $^{a}$ | 0.12 ± 0.05 $^{a}$ | 0.04 ± 0.00 $^{c}$ | 0.03 ± 0.05 $^{d}$ | 0.05 ± 0.00 $^{b}$ | <0.05 |
| C16:1n7 | 0.88 ± 0.32 $^{a}$ | 0.99 ± 0.26 $^{a}$ | 0.82 ± 0.06 $^{a}$ | 0.20 ± 0.05 $^{b}$ | 0.16 ± 0.08 $^{bc}$ | 0.09 ± 0.02 $^{c}$ | <0.05 |
| C17:1n7 | 0.14 ± 0.07 $^{a}$ | 0.12 ± 0.02 $^{a}$ | 0.13 ± 0.02 $^{a}$ | 0.06 ± 0.05 $^{b}$ | 0.04 ± 0.05 $^{c}$ | 0.06 ± 0.05 $^{b}$ | <0.05 |
| C18:1n9 | 71.68 ± 3.47 $^{a}$ | 71.44 ± 2.57 $^{a}$ | 69.45 ± 1.06 $^{b}$ | 59.92 ± 4.72 $^{c}$ | 27.95 ± 2.69 $^{d}$ | 62.61 ± 4.35 $^{c}$ | <0.05 |
| C18:1n7 | 2.01 ± 0.57 $^{c}$ | 2.11 ± 0.39 $^{c}$ | 2.42 ± 0.17 $^{b}$ | 2.68 ± 0.20 $^{a}$ | 0.80 ± 0.13 $^{d}$ | 0.72 ± 0.11 $^{d}$ | <0.05 |
| C20:1n9 | 0.36 ± 0.06 $^{b}$ | 0.30 ± 0.04 $^{c}$ | 0.35 ± 0.02 $^{b}$ | 1.36 ± 0.04 $^{a}$ | 0.23 ± 0.02 $^{d}$ | 1.84 ± 0.42 $^{a}$ | <0.05 |
| C22:1n9 | 0.00 ± 0.00 $^{c}$ | 0.00 ± 0.00 $^{c}$ | 0.00 ± 0.00 $^{b}$ | 0.20 ± 0.14 $^{a}$ | 0.00 ± 0.00 $^{c}$ | 0.12 ± 0.13 $^{ab}$ | <0.05 |
| MUFA | 75.20 ± 2.80 $^{a}$ | 75.07 ± 2.15 $^{a}$ | 73.29 ± 1.02 $^{b}$ | 64.45 ± 4.67 $^{c}$ | 29.20 ± 2.88 $^{d}$ | 65.50 ± 4.58 $^{c}$ | <0.05 |
| C18:2n6 | 7.71 ± 1.72 $^{d}$ | 8.26 ± 1.65 $^{d}$ | 10.09 ± 0.98 $^{c}$ | 18.67 ± 2.34 $^{b}$ | 58.56 ± 3.29 $^{a}$ | 16.09 ± 3.22 $^{b}$ | <0.05 |
| C18:3n3 | 0.66 ± 0.05 $^{b}$ | 0.58 ± 0.05 $^{c}$ | 0.64 ± 0.04 $^{b}$ | 9.05 ± 1.20 $^{a}$ | 0.18 ± 0.04 $^{d}$ | 0.00 ± 0.00 $^{e}$ | <0.05 |
| PUFA | 8.38 ± 1.73 $^{e}$ | 8.84 ± 1.65 $^{e}$ | 10.73 ± 1.01 $^{d}$ | 27.72 ± 4.29 $^{b}$ | 58.73 ± 3.29 $^{a}$ | 16.09 ± 3.22 $^{c}$ | <0.05 |
| IV (g I/100g) | 83.5 ± 1.5 $^{d}$ | 84.1 ± 1.2 $^{d}$ | 86.0 ± 1.1 $^{c}$ | 116.4 ± 5.5 $^{b}$ | 132.8 ± 3.4 $^{a}$ | 87.9 ± 2.5 $^{c}$ | <0.05 |
| SN (mg KOH/g) | 200.2 ± 0.5 $^{a}$ | 200.4 ± 0.4 $^{a}$ | 199.4 ± 0.3 $^{b}$ | 197.3 ± 0.9 $^{c}$ | 199.2 ± 0.3 $^{b}$ | 196.7 ± 0.3 $^{d}$ | <0.05 |

The significant differences between oil groups are indicated by superscript letters (a, b, c, d, e, f) in a row (Kruskal-Wallis and Mann-Whitney U tests, $p < 0.05$). EVOO, extra virgin olive oil; ROO, refined olive oil; POO, pomace olive oil; RSO, rapeseed oil; SFO, sunflower oil; PNO, peanut oil; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids.

In terms of a comparison of EVOO with all other oils, EVOO differed significantly from the other three vegetable oils (RSO, SFO and PNO) for 14 out of 17 FAs. Since lower grade

olive oils are also extracted from olive fruit, they present smaller differences compared to EVOO regarding the FAs composition than compared to other vegetable oils. Nevertheless, EVOO differed significantly from the two lower grade olive oils (ROO and POO) for 4 out of 17 and 10 out of 17 FAs, respectively. Moreover, the three olive oils present significantly higher concentrations of MUFA and lower concentrations of PUFA than the other botanical origin oils.

Since FAs vary in terms of chain length as well as number of double bonds, the values of IV and SN were calculated based on FAs data and are presented in **Table 6.2**. The IV expresses the degree of unsaturation (Stavarache et al., 2007). The IVs of EVOO and ROO do not differ significantly, but they do differ significantly with those of all other oils. The IVs of RSO (116.4 ± 5.5 g I/100g) and SFO (132.8 ± 3.4 g I/100g) are significantly higher than those of the other oils (in the range of 81-92 g I/100g), which indicates a higher unsaturation degree of those two oils. In contrast, the IV of PNO (87.9 ± 2.5 g I/100g) does not differ significantly from the IV of POO (86.0 ± 1.1 g I/100g).

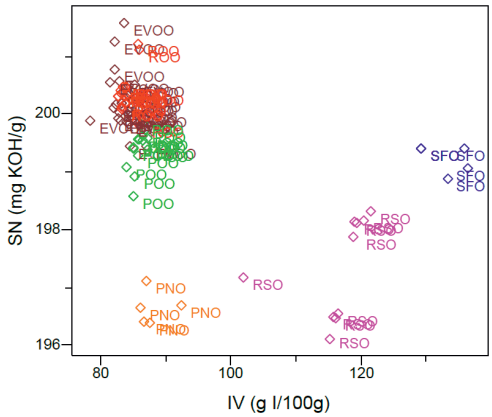


**Figure 6.5.** Scatter plot of IV (iodine values) versus SN (saponification numbers) data of 30 EVOO (extra virgin olive oils), 30 other olive oil grade samples (15 ROO (refined olive oils) and 15 POO (pomace olive oils)) and 20 oils of other botanical origins (10 RSO (rapeseed oils), 5 SFO (sunflower oils) and 5 PNO (peanut oils)).

The value of SN is related to the chain length of FAs, a higher SN implies a short carbon chain length (Stavarache et al., 2007). No significant differences were determined between the SN values of EVOO (200.2 ± 0.5 mg KOH/g) and ROO (200.4 ± 0.4 mg KOH/g), but the SN value of POO (199.4 ± 0.3 mg KOH/g) is significantly lower than that of the other two olive oils. This result is generally consistent with a previous study (Kreps et al., 2017), which reported that high temperature treatment of oils may contribute to polymerisation and formation of long-chain FAs, and this results in decreased SN values. Another possible reason is that POO contains more waxes (long chain FA ester with long chain alcohol, C40-C46) than EVOO as mentioned in the IOC standard (IOC, 2016). The SN values of the three olive oils are significantly higher (in the range of 198.6-201.3 mg KOH/g) than those of RSO (197.3 ± 0.9 mg KOH/g) and PNO (196.7 ± 0.3 mg KOH/g), which implies that olive oils contain shorter

chain length FAs than RSO and PNO. EVOO can be separated from nearly all other types of oil studied here, except ROO, whereas an overlap can be noticed between EVOO and POO at a lower extent, based on IV and SN values in **Figure 6.5**.

Taken together, these results provide important insights into the FAs characteristics of six oil categories.

### *6.3.6. Relationships between ultrasonic velocity, viscosity, density and fatty acid composition*

**Figure 6.6** presents the correlation between velocity, viscosity and density on the one hand and FAs, IV and SN on the other hand. It can be seen that viscosity correlated significantly ($p < 0.05$) with the accumulated saturated and unsaturated FA contents (SFA, MUFA and PUFA). The r values of viscosity and PUFA, viscosity and MUFA are -0.87 and 0.78, respectively. This is in agreement with studies of Santos et al. (2005), which indicated that the viscosity of vegetable oils is more related to the presence of polyunsaturated chains than to monounsaturated chains in an oil/fat mixture. Moreover, viscosity readings correlate more strongly with C18:1n9 and C18:2n6 (r = 0.79 and -0.78, respectively) than with C16:0 (r = 0.69). Results also indicate that the long-chain compounds correlate more strongly with the viscosity. In addition, the position of FAs in triacylglycerol may also influence oil viscosity according to the previous study (Snouber et al., 2019). It is reported that thermal triggered oxidation induces the formation of polar triglyceride oligopolymers, which results in an increase in viscosity.

