# $^{13}$C NMR study of the effect of aerobic treatment of olive mill wastewater (OMW) on its lipid-free content

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**Abstract**

Olive mill wastewater was treated by an aerobic bio-process at different values of pH (with or without addition of lime), for 45 days on a laboratory scale, to evaluate the reduction of the organic load. The lipid content showed an appreciable change in relation to the applied treatment both for total lipids and for the different fractions (neutral lipids, monoglycerides and phospholipids). $^{13}$C NMR spectroscopy was performed on initial and final samples both raw and after lipid extraction. The main spectral differences were observed in the C-alkyl region (0–50 ppm), in the C O-alkyl/N-alkyl region (50–110 ppm), and in the C-carboxylic (160–200 ppm) region, providing information on the alterations occurring in the different biochemical entities composing this complex biomatrix (e.g. lipids and carbohydrates) according to the treatment.

*Keywords:* Olive mill wastewater; Aerobic bio-process; Lime treatment; Lipids; $^{13}$C NMR spectroscopy

## 1. Introduction

Olive mill wastewater (OMW) is the term given to the main liquid by-product generated during the production of virgin olive oils. It is composed partly of the water originally contained in the olives and partly of any other water added during the production process, especially if a three-phase decanter is used [1]. OMW is an acidic matrix (pH 4–5.5) made up of water (83–96%), sugars (1.0–8.0%), nitrogenous substances (0.5–2.4%), organic acids (0.5–1.5%), pectins, mucilage and tannins (1.0–1.5%), lipids (0.02–1.0%) and inorganic substances (0.5–2.0%) [2–4]. OMW shows poor biodegradability, high phytotoxicity due in particular to the presence of phenolic compounds [5–7], free fatty acids (FFA) [1] and inorganic salts (notably potassium) [8–10] and a high microbial load in the form of bacteria, yeasts and fungi [11]. Specifically, the C2–C8 volatile fatty acids (VFA) come from microbial metabolism, whereas the C16–C18 long-chain fatty acids (LCFA) come from the oil originally present in the olives, both showing phytotoxic effects and toxic effects towards microorganisms [1]. For these reasons, OMW have a rather high biochemical oxygen demand (BOD) and chemical oxygen demand (COD), which makes their disposal difficult in view of current regulations [12].

Preliminary treatment of OMW is recommended before its agricultural use. A number of ways to process OMW have been employed in recent years, and can be divided into physico-chemical and biological methods. Physico-chemical methods such as decantation, flocculation, ultrafiltration, thermal concentration or incineration can be very expensive and do not solve the problem completely since the sludge resulting from the process must be disposed of too [13]. Biological methods based on composting or anaerobic and aerobic digestion lead to the production of proteins, poly-hydroxy-β-butyrates, exopolysaccharides, etc. [14,15]. The latter methods have clear benefits due to the potential utilisation of their bio-products [13].

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Table 1
Physico-chemical characteristics of the raw OMW

| Parameter | Value |
|---|---|
| pH | 4.85 ± 0.01 |
| Total solids (TS) (g/L) | 190.45 ± 1.47 |
| Total organic carbon (% TS) | 44.43 ± 1.99 |
| Total Kjeldahl nitrogen (% TS) | 0.62 ± 0.01 |
| C/N ratio | 71.66 |
| Chemical oxygen demand (g/L) | 234 ± 4 |
| Total phenols (g/L) | 0.79 ± 0.11 |
| Total lipids (mg/g TS) | 50.99 ± 1.21 |

Since OMW contains variable quantities of residual oil, depending on the extraction process efficiency, and since this effluent is a putative candidate as a potentially suitable liquid growth medium for microorganisms [16], particularly during its storage either in tanks or aerated lagoons [17], it appears useful to see how the lipid content varies over time.

The aim of the present work was to follow the lipid fraction of the treated olive mill wastewater by chromatography and $^{13}C$ NMR spectroscopy, to study the lowering of the potential toxicity of these effluents as reported by D'Annibale et al. [18] during the aerobic treatment.

