

Optimization of rapid analytical protocols for monitoring the contamination with hydrocarbons of petrogenic origin in the olive oil supply chain

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One of the aims of the current PhD project was to develop, optimize and validate rapid and solvent saving sample preparation protocols aimed at the removal of endogenous interferences from olive oil, in order to perform an accurate and high sensitivity determination of mineral oil hydrocarbons (MOH) contaminants. These were then applied for the analysis of samples taken along the olive oil supply chain in order to identify the main sources of contamination.

Ottimizzazione di protocolli analitici rapidi per il monitoraggio della contaminazione con idrurcarburi di origine petrogenica nella filiera dell'olio di oliva

Uno degli obiettivi del presente progetto di dottorato è stato quello di sviluppare, ottimizzare e validare protocolli di preparazione del campione rapidi e a basso consumo di solvente rivolti alla rimozione di interferenti endogeni presenti nell'olio di oliva, allo scopo di poter determinare in modo accurato e sensibile gli oli minerali (MOH). Questi sono poi stati utilizzati per l'analisi di campioni prelevati lungo la filiera dell'olio di oliva allo scopo di identificare le possibili fonti di contaminazione.

Keywords: mineral oil hydrocarbons; contaminants; olive oil; sample preparation; monitoring; sources.

1. Introduction

In accordance with the PhD thesis project previously described (Menegoz Ursol, 2021), this oral communication reports the main results of the following activities:

- A1) optimization and validation of a sample preparation protocol involving a microwave assisted saponification (MAS) followed by epoxidation, aimed at the removal of triglycerides and of endogenous olefins in olive oil, for the determination of mineral oil aromatic hydrocarbons (MOAH) up to 0.5 mg/kg;
- A2) organization of a sampling involving all the steps of the olive oil supply chain, considering different olive groves and mills distributed throughout the Italian territory, and analysis of the different samples exploiting the sample preparation protocol reported in A1.

2. State of the art

Mineral oil hydrocarbons (MOH) are environmental and processing contaminants of petrogenic origin, which consist of complex mixtures of thousands of hydrocarbon isomers. These compounds can be divided into mineral oil saturated hydrocarbons (MOSH) and aromatic hydrocarbons (MOAH), based on their chemical structure and toxicological relevance. MOSH includes paraffins (linear and branched alkanes) and predominantly alkylated naphthalenes (cyclic alkanes), while MOAH includes mono- or polycyclic aromatic compounds with an alkylation degree greater than 98% (Bratinova and Hoekstra, 2019), which distinguishes them from the polycyclic aromatic hydrocarbons (PAH). Although data are still controversial, the latest evidence have proven MOSH to accumulate in human organs and tissues based on their structure and molecular weight, generating inflammatory states and volume increase in the site of accumulation, while MOAH to carry out carcinogenic, genotoxic and mutagenic actions, with particular reference to polycyclic species with 3–7 rings (EFSA, 2012), which therefore are of greater concern for consumer safety.

According to the recent guidance by the Joint Research Centre (JRC) (Bratinova and Hoekstra, 2019), the instrumental analysis is based on on-line high performance liquid chromatography HP (LC)-gas chromatography (GC), coupled to flame ionization detector (FID), firstly described by Biedermann *et al.* (2009), and currently considered the reference method for MOH analysis. The use of this particular configuration derives from the fact that, due to their different toxicology, MOSH and MOAH need to be quantified separately. Their separation is obtained with the LC, while the actual chromatographic determination of the two individual fractions is performed by the GC. Due to the high amounts of isomers present, the GC is not able to separate every single compound belonging to the mixture of hydrocarbons and for this reason they appear in the chromatographic trace as humps of unresolved peaks. Although vegetable oils can be analyzed directly, reaching the required sensitivity can be tricky if the instrumental determination is not preceded by suitable sample preparation aimed at the removal of both triglycerides and endogenous compounds, the latter behaving as interfering compounds. In particular, even