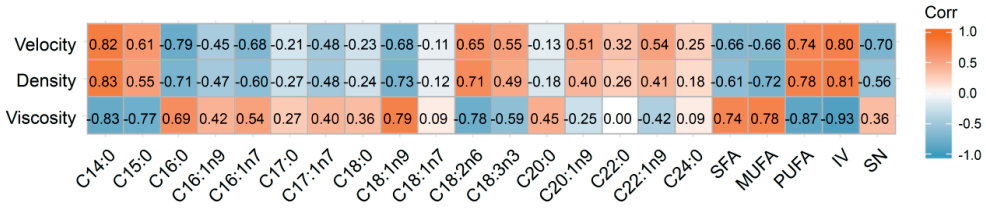


**Figure 6.6.** Pearson correlation coefficients for the correlation of the compositions of the oils and their physical properties. Orange refers to positive correlation; blue refers to negative correlation. The correlation coefficients in each spot, described the strength of the correlation between two variables. The larger the absolute value of correlation coefficient, the darker the colour. Correlation coefficients < -0.22 and > +0.22 are significant (Paired samples t-test, $p < 0.05$).

According to a previous study (Rodenbush et al., 1999), the relationship between viscosity (η) and the ratio of IV over SN can be expressed by equation (4):

$$\log\eta = (-1.4 + 1.25(\mathrm{IV/SN})) + (500 - 375(\mathrm{IV/SN}))/((\mathrm{T} + 140) - 85(\mathrm{IV/SN})) \quad , \quad (4)$$

Where T is the temperature.

A significant negative correlation (r = -0.93, $p < 0.05$) between viscosity and IV is presented in **Figure 6.6**, which confirms that a higher unsaturation degree results in a lower viscosity (Rodenbush et al., 1999; Santos et al., 2005; Schaschke et al., 2006). The presence of

double bonds prevents the same level of intermolecular contact, resulting in an increased capability of the fluid to flow (Abramovic & Klofutar, 1998; Schaschke et al., 2006). Furthermore, viscosity and SN show a weak but significant positive correlation ($r = 0.36$, $p < 0.05$). This result indicates that the long-chain FAs result in a lower viscosity. However, the result is in contradiction with previous studies (Geller & Goodrum, 2000; Rodenbush et al., 1999). In some studies, scientists worked with pure triglycerides (Eiteman & Goodrum, 1993; Geller & Goodrum, 2000). However, natural vegetable oils are complex mixtures of many triglycerides with different chain lengths.

Density correlates significantly ($p < 0.05$) with three abundant FAs (**Figure 6.6**). It correlates also significantly with the sum of (un)saturated FAs: density/SFA ($r = -0.61$), density/MUFA ($r = -0.72$) and density/ PUFA ($r = 0.78$). Generally, the density is expected to correlate with both IV and SN (Rodenbush et al., 1999), according to the Lund equation (5):

$$\mathrm{sg}\,(15/15°\mathrm{C}) = 0.8475 + 0.00030\,\mathrm{SN} + 0.00014\,\mathrm{IV} \quad , \quad (5)$$

where sg is the specific gravity of the vegetable oil at 15°C.

The significant strong positive correlation between density and IV ($r = 0.81$, $p < 0.05$; **Figure 6.6**) confirms that density increases with increasing degree of unsaturation. A significant negative correlation ($r = -0.56$, $p < 0.05$) between density and SN was determined, which means that density increases with the decreasing saponification number, as well as with increasing chain length of FAs.

Those results demonstrate that the FA composition plays an important role when it comes to the viscosity and density of oils. Moreover, the differences in viscosity and density are more strongly related to the unsaturation degree than to the chain length of FAs. According to the correlation results in section 3.6, a higher unsaturation degree of oils results in a lower viscosity and higher density. Moreover, a higher ultrasonic velocity is related to a lower viscosity and a higher density of oils (section 3.4). Consequently, a higher unsaturation degree of oils indirectly results in a higher ultrasonic velocity. This is also supported by the significant positive correlation between velocity and IV ($r = 0.80$, $p < 0.05$; **Figure 6.6**).

### *6.3.7. Prediction of the velocity from the oils' compositions*

PLS regression was applied to determine the capability of FAs for prediction of the ultrasonic velocities. The data were pre-processed involving auto-scaling, mean-centring and range-scaling, and models with the three types of pre-processing were compared. The prediction capabilities of the three corresponding regression models are presented in **Table 6.3**.

In general, a low SEV and high $r^2$ present better prediction ability of a model (Fernandez-Cabanas et al., 2011). For these three variants of PLS regression, best prediction ability is observed with mean-centring pre-processing, although the prediction ability of the

three models varies only to a limited extent. The best model presents an $r^2$ of 0.93 and SEV of 1.01. This regression model is shown in **Figure 6.7**. Therefore, this model allows a reasonable estimation and an indication of the expected velocity for various vegetable oils from their FA compositions.

**Table 6.3.** Performance characteristics of PLS regression models predicting the ultrasonic velocity by fatty acid compositions of 80 vegetable oil samples with leave-one-out cross validation and different pre-processing methods.

| Pre-processing | Factor | SEC | $R^2$ | SEV | $r^2$ |
|---|---|---|---|---|---|
| Mean-centring | 1 | 1.00 | 0.93 | 1.01 | 0.93 |
| Auto-scaling | 3 | 1.01 | 0.93 | 1.07 | 0.92 |
| Range-scaling | 4 | 1.10 | 0.92 | 1.20 | 0.90 |

Factor, optimal factor of each model; SEC, standard error of calibration; $R^2$, correlation coefficient of calibration; SEV, standard error of cross-validation; $r^2$, correlation coefficient of cross-validation.

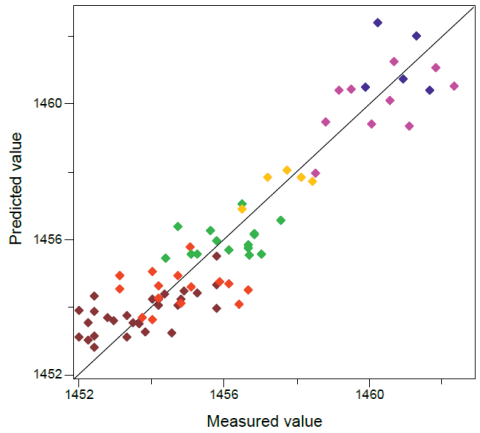


**Figure 6.7.** Regression plot of viscosity with modelling measured value versus predicted value. Grey, extra virgin olive oil; red, refined olive oil; green, pomace olive oil; purple, rapeseed oil; blue, sunflower oil; yellow, peanut oil.



## 6.4. Conclusions and outlook

This study reveals that the developed ultrasonic measurement system is a rapid and non-destructive technique which can be applied for the characterisation of vegetable oils. The ultrasonic measurements are temperature dependent, and the temperature coefficient of the velocity in EVOO is -2.92 $m \cdot s^{-1} \cdot °C^{-1}$. The ultrasonic velocity of EVOO (1453 ± 2 m/s, at 23.5 °C) differed significantly from POO and other vegetable oils, except for ROO. The differences in ultrasonic velocity are primarily due to the level of unsaturation. Prediction of the ultrasonic velocity from the FA composition is promising.

Since the ultrasonic technique is rapid and non-destructive, it is an interesting approach to be included in the EVOO characterisation toolbox. In addition, simultaneous measurement

of the ultrasonic attenuation and temperature coefficients of vegetable oils can offer further valuable information of the ultrasonic properties, which could be another method for the characterisation of vegetable oils.

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# Chapter 7

General discussion

## 7.1. Introduction

With the globalisation of the food supply system, food fraud can have international impacts, sometimes with far-reaching and lethal consequences (Ellis et al., 2012). The extra virgin olive oil (EVOO) trade is a global business, while EVOO fraud scandals happened frequently all around the world for financial gain. It is reported that half of the bottles that are sold as EVOO in Italian supermarkets do not meet the legal requirements for EVOO, while up to 80% of EVOO in the US is fake (Whitaker, 2016). To provide insights for prevention and mitigation of EVOO fraud, there is a need to understand where the vulnerable points in the EVOO supply chain exist and to explore novel, rapid techniques for the authentication of EVOO. Therefore, in **Chapter 2** we evaluated the fraud vulnerability of actors in the EVOO supply chain and their characteristics and identified risk factors contributing to the vulnerability. In **Chapters 3-6** robust confirmatory and screening analysis methods were developed for the assessment of the authenticity of EVOO.