## 2. Materials and methods

### 2.1. Samples and treatments

Liquid effluent was taken from a modern three-phase centrifugation olive-oil production unit in the Marrakech area (Morocco). The physico-chemical characteristics of the raw OMW are represented in Table 1. It was submitted to different treatments (T1 and T2) for 45 days with three replicates for each treatment. Treatments were carried out in glass crystallising dishes. The volume of effluent used was 2 L/dish.

For T1, ammonium nitrate ($NH_4NO_3$) was added to bring the C/N ratio to 50.5, the pH was not adjusted (initial value 4.85). Treatment T2 involved bringing the C/N ratio to 48.08 with $NH_4NO_3$ and bringing the pH to 6.18 by addition of lime $Ca(OH)_2$. For both treatments, run at ambient temperature (~25 °C), aeration was achieved by bubbling compressed air through the liquids. After homogenisation, samples were taken of the initial effluent at time 0 (after addition of ammonium nitrate for T1 and T2 and lime for T2), then after 15, 30 and finally 45 days. Before studying the organic matter, samples were dialysed (Spectra Por membrane MWCO 1000 Da) to eliminate the excess salts, then freeze dried.

### 2.2. Analysis methods

#### 2.2.1. Standard chemical analyses

All chemical analyses were repeated in triplicate and the values reported here are the means. The pH was measured at ambient temperature according to the method of Rodier [19]. The total solids content was measured by drying at 105 °C for 24 h. The total organic carbon (TOC) was determined using Anne's method based on potassium dichromate, as described by Aubert [20]. Total Kjeldahl nitrogen (TKN) was determined using the standard French procedure AFNOR T90-110 [21]. Chemical oxygen demand (COD) was assayed by the method of Rodier [22]. Total phenols were extracted and purified with ethyl acetate, as reported in Macheix et al. [23] and were assayed according to Folin-Cioccalteu [24]. The total phenol concentration was determined using a standard solution of cafeic acid (20 mg per 100 mL methanol). The total lipid concentration was determined using the method of Folch et al. [25].

#### 2.2.2. Lipid assay

*2.2.2.1. Extraction.* Lipid was extracted from 5 to 10 g of fresh OMW using the method of Folch et al. [25]. This involved the OMW being macerated with 60 mL of a 2/1, v/v chloroform/methanol mixture for 24 h at 4 °C. The supernatant was then recovered and filtered through a Durieux filter. This was repeated twice more to ensure maximum recovery of the lipid. The pooled supernatants were then shaken with 60 mL of 1% NaCl to separate the methanol from the chloroform phase containing the lipid. The chloroform phase was then dried over anhydrous sodium sulfate ($Na_2SO_4$) and the total lipid content was determined after evaporation of the chloroform phase at 40 °C.

*2.2.2.2. Lipid speciation.* The separation of neutral lipids (NL), monoglycerides (MN) and phospholipids (PL) was achieved by chromatography on a silica column (Sep-Pak Plus Silica, Waters, Milford, Massachusets) after elution with 25 mL chloroform, 5 mL of a 49/1 chloroform/methanol mixture and 30 mL of pure methanol, respectively [26]. The different fractions of lipid obtained were weighed after evaporation to dryness. All solvents used, anhydrous and of analytical quality, were provided by Carlo Erba-S.D.S., F-13124 Peypin.

#### 2.2.3. $^{13}C$ nuclear magnetic resonance ($^{13}C$ NMR)

$^{13}C$ NMR spectra were obtained on the initial and final OMW and their solvent-treated residues for the two treatments by dissolution of about 150 mg in 3 mL 0.5 M NaOD. The spectra were recorded on a Bruker WB-AM 300 spectrometer at 75.4 MHz with a 10 mm probe head. Chemical shifts ($\delta$) are reported in ppm, relative to 3-(trimethylsilyl)-propane sulfonic acid sodium salt as external reference. To suppress nuclear Overhauser enhancement, a pulse program with inverse gated proton decoupling was used. Spectra were acquired with a repetition time between impulsions of 2.8 s, a sweep width of 16 700 Hz and a pulse width of 5 μs (35°). 50 000–60 000 FID files were accumulated. The FIDs were treated with a 50 Hz line-broadening function. Integration of the spectra was performed with Bruker Win NMR software.