though on one hand oil can be directly injected into the instrument, on the other the column capacity (2 mm × 250 mm silica gel HPLC column) towards the triglycerides is just of 20 mg, significantly limiting the attainable sensitivity. Moreover, the presence of biogenic compounds, like *n*-alkanes in the MOSH fraction and squalene (or other kind of olefins) in the MOAH fraction, disturbs the interpretation of the chromatograms and requires their removal to achieve reliable quantifications. As solutions to these analytical criticalities, several protocols have been proposed in the literature, exploiting either the saponification (Guinda *et al.*, 1996; Koprivnjak *et al.*, 1997) or the elution through fat retainers (Biedermann *et al.*, 2009; Zurfluh *et al.*, 2014) for the elimination of triglycerides and the epoxidation (Biedermann *et al.*, 2009; Nestola and Schmidt, 2017) and the elution through Alox (Fiselier *et al.*, 2009; Moret *et al.*, 2011) to remove olefins and endogenous *n*-alkanes, respectively. However, these protocols have often shown to be solvent and time consuming, and to require significant sample handling, with the risk of introducing contamination.

About the occurrence, the presence of these contaminants in vegetable oils is widespread due to the marked affinity for fatty matrices, given the non-polar character conferred by their chemical structure, and to the high level of mechanization reached for the handling and processing of the raw material. Indeed, the evaluation of the presence of MOH in vegetable oils has already been a matter of study, and data on occurrence as well as assumptions about possible sources of contamination has already been described in the literature (Moret *et al.*, 2009; EFSA, 2012; Brühl, 2016; Purcaro *et al.*, 2016), e.g. environmental pollution, lubricating oils, pesticides etc. Despite this, a specific study along the olive oil production chain, to assess the incidence and the extent of each source, was lacking.

For this reason, the aims of this project were to optimize and validate rapid, highly sensitive and solvent saving methods for MOSH and MOAH quantification in olive oils, and to exploit these methods to analyze samples resulting from different samplings along the entire olive oil supply chain. This was intended to try to identify the critical steps of the supply chain where the contamination occurs and, where possible, to precisely define the source, with the purpose to allow the implementation of strategies aimed at minimizing the contamination in this matrix. As reported above, this paper is focused on activity A1 and A2. About A2, the description will be focused on the first part of the supply chain, which turned out to be the most critical, i.e. the harvesting operation.

3. Materials and methods

For activity A1, two different extra virgin olive oils (EVOOs) were fortified at different levels using two different mineral oils (Gravex and motor oil) and subjected to the analytical protocol to be validated, involving a MAS and an epoxidation. Briefly, 1 g of fortified oil was saponified at 120 °C for 20 min with 10 mL of 1.5 N methanolic potassium hydroxide (KOH), in presence of 10 mL of *n*-hexane, using MAS. A double wash of the hexane phase using milliQ water and methanol, and a 30 min storage at -18 °C between the two washes, were applied. The hexane phase was then evaporated to a volume of 700 µL and epoxidation took place following the Nestola and Schmidt protocol (2017). Recovery, repeatability and LOQ, with a particular focus on the MOAH fraction, were evaluated.

For activity A2, 15 different olive samples were hand-picked directly from the trees in various olive groves located in different Italian regions. From the same olive groves, other 17 samples were collected after the harvesting operations (2 of them were in double since they were harvested using two different harvesting methods and were considered separately), taking them from the containers usually used for their transportation to the mill (e.g. plastic bins, trailers etc.). Olive oil was physically extracted from the olive samples using an Abencor system, a small laboratory plant for the milling of olives, in order to resemble the milling occurring in a real plant and obtain comparable data.