## 7.2. Vulnerable points in the EVOO supply chain

In order to investigate the vulnerable points, the fraud vulnerability of the EVOO supply chain was assessed in **Chapter 2**. Moreover, the similarities and differences in fraud vulnerability according to group characteristics were evaluated. The overall perception of the companies is that the EVOO supply chain is highly vulnerable to fraud. Eleven risk factors contributed most to this vulnerability (orange + red > 80% in **Figure 7.1**). These high-risk factors included five factors related to technical opportunities, three factors related to economic drivers, one factor related to technical control measures and two factors related to managerial control measures.

It is striking that all technical opportunities were rated as high risk. For other products, lower risk scores were determined for these kinds of opportunities previously, e.g. for spices (Silvis et al., 2017) and milk (Yang et al., 2019) supply chains. Moreover, 3 out of 11 factors associated with economic drivers were rated as high vulnerability. A similar level of vulnerability was found for spices previously (Silvis et al., 2017). The prices of EVOO vary considerably, which may be due to the fact that EVOO is a seasonal product and the quality is affected by multiple factors, such as climate and harvesting (Food Standards Agency, 2016; IOC, 2018; Santini et al., 2018). In the past decade, the prices of EVOO surged frequently due to disease, drought and frost effecting olive trees in Italy and Spain (Butler, 2015; Macdonald, 2017; Terazono, 2019). EVOO also has special properties, because the value of EVOO is largely determined by its processing method, country of origin, concentration of antioxidants and special organoleptic properties (Kalogeras et al., 2009). Furthermore, the level of competition across the EVOO supply chain is high, which in turn could trigger food fraud as mentioned by Manning (2016). This finding is also in agreement with the study of Santini et al.

(2018), who reported that growing competition has appeared in the EVOO sector. In this group of prime drivers and enablers, both opportunity and motivation related fraud factors are included, which is a toxic combination because it implies that there is a suitable target as well as a likely offender at hand. The combination in the absence of a capable guardian is the perfect recipe for fraud according to the conceptual framework of van Ruth et al. (2017), which is based on the criminological Routine Activities Theory (Cohen & Felson, 1979). Therefore, the availability of accurate control measures will be key to mitigate fraud in this supply chain.

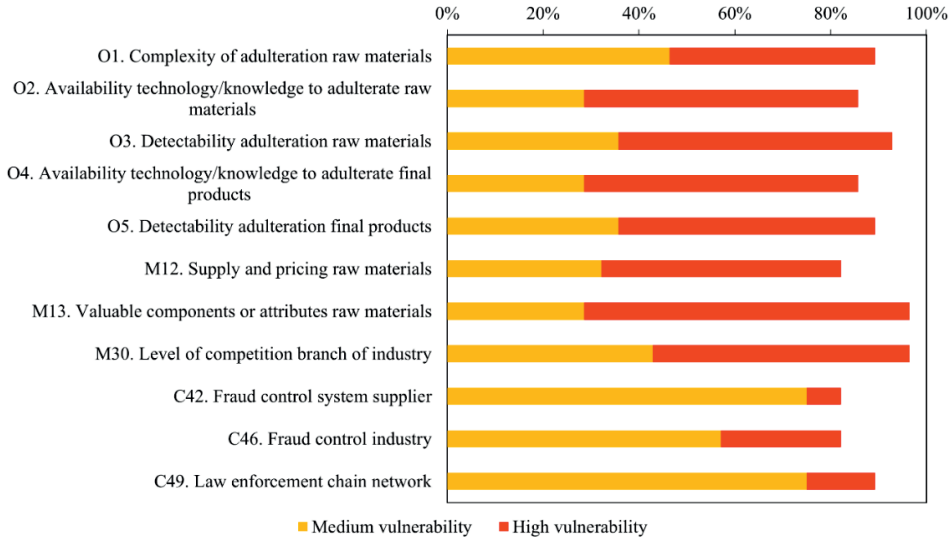


**Figure 7.1.** Relative medium-high vulnerability frequencies of the answers of 28 interviewees for 11 high vulnerability (orange + red > 80%) factors in the extra virgin olive oil supply chain (SSAFE Food fraud vulnerability assessment tool). O refers to opportunities; M refers to motivations; C refers to control measures; numbers next to capital letters refer to the question number in the questionnaire.



Regarding control measures, three factors are rated high vulnerability. This concerned the lack of a well-established fraud control system by suppliers, which is related to the technical control measures and two managerial control measures. With regard to the managerial measures, most of the respondents mentioned that the guidance for fraud prevention is lacking across the supply chain and fraud related enforcement practices are lacking at most stages across the international supply chain. This is a similar result as previously found for spices (Silvis et al., 2017). On the contrary, these two factors appear to contribute less to fraud vulnerability in the Dutch milk supply chain (Yang et al., 2019). Considering the vulnerabilities and limited control measures in place, mitigation should receive more attention.

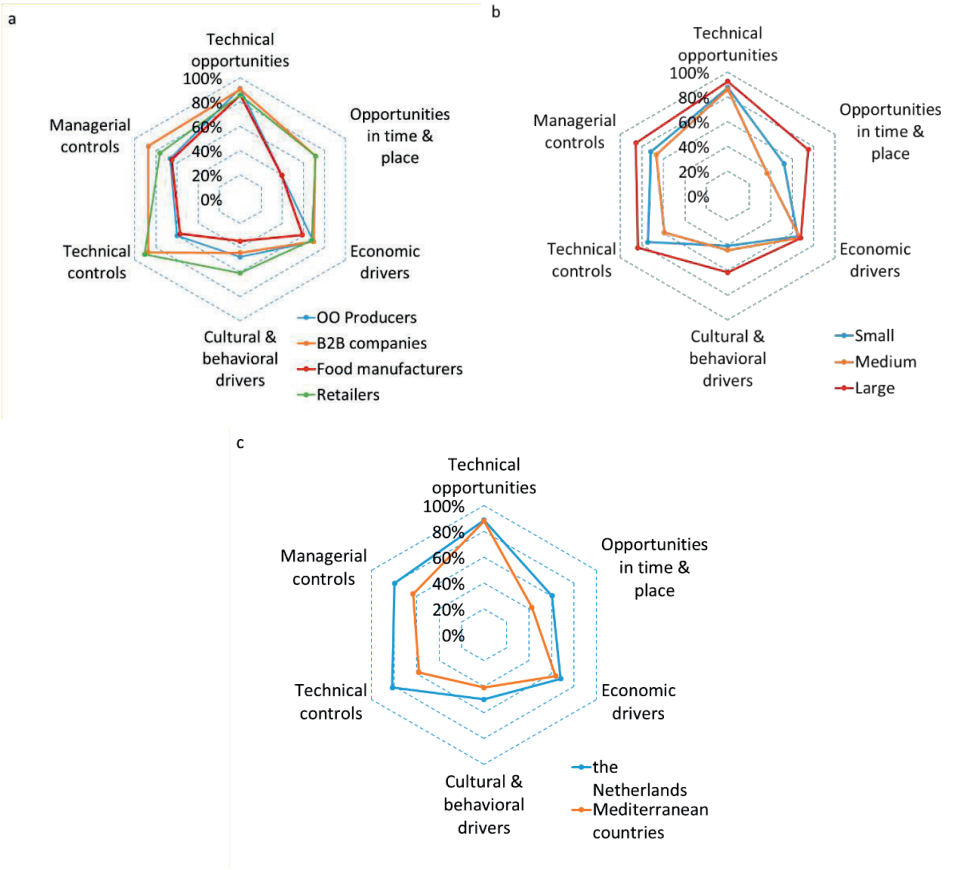


**Figure 7.2.** Radar plot of the medium & high vulnerability frequencies of answers for the six categories of the fraud vulnerability assessments for a) four tier groups (OO producers, B2B companies, food manufacturers and retailers); b) three groups presenting businesses of different scales (small, medium and large); c) two location groups (the Netherlands and Mediterranean countries). B2B refers to business-to-business; OO refers to olive oil.

Company characteristics appear to affect fraud vulnerability. The effects of the position/role in the chain, the scale of the company and its location are presented in **Figure 7.2.** The latter shows the medium+high vulnerability frequencies of answers to the six fraud factor categories for the various groups. In **Figure 7.2a**, retailers and business-to-business (B2B) companies show similar fraud vulnerability patterns, and they appear more vulnerable to fraud than the other two tier groups. Specifically, the significant differences between these tier groups are found in the opportunities in time and place, technical controls and managerial controls. This is in line with results in five supply chains, in which the wholesaler/trader group was also the most vulnerable one (van Ruth et al., 2018). Large companies appear more vulnerable to fraud than small and medium scale companies due to the more extensive opportunities in time and place, cultural and behavioural drivers, as well as greatest lack of control measures (**Figure 7.2b)**. This is somewhat surprising, since large companies may have more resources to

implement a fraud mitigation plan than small and medium companies (Silvis et al., 2017). A possible reason is that small and medium companies are primarily producers and food manufacturers, and interviewees from these companies are overconfident about themselves. The larger companies appear later in the chain. While this interaction might lead to bias. Furthermore, Dutch companies seem more vulnerable than their Mediterranean counterparts, which is primarily due to the increased opportunities in time and place, as well as lack of control measures (**Figure 7.2c**). These aspects may be due again to intertwinement of location and position/role in the chain causing bias, since the Dutch companies are primarily retailers and B2B companies. They are obviously not olive growers. Taken together, it is evident that both the scale and location of the companies in the supply chain affected the vulnerability of the tier groups. Moreover, it appears that the companies that are the intermediaries (retailers and B2B companies) between producers and customers are more vulnerable to fraud.