## 3. Results and discussion

### 3.1. Levels of total lipids and of the different lipid fractions

The variation of the levels of total lipids during processes 1 and 2 is presented in Fig. 1. It can be noted that there is a steady increase in total lipids for process 1: the levels rose from 50.99 to 64.59 mg/g TS after 45 days of treatment. This can be related to the exponential growth phase of the microbial community that occurs during biological treatment [27]. For treatment 2 the

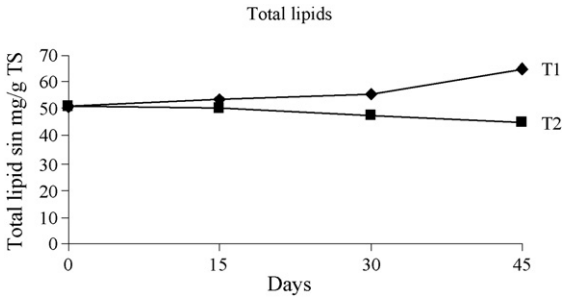


Fig. 1. Concentrations of total lipids at different stages of treatment 1 (T1) and treatment 2 (T2).

level of total lipids decreased, falling from 50.80 mg/g TS at the start of treatment to 45.19 mg/g TS at the end. This decrease is due to the regularly reported decomposition of the lipids of plant and microbial origin [28,29].

The concentrations of the different lipid fractions (NL, PL and MN) at different stages of treatments 1 and 2 are presented in Table 2. It can be seen that notable differences occur in the initial lipid profile between processes 1 and 2, in particular, strong losses of phospholipid which is more sensitive to lime-induced hydrolysis (1.43 mg/g TS vs. 7.37 mg/g TS). The products of this hydrolysis can be partly found in the strong increase of the MN group observed in process 2.

Concerning the evolution of the groups of lipids with time, the variations in the neutral lipids, mainly composed of triglycerides, follows that of total lipid, i.e. an increase for treatment 1 and a decrease for treatment 2, in agreement with the microbiological growth observed. For phospholipids, a certain level of stability was noted for treatment 1 while for treatment 2, after having undergone heavy losses (due to lime-induced hydrolysis) the PL increased owing to the growth of a new population of microorganisms.

### 3.2. $^{13}C$ NMR

$^{13}C$ NMR spectra of the OMW at the initial and final stages of each process (treatments 1 and 2) before and after lipid extraction are presented in Figs. 2 and 3. These spectra were interpreted on the basis of the studies of Preston [30]; Barančíková et al. [31], Almendros et al. [32], Kögel-Knaber [33], Ait Baddi et al. [34], Amir et al. [35] and Hafidi et al. [13]. The spectra were characterized by the presence of many signals in the area of paraffinic carbon in alkyl chains (0 and 50 ppm), aliphatic carbon substituted by oxygen and nitrogen and including the methoxyl groups of aromatic ethers (50 and 110 ppm), double bonded or aromatic carbon (110 and 160 ppm) and carboxylic carbon in ester or amide (160–200 ppm).