4. Results and discussion

4.1 Validation of the MOAH protocol

Two EVOOs were spiked at different levels with two different mineral oils (figure 1A) and quantitative recoveries as well as good repeatability (RSD% always below 20% for both of them), were obtained for all the fortification levels (2.0-40.7 mg/kg for MOSH and 0.5-9.9 mg/kg for MOAH), even at concentrations of added MOAH close to the LOQ (0.5 mg/kg for the total hump, 0.2 mg/kg for each single C-fraction). MOAH recovery data are reported in table 1. Also linearity was confirmed in all the tested range of concentrations by the coefficients of determination (R^2) always above 0.998. This range was chosen in order to cover the range of contaminations usually found in this type of oil. The validated method, also applied for MOSH determination (C-fraction LOQ: 0.5 mg/kg, total hump LOQ: 1.0 mg/kg), gave excellent results despite the presence of endogenous *n*-alkanes, since by finding a compromise between sensitivity and resolution it was possible to avoid the signal overload due to their presence and to directly evaluate the MOH contamination even without their removal.

Table 1 Recovery and RSD at different fortification levels for each internal standard.

Sample	Type of mineral oil	Number of replicates	MOAH added (mg/kg)	Recovery % (mean)				RSD (%)		
				5B	1-MN	2-MN	TBB	5B	1-MN	2-MN
EVOO1	motor oil	6	0.5	96.4	104.7	105.8	81.9	9.4	9.4	9.2
		4	1.0	99.2	105.2	106.0	84.8	5.1	3.5	3.3
		4	1.5	96.6	102.9	103.8	83.4	8.1	7.0	6.6
		4	2.0	97.3	104.3	105.2	83.8	4.8	2.6	2.2
		4	4.9	100.7	106.7	107.4	87.2	2.4	3.0	3.3
		4	9.9	102.4	109.8	110.4	87.8	2.6	4.7	5.0
EVOO2	Gravex	6	0.8	93.4	99.7	101.7	79.4	5.0	4.2	4.8
		6	1.4	94.6	102.4	102.6	79.8	2.2	2.7	2.9
		6	2.8	100.6	106.0	108.0	83.3	5.8	5.7	6.1
MEAN RECOVERY*				97.9	104.6	105.7	83.5			

*all replicates at different spiking levels

Different recoveries were observed for the different internal standards (table 1 and figure 1B). This turned out to depend on their different partition between the aqueous/alcoholic phase and the *n*-hexane phase (already visible in step 2 when adding the KOH solution, before the MAS procedure), and on the fact that part of the *n*-hexane phase remained in the aqueous/alcoholic phase, concentrating the standards in the organic solvent (this effect is well visible in step 3 for TBB). This required the introduction of correction factors for the recovery, to be applied to the analytical data based on the standard used to perform the quantification.

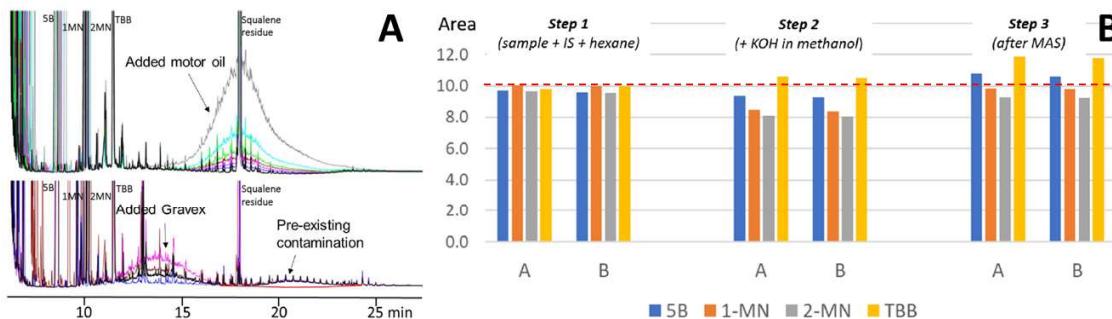


Figure 1 (A) Overlay of the LC-GC-FID traces of the MOAH fractions of the two fortified EVOOs. The overlay of chromatograms starts from the unspiked matrix and the humps with increasing area refer to the different fortification levels reported in table 1. (B) Behaviour of the internal standards of the MOAH fraction in different steps of the MAS procedure.