## 7.3. Evaluation of the new fraud detection methods

Although fraud detection will not prevent fraud, fraud detection is an inseparable part of fraud mitigation (van Ruth & Granato, 2017), which means that advanced detection methods will limit the impact of fraud. In this thesis, four novel fraud detection methods were developed for the authentication of EVOO (**Chapters 3-6**). In order to assess these four techniques, twelve indicators were selected as evaluation criteria. These indicators can be divided into three main groups, including technical evaluation (analytical parameters, screening or confirmation test, targeted or non-targeted method, sample preparation, sample analysis time, destructive or non-destructive nature of the technique and maturation stage) (McGrath et al., 2018), performance evaluation (discriminatory power) (Malegori et al., 2017) and application evaluation (lab or field test, price of the instrument, ease of validation and ease to be circumvented by fraudsters) (Rambla-Alegre et al., 2012). **Table 7.1** shows the comparison of the four novel fraud detection techniques for the discrimination of EVOO from lower grade olive oils according to the evaluation of these twelve indicators. The differences are discussed for each indicator below.



### *7.3.1. Technical evaluation*

**Analytical parameters.** The four techniques focus on different features, which include chemical properties, physical properties and other data derived from a combination of both.

The first set of parameters concern the group of 2-/3-monochloropropanediol (2-/3-MCPD) esters and glycidyl esters (GEs) which are food processing contaminants most commonly occurring in preliminary heat treatment and the refining process of foods (Baer et al., 2010). Since these compounds are formed during the deodorisation step of oils, it seemed an interesting group of markers to detect the presence of refined oil (**Chapter 3**). AOCS released an official method to detect those processing derived contaminants using gas chromatography-mass spectrometry (GC-MS) (AOCS, 2013). GC coupled to tandem mass

spectrometry (MS/MS) was used to conduct the measurement in the present study instead of GC-MS, since it can improve the sensitivity of the method.

The second parameter chosen was the group of volatile organic compounds (VOCs). EVOO retains its characteristic aroma (Vichi, 2010) due to the mechanical processing. Non-EVOO grade oils have less and different aromas than EVOO (Fullana et al., 2004). Therefore, VOCs seemed an interesting marker group. A GC-MS set-up is commonly used to separate, qualify, and/or quantify relatively volatile compounds of olive oils (Perestrelo et al., 2017; Romero et al., 2015). Proton transfer reaction-quadrupole ion guide time of flight-mass spectrometer (PTR-QiToF-MS) is a technique that measures volatiles by their masses very rapidly in the headspace of food products, and it was selected as a suitable technique for the quantitative characterisation of the olive oil VOCs (**Chapter 4)**.

Many developments in the area of rapid, non-destructive and on-site measurement technology have been seen in the infrared spectroscopy area. Near infrared spectroscopy (NIR) is based on the absorption of electromagnetic radiation and has shown its value in the discrimination of vegetable oils (Casale & Simonetti, 2014). Handheld NIR has been widely adopted due to its small size, cost-effectiveness, exceptional performance and ease of use (Viavi, 2015). Considering its rapid nature and the potential laymen use of the portable variants of the technique, handheld NIR was examined for its potential to discriminate EVOOs from other grade olive oils (**Chapter 5**).

In this study, a very new approach based on ultrasonic measurements was developed too. Measurements of ultrasonic velocity have been employed for quality assessments of oils, often as a measure calibrated against reference methods (Alouache et al., 2015; Alouache et al., 2018). However, it had not been used for authentication purposes. Therefore, an ultrasonic pulse-echo system was developed and examined for its capabilities to distinguish EVOO from other oils (**Chapter 6**).

**Screening or confirmatory test.** The MCPD esters analysis is a confirmatory test and is based on the measurement of the concentrations of processing contaminants, which should be (nearly) absent in EVOO. GC-MS/MS was utilised to conduct quantitative analysis of these processing contaminants (**Chapter 3**). On the other hand, the other three methods (VOCs analysis by PTR-QiToF-MS, spectral analysis by handheld NIR and ultrasonic velocity analysis using a pulse-echo system analysis) are screening tests, which generates a full mass spectrum (**Chapter 4**), a full spectrum (**Chapter 5**) and a monopolar electrical pulse signal (**Chapter 6**), respectively.

**Table 7.1.** Comparison of the four fraud detection methods developed in this thesis for the discrimination of EVOO from lower grade olive oils.

| Techniques | MCPD esters analysis by GC-MS/MS | VOCs analysis by PTR-QiToF-MS | Spectral analysis by handheld NIR | Ultrasonic velocity analysis by ultrasonic pulse-echo system |
|---|---|---|---|---|
| ***Technical evaluation*** | | | | |
| Screening or confirmation test | Confirmation | Screening | Screening | Screening |
| Targeted or non-targeted | Targeted single markers | Non-targeted fingerprinting | Non-targeted fingerprinting | Targeted single markers |
| Sample preparation | Yes, chemical treatment and 16 hours incubation | Yes, equilibration for 30 mins in water bath | No | No |
| Sample analysis time | > 30 min | ~1 min | 0.25-0.5 s | < 30 s |
| Destructive or non-destructive | Destructive (Chemical extraction) | Non-destructive | Non-destructive | Non-destructive |
| Maturation stage | Mature | Developing | Developing | Infancy |
| ***Performance evaluation*** | | | | |
| Discriminatory power | Good<br>ROO and POO admixtures to EVOO are detectable at 2% w/w levels based on 3-MCPD esters, at 5% based on 2-MCPD esters and at 13% (ROO) and 14% (POO) based on GEs. | Good<br>EVOO were 100% correctly identified. POO were efficiently discriminated from EVOO, but 9% ROO were predicted incorrectly. | Good<br>EVOO were 100% correctly identified. POO were efficiently discriminated from EVOO, but 5 out of 40 ROO were predicted incorrectly. | Poor<br>The ultrasonic velocity of EVOO significantly differed from other vegetable oils, except from ROO. |
| ***Application evaluation*** | | | | |
| Lab or field test | Lab | Lab | Field | Field |
| Price of the instrument | > € 50,000 | > € 100,000 | < € 5,000 | < € 2,000 |
| Ease of validation | Easy<br>Routine strategies [a]:<br>1) Evaluation of bias and precision;<br>2) Systematic assessment;<br>3) Testing method robustness;<br>4) Interlaboratory comparisons;<br>5) Comparison of results achieved with other validated methods;<br>6) Evaluation of measurement uncertainty of the results. | Difficult<br>Proposed scheme [b]:<br>1) Data preparation, including pre-processing and pre-treatment;<br>2) Model optimisation by internal validation;<br>3) Model testing by external validation;<br>4) Stability testing of the model by system challenges. | Difficult<br>Proposed scheme:<br>1) Data preparation, including pre-processing and pre-treatment;<br>2) Model optimisation by internal validation;<br>3) Model testing by external validation;<br>4) Stability testing of the model by system challenges. | Easy<br>Routine strategies:<br>1) Evaluation of bias and precision;<br>2) Systematic assessment;<br>3) Testing method robustness;<br>4) Interlaboratory comparisons;<br>5) Comparison of results achieved with other validated methods;<br>6) Evaluation of measurement uncertainty of the results. |

| Techniques | MCPD esters analysis by GC-MS/MS | VOCs analysis by PTR-QiToF-MS | Spectral analysis by handheld NIR | Ultrasonic velocity analysis by ultrasonic pulse-echo system |
|---|---|---|---|---|
| Ease to be circumvented by fraudsters | Difficult<br>1) Difficult to completely eliminate these contaminants;<br>2) Most of the mitigation strategies are not implemented by industries yet;<br>3) Financial investment for the mitigation strategies is big. | Difficult<br>1) No single marker could be traced by fraudsters;<br>2) The combination of the complex data and advanced statistical analysis makes it difficult for fraudster to catch up the method. | Difficult<br>1) No single marker could be traced by fraudsters;<br>2) The combination of the complex data and advanced statistical analysis makes it difficult for fraudster to catch up the method. | Difficult<br>1) Newly developed method for vegetable oil characterisation;<br>2) Complex mechanism makes it difficult to catch up by fraudsters. |

[a] ISO (2017); [b] Riedl et al. (2015); (Alewijn et al., 2016). EVOO, extra virgin olive oil; GC-MS/MS, gas chromatography-tandem mass spectrometry; GEs, glycidyl esters; MCPD esters, monochloropropanediol esters; NIR, near-infrared spectroscopy; POO, pomace olive oil; PTR-QiToF-MS, proton transfer reaction-quadrupole ion guide time of flight-mass spectrometry; ROO, refined olive oil; VOCs, volatile organic compounds.