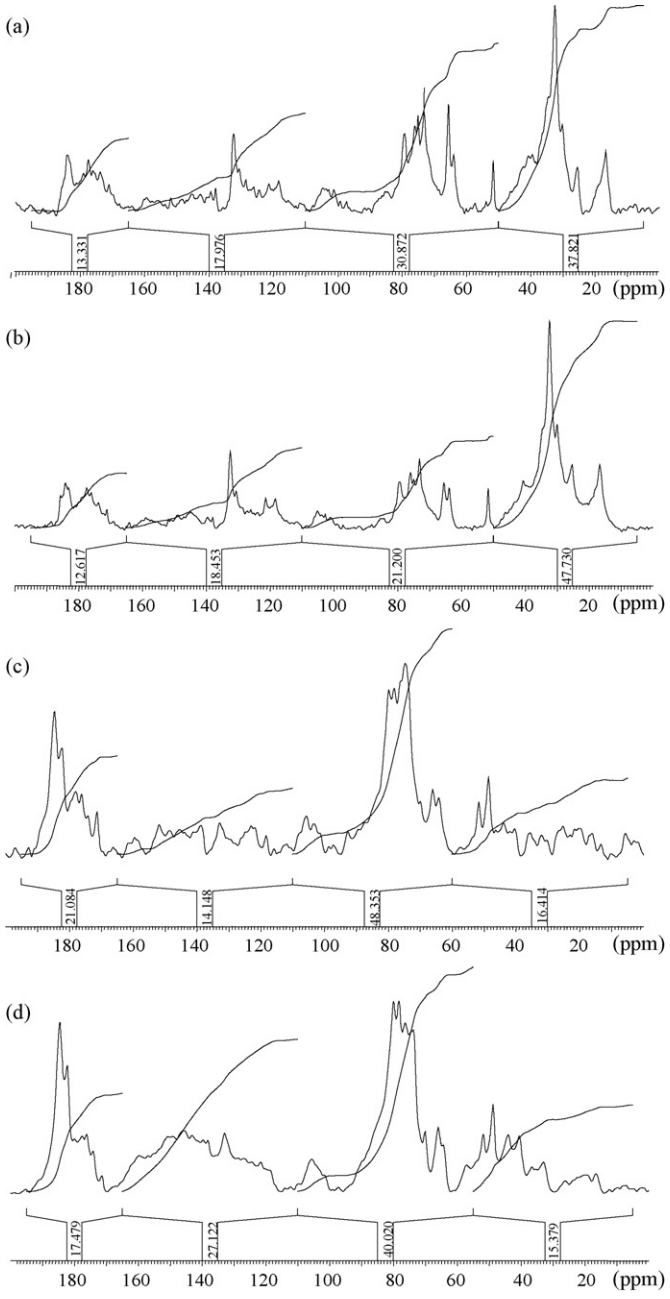


Fig. 2. $^{13}C$ NMR spectra of OMW before and after solvent lipid extraction at initial and final stages of treatment 1. (a) 0 days before solvent lipid extraction; (b) 45 days before solvent lipid extraction; (c) 0 days after solvent lipid extraction; (d) 45 days after solvent lipid extraction.

Table 2
Concentrations of neutral lipids, monoglycerides and phospholipids (mg/g TS) at different stages of the two treatments

| | Treatment 1 (days) | | | | Treatment 2 (days) | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 15 | 30 | 45 | 0 | 15 | 30 | 45 |
| Neutral lipids (mg/g TS) | 42.38 | 42.30 | 48.73 | 55.14 | 46.47 | 42.50 | 41.77 | 42.45 |
| Monoglycerides (mg/g TS) | 1.23 | 2.64 | 2.15 | 1.58 | 2.86 | 3.51 | 2.83 | 0.69 |
| Phospholipids (mg/g TS) | 7.37 | 8.81 | 8.30 | 7.88 | 1.43 | 3.94 | 2.83 | 2.05 |