In conclusion, a rapid and solvent-saving method, based on MAS followed by epoxidation and LC-GC-FID, was optimized and validated for high-sensitivity determination of MOAH in EVOO.

4.2 Monitoring along the supply chain

As reported in paragraph 3, different olive samples were sent to our laboratory from different producers operating in the olive oil supply chain. The investigation had the purpose of tracing the contamination, starting from the trees up to the oil extracted at the mill, into a fairly large number of different Italian production realities, located in various conditions of potential contamination. However, this contribution is specifically focused on the harvesting phase, which turned out to be the most critical step. In addition to the olives collected from the trees and from the containers for their transport to the mill (after the harvesting operations), in some cases it was also possible to obtain samples of grease/lubricating oil/hydraulic oil used for harvesting operations, with the purpose of comparing their chromatographic profile and the MOSH/MOAH ratio, with that possibly found in the olives.

Figure 2 shows MOSH and MOAH levels of samples collected directly from the tree and after the harvesting operations. At this point of the supply chain, two possible sources of contamination were taken into consideration: environmental contamination (evaluated as closeness to possible source of contamination such as urban areas, industrial sites and vehicular traffic, etc.) and phytosanitary/fertilizing treatments.

Except for two samples (TB4 and I2), 13 out of the 15 hand-picked olives samples showed MOSH ranging from <LOQ to 2.7 mg/kg, in line with the background levels normally found in olives collected at this point of the supply chain (Menegoz Ursol *et al.*, 2022). Only in one case (SI1) a MOAH contamination of 1.1 mg/kg was highlighted, even though the origin remained unknown. More in detail, 7 out of 8 different samples collected very close to roads with medium-high traffic (<700 m) or urban areas (<3 km) (TB5, T1, IB1, IB2, IB3 and I1) showed contamination levels below the LOQ for MOSH, and only T2 showed levels a little bit higher (1.6 mg/kg). On the contrary, 3 out of 6 samples located more distant from possible contamination sources (TB1, IB4 and SI1) reported levels between 1.6 and 2.7 mg/kg. Again, the other 3 did not show contamination above the LOQ (TB2, TB3,

TIB1). Based on these data, no correlation was found between the levels of contamination and the position of the olive grove with respect to potential sources. No correlation was found even with respect to the carrying out of phytosanitary/fertilizing treatments, as no substantial differences were highlighted among samples from olive groves which underwent or not treatments, or between samples from conventional and biological farming. Finally, for the two samples where the contamination was significantly higher, the explanation was found in the possible contamination occurred during sample collection/manipulation or, in the context of the treatments, related to the addition of mineral oil-based products to the atomizer to allow a better dispersion/adhesion of the active principle to the plants (a not declared practice), or to a leak of lubricating oil from its mechanical components (e.g. the pump). Therefore, based on these data, no particular criticalities were highlighted in relation to the presence of mineral oils in the olives collected from the trees.