**Targeted (single markers) or non-targeted (fingerprinting) method.** Traditional strategies for food fraud identification mainly focus on the determination of the amount of marker(s) (Ahad & Nissar, 2017). It can be noticed that the first and the fourth methods (**Chapter 3** and **6**) focused on the detection of single features, i.e. 2-MCPD esters, 3-MCPD esters, GEs (**Chapter 3**) and ultrasonic velocity (**Chapter 6**). In addition, the non-targeted fingerprinting approach has been developed and evaluated for food analysis based on the measurement and the evaluation of a large number of compounds at once. Since these 'composite' fingerprints contain relevant information on features of the authentic products and sometimes also the adulterants in the foodstuff. Due to its complexity, this information is extracted with chemometric methods (Ellis et al., 2012). In this thesis, the non-targeted fingerprinting approach was applied for the discrimination of EVOO from its lower grade counterparts with the second and third methods (**Chapter 4** and **5**). Furthermore, non-targeted fingerprinting approaches are more likely to provide rapid and high-throughput analysis, since they are able to reliably identify those that are potentially non-compliant in large numbers of samples before confirmatory methods are performed (Ahad & Nissar, 2017).

**Sample preparation.** In terms of the sample pre-treatment prior to analysis, the four methods differ considerably. Working according to the official AOCS method, laborious sample pre-treatment using chemical reagents is needed. In particular, samples need to be incubated at 40°C for 16 h prior to analysis (AOCS, 2013). Generally, this procedure is complex, time consuming and less environmentally friendly (**Chapter 3**). There is no considerable sample preparation for the other three methods. The headspace air of the sample is equilibrated for 30 min at 30 °C in a water bath prior to the VOCs analysis by PTR-QiToF-MS (**Chapter 4**), but this can be carried out while other samples are running. In relation to the spectral analysis by handheld NIR (**Chapter 5**) and ultrasonic velocity analysis with the pulse-echo system analysis (**Chapter 6**), samples can be analysed directly without any sample preparation.

**Sample analysis time.** For the first method (**Chapter 3**), it takes more than 30 min per sample for separating and analysing the particular contaminants by GC-MS/MS (Yan et al., 2018). The time required to characterise a single sample with PTR-QiToF-MS is only about 1 min per sample (**Chapter 4**). With regard to the spectral analysis and ultrasonic velocity analysis (**Chapter 5-6**), the typical measurement time for one sample is less than 30 s (Yan et al., 2019a; Yan et al., 2019b). In terms of the total analysis time (including sample preparation and sample analysis), the spectral analysis by handheld NIR is the fastest method (no sample preparation and less than 1 s analysis time per sample), followed by ultrasonic pulse-echo system (no sample preparation and ~30 s per sample), PTR-QiToF-MS (30 min sample preparation and ~30 s analysis time per sample) and GC-MS/MS (> 1 day sample preparation and > 30 min analysis time per sample). Therefore, handheld NIR and the ultrasonic pulse-echo system would be the best choice if rapid analysis without any sample preparation is needed and when considering sample preparation and analysis time only.

**Destructive or non-destructive nature of the method.** In the MCPD esters analysis (AOCS, 2013), samples are destroyed by the chemical solvents for the extraction of the processing contaminants of interest (**Chapter 3**). With regard to the other three techniques (**Chapter 4-6**), no chemicals are applied and samples are not destroyed, which means those samples can be recycled. Those results also indicate that the MCPD esters analysis is less environmentally friendly as it involves the usage of chemicals, such as n-heptane and methanol.

**Maturation stage.** For the first technique, the MCPD esters analysis by GC-MS/MS is considered a matured method (**Chapter 3**). MCPD esters have been well known as food processing contaminants since 1978 (Weißhaar, 2008). They are present in many foodstuffs like vegetable oils, acid-hydrolysed vegetable protein, soy sauces, crackers, bread, toast as well as other bakery products and soups (Hamlet et al., 2002). Due to its broad spread across foods and its health concerns, analysis of MCPD esters has been standardised. For instance, the AOCS has released an official method for the measurement of these processing contaminants in edible oils using GC-MS (AOCS, 2013).

PTR-MS technology was introduced for on-line measurements of trace components at ppb levels about 25 years ago (Hansel et al., 1995; Lagg et al., 1994). It has been proven to be a highly sensitive and efficient method to detect most of the VOCs (Lindinger et al., 1998). About ten years ago, the PTR-(Qi)ToF-MS variant was launched and applied for on-line breath analysis in 2009 (Herbig et al., 2009). After this application, this technique was applied to many food analyses (Fabris et al., 2010; Marone et al., 2017; Sabbatini et al., 2018; Schuhfried et al., 2016; Taiti et al., 2015; Yener et al., 2014). Although the PTR-MS technology has been widely applied by scholars and the food industries, no standardised methods exist. Therefore, we consider this technique to be at a developing stage (**Chapter 4**).

NIR has received particular attention due to the pioneering work of Norris (1965), who is the founder of modern NIR analysis. Moreover, the on/in-line NIR instrumentation has a proven record of 40 years for food analysis due to the robustness and simplicity of instrumentation, as well as its highly precise and efficient nature (Ellis et al., 2012; Osborne, 2006). With the increasing miniaturisation and affordability of NIR instruments, the MicroNIR (Viavi, 2013), SCIO (Consumer Physics, 2017) and Telspec (Tellspec Inc., 2015) surfaced on the market. These devices can be applied in the field, at the farm, or anywhere throughout the supply chain. They have been successfully applied in food analysis with high accuracy (Correia et al., 2018; Grassi et al., 2018; Liu et al., 2018; O'Brien et al., 2013; Ribeiro et al., 2016; Sun et al., 2016). Despite these scientific applications, there are very few fully, formally validated and no standardised procedures exist for these portables yet. Hardware is also still developing rapidly. Therefore, this technique is considered to be at a developing stage (**Chapter 5**).

The ultrasonic behaviour of edible oils was first reported in 1985. The correlation between ultrasonic properties and rheology was investigated (Gladwell et al., 1985). Some applications for fat quality assessments have been developed as well. However, the technology

had not been applied for authentication purposes. Currently, the technique is still in its infancy for these kind of application (**Chapter 6**). It is believed that further applications of this approach in food analysis will be developed in the future.

Thus, the MCPD esters analysis by GC-MS/MS is best established, followed by the VOCs analysis by PTR-QiToF-MS and spectral analysis by handheld NIR. The most novel technique and least established is the ultrasonic velocity analysis with the pulse-echo system.

### *7.3.2. Performance evaluation*

**Discriminatory power.** The MCPD esters analysis by GC-MS/MS showed good repeatability and linearity (**Chapter 3**). Moreover, the results predicted a good discrimination performance, which is that refined olive oil (ROO) and pomace olive oil (POO) admixtures to EVOO are detectable at 2% w/w levels based on 3-MCPD esters, at 5% based on 2-MCPD esters and at 13% (ROO) and 14% (POO) based on GEs.

With regard to the VOCs analysis, the fingerprinting approach combined with chemometrics showed good discrimination performance (**Chapter 4**). POO was efficiently discriminated from EVOO, however, the identity of 9% of the ROO samples was predicted incorrectly. The good discriminatory power of PTR-QiToF-MS observed in the current research is in keeping with the result of Taiti and Marone (2017). These scientists reported a study in which EVOO PTR-ToF-MS data were correlated with panel test results. The instrument seemed to show similar specificity as the sensory panel.

The spectral analysis by handheld NIR also presented high discrimination performance (**Chapter 5**). POO samples were fully discriminated from EVOO, but for 12% of the ROO samples, the identity was predicted incorrectly. This finding is consistent with that of Yang and Irudayaraj (2001) who found that POO could be 100% discriminated from EVOO by benchtop NIR. It also indicates that this miniature device may be an alternative to the conventional NIR for this application.

The performance of the fourth technique is still relatively weak (**Chapter 6**). The ultrasonic velocity of EVOO samples differed significantly from those of other botanical vegetable oils and POO samples, but not from ROO samples. However, the application of this technique for authentication is at an early stage. Additional work may improve its discriminatory power. For instance, the investigation of ultrasonic attenuation as an additional authentication parameter (Ju et al., 2010). Considering the discriminatory power of these four techniques, the MCPD esters analysis by GC-MS/MS presented the best discriminatory power, followed by the VOCs analysis by PTR-QiToF-MS, the spectral analysis by handheld NIR and the ultrasonic velocity analysis by pulse-echo system. For the latter three methods, it was consistently the ROO samples that caused difficulties.