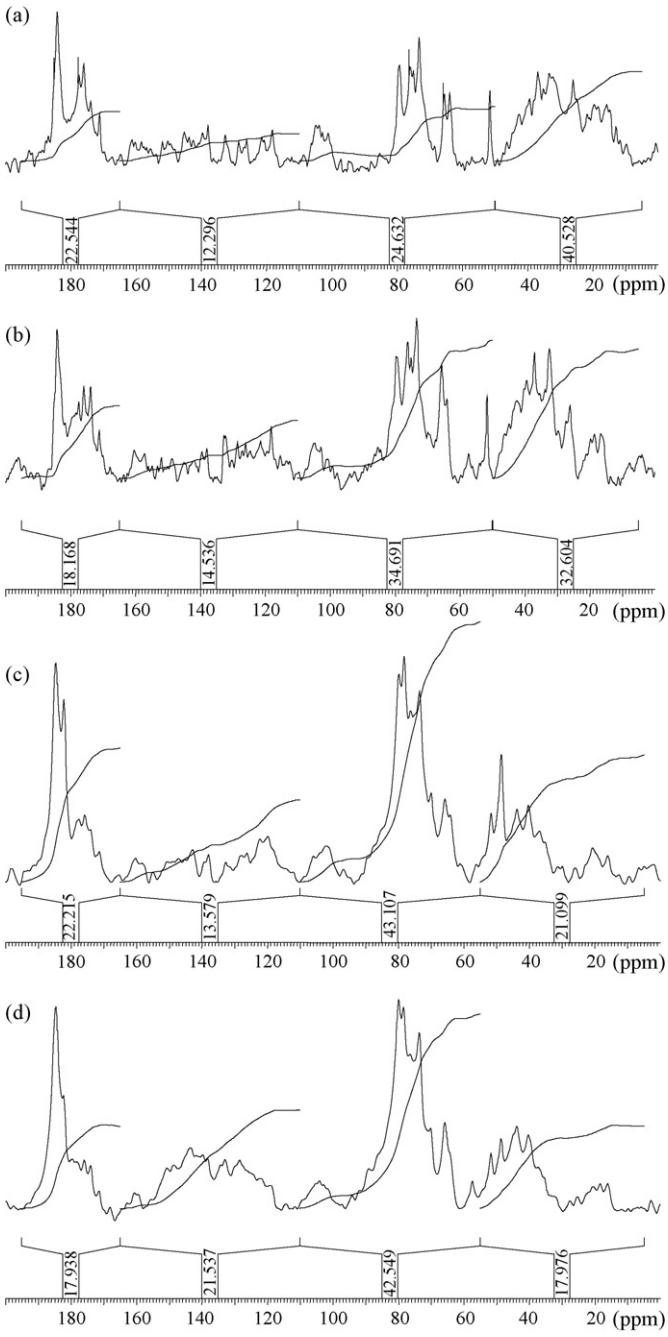


Fig. 3. $^{13}C$ NMR spectra of OMW before and after solvent lipid extraction at initial and final stages of treatment 2. (a) 0 days before solvent lipid extraction; (b) 45 days before solvent lipid extraction; (c) 0 days after solvent lipid extraction; (d) 45 days after solvent lipid extraction.

In the area of alkyl carbons, the signal at 16.5 ppm was characteristic of the final methyl group (–$CH_3$). Signals from 20 to 32 ppm were attributed to $CH_2$ groups in the long fatty acid chains by Preston and Schnitzer [36], whereas the signal at 37 ppm was characterized as $(CH_2)_n$ in the long chains of other alkyls [37]. However, Ouatmane [38] attributed the presence of the latter peak to ramified aliphatic structures and/or long chains in humic acids.

The resonances observed between 40 and 105 ppm were generated by carbons bound directly to an oxygen heteroatom as in alcohols and carbohydrates or nitrogen as in amines and amino acids. These resonances could also be attributed to carbons bound to groups such as aldehydes and ketones [39]. The signals at 66, 70–80 and 105 ppm were found to correspond to the β-D-glucopyranose units present in cellulose [39] and in oleuropeine, a compound of interest in OMW.

The aromatic region (110–160 ppm) can be divided into three parts, one between 110 and 130 ppm for unsubstituted aromatic carbons, another between 130 and 145 ppm for C-substituted aromatic carbons and the last between 145 and 160 ppm for O or N substituted aromatic carbons [35]. The signal in the 141–159 ppm range is assigned to phenolic carbons in lignin units [40]. This region can also be attributed to the resonance of carbons in steroids such as cholesterol (121.3; 141.2 and 160.5 ppm) and ergosterol (119.4; 136.0 and 140.7 ppm) [41].