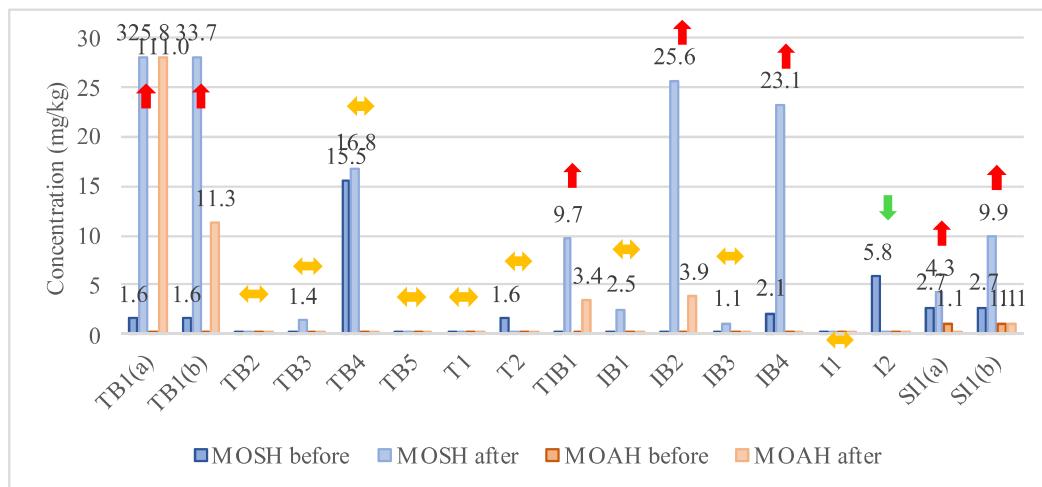


Figure 2 Comparison of MOH concentrations in the oils extracted from olives sampled before (hand-picked from the trees) and after the harvesting operations. Absence of data labels indicates levels below the LOQ (1.0 mg/kg for MOSH and 0.5 mg/kg for MOAH).

On the contrary, a significant increase in contamination was witnessed for 7 samples out of 17 due to the harvesting operations, highlighting their significant contribution on the final contamination (figure 2). For almost all of them it was possible to clearly identify the source thanks to the comparison of the profile of the contamination with those of the lubricants used in the machinery. An example of source identification for sample IB4 is now reported. For IB4 sampling, whose olives were harvested with a straddle harvester, the correspondence was found in the grease used to lubricate the mechanical parts of the latter. In fact, as visible in figure 3, the *n*-C₂₁₋₅₀ distribution, centered on *n*-C₃₃, matched the contamination found in the olives. Moreover, the absence of MOAH (just below the LOQ), in presence of a significant MOSH contamination, fitted with the classification of this grease as a food-grade lubricant (refined to remove/minimize the aromatic fraction, and in the specific case containing 2% of MOAH). For confirmation, also the oil from the hydraulic circuit of the same machinery was sampled, which however showed a molecular weight distribution located at earlier retention times, i.e. centered on *n*-C₂₈ and covering the range *n*-C₁₇₋₄₄, and therefore not matching with the contamination into the olives. The source was therefore determined unambiguously.

According to the same reasoning, the source was also identified for almost all the other samples reporting a significant increase in MOH contamination.

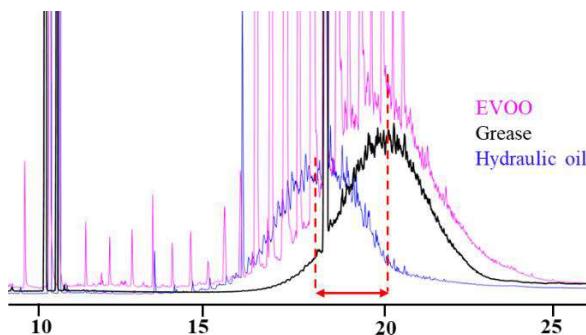


Figure 3 Overlay of chromatograms of the mineral oils used in the harvesting machinery and the contaminations found in the olives sampled after the harvesting operations for sampling IB4.

However, as evident from the bar diagram (figure 2), a wide number of samples did not report any significant increase in the contamination level, excluding problems in the harvesting step.

In conclusion, what was found to be different for these samples, compared to the others, was the lower level of mechanization in the harvesting phase. The contamination preferably occurred on olives harvested with big machinery, such as trunk shakers, olive harvesters or similar, rather than with smaller equipments like hand-held combs. As a direct consequence, the type of cultivation also appeared to be a discriminating factor. Indeed, olives from olive groves where the production density is high, and where the age and the size of the trees allow it, as in the case of intensive or super-intensive cultivation, are more suitable for mechanized harvesting (Lo Bianco *et al.*, 2021) and therefore more prone to be contaminated by mineral oils. Anyway, the harvesting resulted to be a step to be kept under control in order to minimize contamination on the finished product.

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