### *7.3.3. Application evaluation*

**Lab or field test.** The GC-MS/MS and PTR-QiToF-MS can only be used in the lab due to their large size and the critical experimental conditions. In order to allow for industrial applications, large, stationary analytical instruments need to be transformed into lightweight tools (Crocombe, 2004). The handheld NIR device is already at that stage. In terms of the pulse-echo system, the size of the sample platform is 80 × 100 × 200 mm (**Chapter 6**). The oscilloscope (Tektronix TDS 210) is only 120 mm from front to back and weighs 1.5 kg, which allows for portability. Furthermore, the size of the pulse generator (Panametrics-NDT Model 5800) is 419 × 88.9 × 315 mm and the weight is 6 kg (Olympus NDT, 2008). However, much smaller ultrasonic systems are available. Therefore, the pulse-echo system could also be applied for field tests. Considering the scope of the application, the handheld NIR and ultrasonic pulse echo system could be more widely applied than GC-MS/MS and PTR-QiToF-MS, since the first two devices can be utilised anywhere along the supply chain.

**Price of the instrument.** The price is an important aspect for companies and authorities when considering the purchase of an analytical instrument. To meet all companies' requirement, an important development on the analytical equipment is the reduction in the manufacturing costs and subsequent market prices. The price of the two laboratory devices is more than € 50,000. While the price of the other two devices is significantly lower than the two laboratory equipment, i.e. less than € 5,000. Since the high price of GC-MS/MS and PTR-QiToF-MS is beyond the affordability of small food companies, these devices are more likely to be applied by large companies and large research centres. Handheld NIR and the ultrasonic pulse echo system are affordable for nearly all companies. In terms of the price, handheld NIR and ultrasonic pulse echo system could be more widely applied by any types of companies than the laboratory-based devices (GC-MS/MS and PTR-QiToF-MS). Obviously, when considering finances, not only the purchase of the equipment but also the running costs and labour must be considered.

**Ease of validation.** For routine analysis, analytical procedures must be validated to make it legally incontestable (Riedl et al., 2015). To conduct a validation, the analytical requirement will be described and the performance capability will be evaluated (Khodabocus & Balgobin, 2011). With regard to the classical targeted analysis, some official routine strategies for the validation of the method are available (Commission of the European communities, 2002; European Commission, 2015; ISO, 2017). Methods can be easily validated and accredited for official use due to the standardised procedures for validation of single feature methods. Therefore, the MCPD esters analysis by GC-MS/MS and the ultrasonic velocity analysis by pulse-echo system are easily validated.

On the other hand, there are no equal guidelines for methods of validation concerning non-targeted fingerprinting methods. Researchers (Alewijn et al., 2016; Riedl et al., 2015) proposed practical guidance documents for the validation of non-targeted fingerprinting

approaches. However, there is still a lot of debate over which performance characteristics would need to be considered. Therefore, it would not be easy to sufficiently validate these methods for official use. This holds for the VOCs analysis by PTR-QiToF-MS and the spectral analysis by handheld NIR.

**Ease to be circumvented by fraudsters.** The four analysis methods are not easy to be circumvented by fraudsters. Considering the 3-MCPD esters analysis, the refining process will trigger the formation of these processing derived contaminants (Hrncirik & van Duijn, 2011; Smidrkal et al., 2016; Weisshaar & Perz, 2010). Weißhaar (2008) reported that no or only a small amount of 3-MCPD esters can be detected in unrefined oils, but concentrations of 3-MCPD esters in nearly all refined oils are in the range of 0.2-20 mg/kg. Nowadays, many research papers reported that the improved refining technology is able to mitigate the formation of 3-MCPD and glycidyl esters, such as water degumming instead of dry degumming (Zulkurnain et al., 2013), alkali neutralisation by adding sodium carbonate or sodium bicarbonate (Freudenstein et al., 2013), wet bleaching followed by use of magnesium silicate (Zulkurnain et al., 2013), double deodorisation (Shimizu et al., 2013), addition of various antioxidants (Cheng et al., 2017), or use of lower deodorisation temperatures (Gallina-Toschi, 2018; Matthäus & Pudel, 2013), but it cannot completely eliminate these contaminants. Furthermore, most of the mitigation strategies are reported by academic and research organisations. It is unknown to what degree the industry implements these strategies. After the mitigation methods are successfully operated in laboratories, a scale-up of research and operation costs are required to implement the mitigation strategies in production plants (Oey et al., 2019). In our studies, these contaminants were found in all refined olive oils analysed above the EVOO levels so far. Thus, currently the detection method in practice appears to be not easily circumvented by fraudsters.

The VOCs analysis by PTR-QiToF-MS and the spectral analysis by handheld NIR are both fingerprinting methods, which means that a full (mass) spectrum is used in the analytical method. Therefore, the combination of complex data and advanced statistical modelling techniques makes it difficult for a fraudster to catch up with the method (van Ruth & Granato, 2017).

With regard to the ultrasonic velocity analysis by the pulse-echo system, it is a newly developed method for vegetable oil characterisation and has a complex mechanism since it is related to physical and chemical properties. Therefore, this methodology is also difficult to be circumvented by fraudsters. Therefore, all four detection techniques are fairly resistant in terms of potential fraudster manipulation.

In summary, each method has its own intrinsic strengths and weaknesses:

1) In regard to the technical evaluation, the four methods rank as follows from high to low: (1a) the VOCs analysis by PTR-QiToF-MS, (1b) the spectral analysis by

handheld NIR and (1c) the ultrasonic velocity analysis using the pulse echo system; (2) the MCPD esters analysis by GC-MS/MS.

2) Regarding performance, the ranking of the methods is as follows from high to low: (1) the MCPD esters analysis by GC-MS/MS; (2a) the VOCs analysis by PTR-QiToF-MS and (2b) the spectral analysis by handheld NIR and (3) the ultrasonic velocity analysis using the pulse echo system.
3) The ranking of the methods according to their application evaluation is: (1a) the spectral analysis by handheld NIR and (1b) the ultrasonic velocity analysis using the pulse echo; (2a) the VOCs analysis by PTR-QiToF-MS and (2b) the MCPD esters analysis by GC-MS/MS.

Therefore, the selection of the suitable method for any situation is based on the intended purpose.

### 7.4. Practical implications

In order to prevent and mitigate frauds, the following pipeline is proposed: (1) an initial search for the weak spots by an assessment of the fraud vulnerabilities, (2) on site screening and (3) subsequent more elaborative screening and confirmatory tests in a laboratory setting **(Figure 7.3)**. The four stages are detailed below.

**1) Fraud vulnerability assessments**. Food companies can use any of the available food fraud vulnerability assessment tools to conduct a self-assessment and assess their vulnerability to fraud. A low fraud vulnerability would require low levels of mitigation. However, if the company appears vulnerable, mitigation is recommended. This mitigation can include fraud monitoring in raw materials and final products.

**2) On-site rapid screening**. Rapid screening methods (e.g. handheld NIR analysis, **Chapter 5**) could be employed as a front-line testing method to ensure the quality and authenticity of commercial EVOO since the analysis can be conducted on-site, is non-destructive and rapid. B2B companies (distributors and wholesalers), retailers (supermarkets and groceries) and law enforcers, who want a cost efficient device, can employ this technique (**Figure 7.4**). If the analysis results shed doubt on the identity of the sample, it is time to send it to the laboratory.