The carboxylic region (160–200 ppm) can be divided into two parts, one between 160 and 184 ppm for carboxylic acids (–COOH) mainly organic acid that are free or involved in esters or amides, and the second between 184 and 200 ppm for the carbonyl group (–C=O) present in aldehydes, ketones and organic acids [42]. In the present case, this region is of interest for the different fatty acids and particularly for the phospholipids characterized by a carbonyl band between 173.0 and 173.4 ppm [43]. Other compounds such as pectin polysaccharides mainly present in the cell wall pulp (hexuronic acids) [44], which also present a carbonyl group, could resonate at 176.8 and 177.4 ppm [45].

The NMR spectra in samples 1 and 2 (spectra 2-a and 2-b; spectra 3-a and 3-b) presented the same general pattern at the start and at the end of the treatments suggesting that neither treatment greatly modified the biochemical entities initially present in the samples. With respect to spectrum 2a, which can be considered as a reference for the study, large variations can be seen for the peaks at 16.7 and 32.5 ppm as well as for the peak at 184.2 ppm in spectrum 3-a, which follows initial treatment with lime. This results from the hydrolysis of esters, especially of fatty acid esters, to give carboxylic acids. The same changes (spectra 2-c and 3-c) were observed after solvent treatment, confirming that the lipid part of the samples was easily accessible to external actions (chemical removal or solvent extraction). For all samples treated with solvent (spectra 2-c and 2-d; spectra 3-c and 3-d), amplification is seen to occur in the continuum between 73 and 79 ppm which corresponds to the simultaneous resonance of C-2, C-3 and C-5 of polysaccharides such as the resonance of pyranosides in cellulose and hemicellulose [46]. This part of the spectrum is in agreement with the fact that OMW could represent a source of simple and complex sugars, which might be a basis for fermentation processes [47].

Integration of the C-alkyl region of the initial and final samples proved to give identical values for the two treatments (Tables 3a and 3b), indicating that for the first treatment, there was an increase in the percentage of C-alkyl derivatives attributed to the C-aliphatic of the fatty acids, while in treatment 2, there was a decrease. In the C-carboxylic region, while the decreases observed for the untreated samples were rather low, solvent treatment showed a notable decrease (4%) in C-carboxylic, which could indicate the occurrence of a certain level of mineralisation of the organic matter, particularly from

Table 3a
Carbon distribution at the initial and final stages for treatment 1 before and after solvent lipid extraction

| Stage | C-alkyl[a] | O-alkyl/N-alkyl[a] | Aromatic[a] | Carboxylic[a] |
|---|---|---|---|---|
| 1–0 d | 37.82 | 30.87 | 17.98 | 13.33 |
| 1–45 d | 47.73 | 21.20 | 18.45 | 12.62 |
| 1–0 d s | 16.41 | 48.35 | 14.15 | 21.08 |
| 1–45 d s | 15.38 | 40.02 | 27.12 | 17.48 |

d: days; s: solvent lipid extraction.
[a] Values are expressed as percentages of the whole spectral area.

Table 3b
Carbon distribution at the initial and final stages for treatment 2 before and after solvent lipid extraction

| Stage | C-alkyl[a] | O-alkyl/N-alkyl[a] | Aromatic[a] | Carboxylic[a] |
|---|---|---|---|---|
| 2–0 d | 40.53 | 24.63 | 12.30 | 22.54 |
| 2–45 d | 32.60 | 34.69 | 14.54 | 18.17 |
| 2–0 d s | 21.10 | 43.11 | 13.58 | 22.21 |
| 2–45 d s | 17.98 | 42.55 | 21.54 | 17.94 |

d: days; s: solvent lipid extraction.
[a] Values are expressed as percentages of the whole spectral area.

the lipid part in treatment 2. In contrast, for C-aromatic, there was a sharp increase in the signal, confirming the increase in aromaticity of the final products, as previously reported concerning the transformation of polyphenols into polymerised aromatic compounds [7].