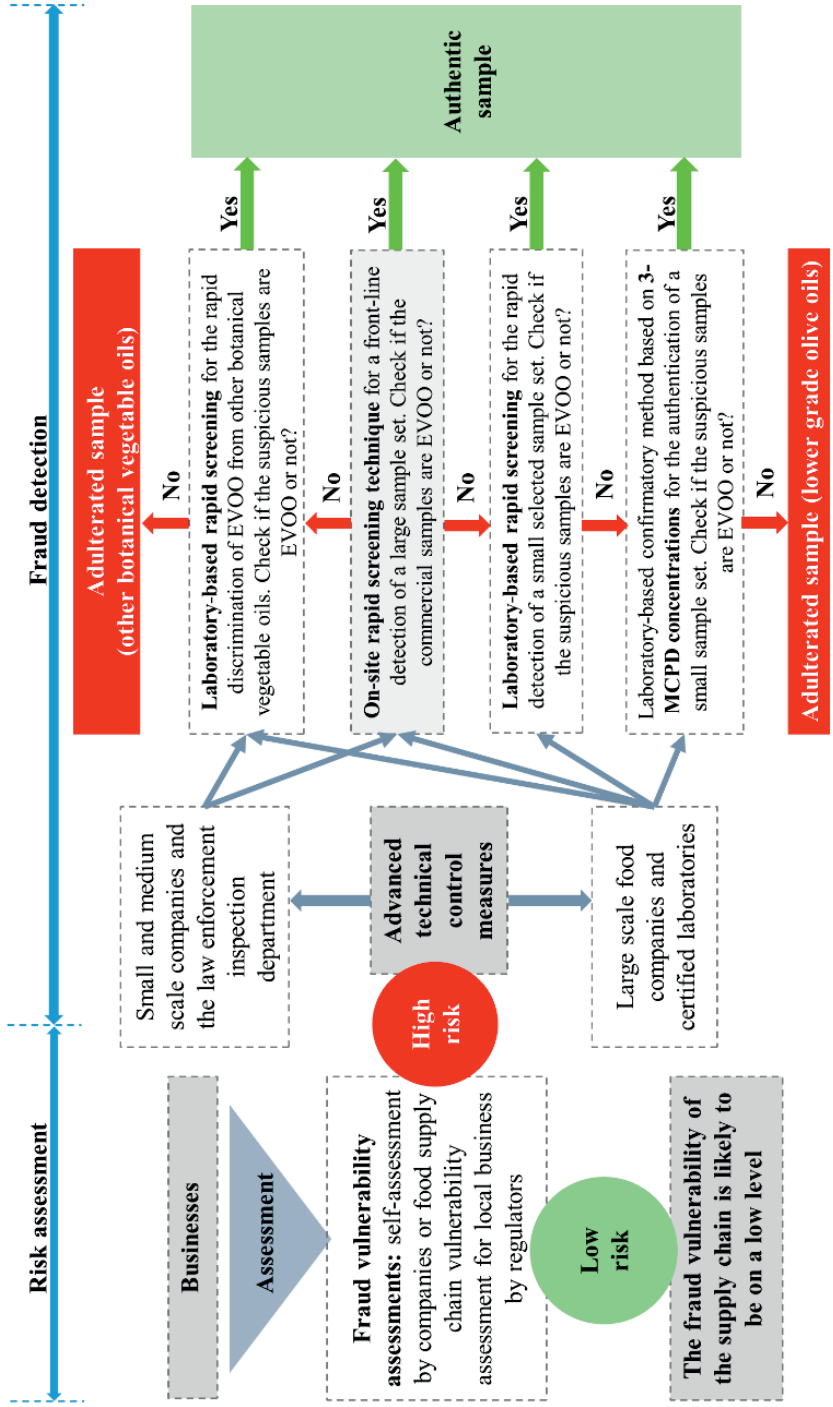


**Figure 7.3.** Food fraud monitoring strategies for regulators and food companies. EVOO, extra virgin olive oil.

**3) Laboratory-based rapid broad anomaly screening**. After the on-site screening, there are two ways to go if we consider the developments in this thesis. The ultrasonic velocity analysis using a pulse echo system mentioned above as potential on-site method (Yan et al., 2019b) could also be applied as a laboratory method for rapid discrimination of EVOO from other botanical origin vegetable oils. Another rapid option is VOCs analysis by PTR-QiToF-MS. Due to the high price of PTR-QiToF-MS, only large-scale food companies and advanced laboratories, can afford this kind of device (**Figure 7.4**). One could also skip the second screening and progress with the confirmatory analysis.

**4) Laboratory-based confirmation**. For confirmatory analysis, the MCPD esters analysis by GC-MS/MS can be applied. However, the 3-MCPD analysis is a tedious and time-consuming method (**Table 7.1**) and also requires advanced equipment. On the other hand, it performs very well and has a low detection limit. Therefore, this method could be considered as the final step to confirm the authenticity of commercial EVOO. Due to the high price of GC-MS/MS and the tedious sample preparation of this method, this technique can only be applied by large scale food companies and advanced laboratories (**Figure 7.4**).

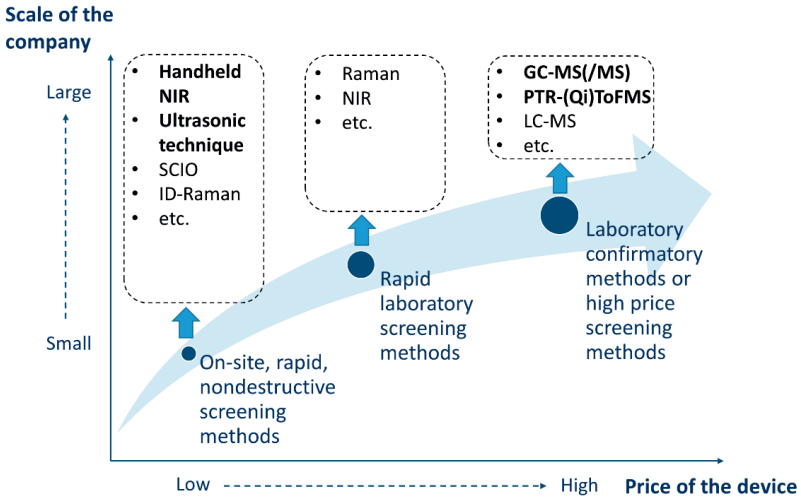


**Figure 7.4.** The application of the advanced fraud detection techniques by food companies of different scales. GC-MS/MS, gas chromatography-tandem mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; NIR, near-infrared spectroscopy; PTR-QiToF-MS, proton transfer reaction-quadrupole ion guide time of flight-mass spectrometry.

The purpose of the proposed pipeline is to improve food quality control, reduce costs and make better and faster decisions. To achieve this goal, on-site screening, followed by laboratory-based broad anomaly testing combined with a multivariate statistics approach is

proposed. Then, a selection of samples will only be analysed in the laboratory for a reliable, validated and possibly accredited confirmation.

### 7.5. General conclusions

The overall objective of this thesis is to develop strategies to combat fraud in the EVOO supply chains through knowledge about weak spots and underlying risk factors and the development of novel detection methods. The weakest points in the EVOO supply chain are, according to the present study, the companies in the middle and end of the supply chain (B2B companies and retailers) due to the higher number of opportunities to commit fraud, as well as greatest lack of food fraud control measures. In addition, both the scale and location of the companies in the supply chain affect the fraud vulnerability of olive oil supply chain actors.

In the current study, four new methods were developed, which cover a wide range of points of use and differ in maturation stage. Each method has its own strengths and weaknesses. More specifically, the MCPD esters analysis by GC-MS/MS is a confirmatory lab-based method that presented the highest discriminatory power, but it is time consuming and is not an eco-friendly method. The VOCs analysis by PTR-QiToF-MS is a rapid, non-destructive, lab-based screening method and is eco-friendly, but the instrument is fairly expensive. The spectral analysis by handheld NIR could be utilised in field tests for rapid and non-destructive analysis, but it presents a slightly poorer performance than the MCPD esters analysis. The newly developed ultrasonic pulse-echo system presents a new idea to discriminate EVOO from other oils based on the sound properties of oils. Although it could discriminate EVOO from most other oils, it showed a poor discrimination between EVOO and ROO. ROO appeared also the most challenging sample group for the other methods. Taken together, each method has its own characteristics, therefore, the selection of the most suitable method for any situation needs to be based on the intended purpose. There is no one size fits all. The newly developed methods could be implemented in particular at the weak spots identified in the current thesis.



### 7.6. Research limitations and recommendations

There are three limitations in this study that could be addressed in future research. Firstly, the variety of samples studied has introduced some bias. Samples in this study are all from European countries, whereas non-Europe olive oils were not considered. Indeed, the main purpose of this study was intended to provide a fraud prevention strategy based on the European olive oil market. However, due to the expansion and globalisation of the olive oil market, it is important to take non-European olive oil production into account as well. In future research the sample set needs to be expanded in general to include more samples from each source. The second limitation is related to the adulterated samples, all methods were applied successfully to

differentiate EVOO from its lower grade counterparts with relatively high sensitivity, specificity and accuracy. However, mixtures of olive oils have not been considered in this study, which needs to be further investigated, since most frauds in practice are admixtures rather than full replacements. The third limitation is the lack of full validation of the methods developed. In this study, the calibration or evaluation of bias and precision using reference standards or reference materials have been conducted. However, the evaluation of some other performance characteristics has not been considered yet. This concerns e.g. a systematic assessment of the factors influencing the result, interlaboratory comparisons and the evaluation of measurement uncertainty of the results based on an understanding of the theoretical principles of the method and practical experience of the performance of the sampling or test method (ISO, 2017). Similarly, the validation of the fingerprint methodology could be extended. Therefore, in order to apply these techniques in practice, a full method validation should be performed to provide evidence that methods are fit for the intended purpose in practice.