Based on the NMR determination of the carbon functions, the different biochemical entities present in the organic matter were evaluated as follows (Tables 4a and 4b):

$$\text{C-alkyl}x14/12;\ \text{O-alkyl/N-alkyl}x30/12;\ \text{C-aromatic}x13/12$$

$$\text{and C-aliphatic}x44/12.$$

This enables an estimation to be made of their weight contribution to the biomatrix. The resulting glycosidic carbon contribution in this study (30–52%) was of the same order as that reported elsewhere [47].

Table 4a
Carbon contribution to the biomatrix at the initial and final stages for treatment 1 before and after solvent lipid extraction

| Stage | C-alkyl | O-alkyl/N-alkyl | Aromatic | Carboxylic |
|---|---|---|---|---|
| 1–0 d | 23.27 | 40.69 | 10.27 | 25.77 |
| 1–45 d | 31.83 | 30.30 | 11.42 | 26.45 |
| 1–0 d s | 8.23 | 51.96 | 6.59 | 33.22 |
| 1–45 d s | 8.49 | 47.31 | 13.89 | 30.31 |

d: days; s: solvent lipid extraction.

Table 4b
Carbon contribution to the biomatrix at the initial and final stages for treatment 2 before and after solvent lipid extraction

| Stage | C-alkyl | O-alkyl/N-alkyl | Aromatic | Carboxylic |
|---|---|---|---|---|
| 2–0 d | 23.08 | 30.06 | 6.51 | 40.35 |
| 2–45 d | 18.36 | 41.87 | 7.60 | 32.16 |
| 2–0 d s | 10.77 | 47.16 | 6.44 | 35.63 |
| 2–45 d s | 9.69 | 49.14 | 10.78 | 30.39 |

d: days; s: solvent lipid extraction.

This approach enables us to confirm the results of the method based on the integration areas, i.e. the differentiation between the two treatments for the C-alkyl and the increase in the degree of aromaticity. The variations in the O-alkyl/N-alkyl region (50–110 ppm) indicate that the lime treatment caused a fall in the initial levels of O-alkyl/N-alkyl derivatives (drop from 40.7 to 30.1%) to the advantage of C-carboxylic derivatives (rise from 25.8 to 40.4%), also affecting the C O-alkyl/C-carboxyl ratio for samples 1-0d/2-0d which fell from 1.6 to 0.7%. This suggests the degradation of certain carbohydrate derivatives such as pectins to give uronic acids [44]. For treatment 1, comparison of the initial, final and treated spectra showed a decrease in the polysaccharides of about 5–10%, this source of carbon being available for the synthesis of lipid. The difference between the two treatments for the production of lipid is therefore confirmed. Thus, for treatment 1, the increase in the C-alkyl and the constant C-carboxylic levels, giving rise to an increase in the C-alkyl/C-carboxylic ratio between the initial and the final states, clearly confirms the conservation of the ester bonds of the fatty acids. For treatment 2, the decrease in the C-alkyl derivatives is paralleled by a similar decrease in C-carboxylic, leading to a stable ratio of 0.6, confirming that the lime treatment also altered the lipid entities.

## 4. Conclusion

Monitoring the behaviour of lipids during the aerobic treatment of olive mill wastewater for 45 days shows notable variations for total lipid and its component fractions (neutral lipids, monoglycerides and phospholipids) depending on whether lime was added before microbial degradation or not.

$^{13}$C NMR spectroscopy carried out on the initial and final samples before and after solvent extraction revealed large differences in the spectrum between the initial sample (1-0d) and its lime-treated counterpart (2-0d). The main spectral differences were observed in the C-alkyl region (0–50 ppm), in the C O-alkyl/N-alkyl region (50–110 ppm), and in the C-carboxylic (160–200 ppm) region, providing information on the alterations occurring in the different biochemical entities composing this complex biomatrix (e.g. lipids and carbohydrates) according to the treatment.

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