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# Summary

Food fraud is a crime, an intentional act for economic gain. Extra virgin olive oil (EVOO) is considered one of the most frequently reported commodities, suffering from fraud. Knowledge about risk factors and precise laboratory and broad on-site screening methods will help to combat fraud in the EVOO supply chain network. The main objective of this thesis is to develop strategies to combat fraud in the EVOO supply chains through knowledge about weak spots in the supply chain and underlying risk factors and the development of novel detection methods. To achieve these goals, firstly, the EVOO supply chain was assessed for their vulnerability using the SSAFE food fraud vulnerability assessment tool (**Chapter 2**). These assessments indicate that the EVOO supply chain is fairly vulnerable. B2B companies and retailers in the EVOO supply chain are more vulnerable to fraud than olive oil producers and food manufacturers due to the additional vulnerability related to opportunities in time and place and a lack of control measures. Fraud vulnerability across the EVOO supply chain was not only determined by the place of the actor in the chain (node), but also by the scale and location of the companies.

Four novel methods were developed in this thesis for EVOO authentication. Monochloropropanediol (MCPD) esters and glycidyl esters (GEs) analysis by gas chromatography-tandem mass spectrometry (GC-MS/MS) was applied to defect EVOO adulteration with lower grade oils (**Chapter 3**). The limit of fraud detection of lower grade olive oils in EVOO was 2% when using 3-MCPD esters, 5% for 2-MCPD esters and 13–14% for GEs. These results imply that the method is fairly useful for confirmatory analysis. However, 3-MCPD analysis by GC-MS/MS is currently a tedious and time-consuming method, it is not recommended to use this method to analyse a large number of suspect samples when a quick response is required. In addition, three rapid and non-destructive techniques were developed. The volatile organic compounds (VOCs) fingerprint analysis by proton transfer reaction-quadrupole ion guide time of flight-mass spectrometry in combination with multivariate statistics proved to be a promising screening methodology for the distinction of EVOO from its lower grade counterparts, as well as from other vegetable oils that are potential adulterants (**Chapter 4**). In the one class classification evaluation, the k-nearest neighbours model presented the best results, which showed that more than 95% of oil samples were correctly predicted. For this most successful model, formic acid, dimethyl sulphide and hexenal are key compounds for the distinction of EVOO from the other oils. Except for the VOCs analysis, the spectral analysis by handheld near infrared spectroscopy combined with multivariate statistics also proved to be good methodology to discriminate EVOO from its lower grade counterparts (**Chapter 5**). The EVOO samples were 100% correctly identified. Pomace olive oil (POO) was efficiently discriminated from EVOO, but 7% of the refined olive oil samples were predicted incorrectly. Furthermore, it was found that the relevant spectral information for the distinction of the oils strongly correlated with the degree of unsaturation of the oils as well as their levels of chlorophylls, carotenoids and moisture. In addition, a newly developed ultrasonic pulse echo system appeared to be a rapid and non-destructive method for the characterisation of vegetable

oils (**Chapter 6**). The ultrasonic velocity of EVOO differed significantly from those of POO and the oils of other botanical origin, but not from the velocity of refined olive oil. Furthermore, it was found that the underlying reason for the ultrasonic velocity differences between oils was the variation of the density and viscosity of the oils.

In conclusion, this thesis shows that the intermediaries between producers and consumers are more vulnerable to fraud due to the opportunities to commit fraud, as well as the greatest lack of adequate food fraud control measures. The results of this thesis also show that the newly developed methods cannot easily to be circumvented by fraudsters and they can be effectively applied for the distinction of EVOO from lower grade counterparts and some vegetable oils. The insights in the weak spots in the EVOO supply chain network in combination with the newly developed fraud methods add to and reinforce the strategies to combat fraud in the EVOO supply chain. This all will help to ensure that consumers get what they are paying for and to fight unfair competition.

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*This is not the end. It is not even the beginning of the end.*

*But it is, perhaps, the end of the beginning.*

*(Quote by Winston S. Churchill)*

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# About the author

## Curriculum Vitae

Jing Yan was born in Dujiangyan, Sichuan, China, on the 15th of January 1989. In 2012, she obtained her bachelor's degree in Food Quality and Safety Control from College of Food Science affiliated to Sichuan Agricultural University in Sichuan, China. Three years later, she obtained her master's degree in the field of Food Processing and Preservation Engineering from the same college. Her master thesis focused on the geographical identification of Long An pomelo based on its chemical and physical characteristics. Due to the financial support of China Scholarship Council, she moved to the Netherlands where she performs a doctoral research in Wageningen University and Research since 2015. Her research topic is to investigate fraud in the extra virgin olive oil supply chain, which includes the identification of vulnerable points and development of novel fraud detection methods. The results of this research are described in this thesis.

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## List of publications

Yan, J., Erasmus, S. W., Aguilera, M., Huang, H., & van Ruth, S. M. (2020). Food fraud: Assessing fraud vulnerability in the extra virgin olive oil supply chain. *Food Control, 111*: 107081.

Yan, J., van Stuijvenberg, L., & van Ruth, S. M. (2019). Handheld near infrared spectroscopy for distinction of extra virgin olive oil from other olive oil grades substantiated by compositional data. *European Journal of Lipid Science and Technology*, *121*: 1900031.

Yan, J., Wright, W. M. D., O'Mahony, J. A., Roos, Y., Cuijpers, E., & van Ruth, S. M. (2019). A sound approach: Exploring a rapid and non-destructive ultrasonic pulse echo system for vegetable oils characterisation. *Food Research International, 125*: 108552.

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Yan, J., Wright, W. M., Roos, Y., & van Ruth, S. M. (2019). Evaluation of food-grade vegetable oils using ultrasonic velocity measurement and fatty acid composition. In 2019 IEEE International Ultrasonics Symposium (IUS) (pp. 2435-2438). IEEE. (Conference publication)

## Overview of completed training activities

**Discipline specific activities**

- EVOO research's got talent (2020). Bari, Italy. Oral presentation.
- 13th China International Food Safety and Quality Conference (2019). Beijing, China. Oral presentation.
- 2nd Postgraduate Symposium on Food Fraud (2018). Prague, Czech Republic. Oral presentation.
- 132nd AOAC Annual Meeting & Exposition (2018). Toronto, Canada. Oral presentation.
- MVO Networking Event (2018). Wageningen, The Netherlands. Oral presentation.
- 16th Euro Fed Lipid Congress (2018). Belfast, UK.
- 8th International Symposium on Recent Advances in Food Analysis (2017). Prague, Czech Republic. Oral presentation.
- 11th China International Food Safety and Quality Conference (2017). Beijing, China. Oral presentation.
- The Nordic Lipid Forum Academy 2017 an introductory course to Lipid Science and Technology, Uppsala, Sweden. Poster presentation.
- Advanced Food Analysis (2017), Wageningen, The Netherlands.
- 1st Summer School on Smartphone-Based Food Analysis (2017), Wageningen, The Netherlands.
- 15th Euro Fed Lipid Congress (2017). Uppsala, Sweden. Poster presentation.
- Symposium on MCPD Esters and Glycidyl Esters (2017). Berlin, Germany.
- 1st Postgraduate Symposium on Food Fraud (2016). Wageningen, The Netherlands. Poster presentation.
- 9th International Conference on Broadband Dielectric Spectroscopy and its Applications (2016). Pisa, Italy.
- 14th Euro Fed Lipid Congress (2016). Ghent, Belgium. Poster presentation.
- Sensory Perception & Food Preference: Affective Drivers of Food Choice (2016), Wageningen, The Netherlands.

**General courses**

- Career Perspectives (2018). VLAG, Wageningen, The Netherlands.
- The Essentials of Scientific Writing and Presenting (2017). WGS, Wageningen, The Netherlands.
- Chemometrics (2017). VLAG, Wageningen, The Netherlands.
- Workshop cross cultural communication (2017). WFSR, Wageningen, The Netherlands.
- Philosophy and Ethics of Food Science and Technology (2016). WGS, Wageningen, The Netherlands.

- VLAG PhD week (2016). VLAG, Baarlo, The Netherlands.

**Optional courses and activities**

- Expertise group/business unit meetings (2015-2019). WFSR, Wageningen, The Netherlands.
- Colloquia (2015-2019). FQD, Wageningen, The Netherlands.
- Preparation of research proposal (2015-2016). FQD, Wageningen, The Netherlands.
- FQD PhD trip to Italy (2016). FQD, Wageningen, The Netherlands.

**Teaching obligation**

- Mentoring MSc- and BSc-students and internships (2016-2019). Wageningen University and Research, Wageningen, The Netherlands.
- Food fraud and mitigation (assistant), FQD-36306 (2018/2019). FQD, Wageningen, The Netherlands.

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# Propositions

1. Fraud vulnerability assessments and fraud detection methods ensure food authenticity and identify food fraud.

(this thesis)

2. Novel fraud detection methods enhance the probability of fraudsters being caught.

(this thesis)

3. Food fraud becomes food crime when public safety is threatened.

4. A fraudster could be anyone around you.

5. Broaden your view, you will find a new world.

6. Stay curious and objective, you may end up as a scientist.

Propositions belonging to the thesis, entitled:

Fraud investigation in the extra virgin olive oil supply chain: Identification of vulnerable points and development of novel fraud detection

Jing Yan

Wageningen, 10 June 2020