https://doi.org/10.17221/81/2019-SWR

^{13}C-NMR spectroscopy of humic substances isolated from the agricultural soils of Puchuncavi (El Melón and Puchuncavi areas), central Chile

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Citation: Alekseev I., Abakumov E. (2020): ^{13}C-NMR spectroscopy of humic substances isolated from the agricultural soils of Puchuncavi (El Melón and Puchuncavi areas), central Chile. Soil & Water Res., 15: 191–198.

Abstract: Chile is a well-developed agricultural country, which is faced with the problem of agricultural soil contamination with metals, such as Cu, Cd, Pb and Zn, and metalloids (As). These elements can be retained by soils through different mechanisms, i.e., complex-forming with organic matter or occlusion within organic matter. That is why studying soil contamination should also be accompanied by detailed investigations of the soil organic matter composition. Soil organic matter is crucial for plant growth since its decomposition releases nitrogen, phosphorus and other nutrients available for the plants. ^{13}C nuclear magnetic resonance spectroscopy, providing crucial data on carbon functional structures diversity, can also be used to study changes in the soil organic matter (SOM) during decomposition and humification. This study is aimed at investigating the molecular composition of the soil organic matter in the agricultural soils of urbanised areas of central Chile using ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The studied soils were characterised by almost neutral pH values and organic carbon contents from 1.7% to 5.2%. The results showed that soils with an increased content of the total organic matter demonstrated an increased portion of aromaticity and a decreased portion of aliphaticity. Most of the investigated humic acids show the highest peaks centred at 125 ppm, which can be tentatively assigned to aromatic alkene structures. The relatively high stabilisation rate of the organic matter in the studied soils can be explained by the mineralisation of its peripheral part, which, in turn, is explained by the decreasing C/N ratio values. The humic substances of the studied agricultural soils of central Chile showed a high average content of aromatic carbon, which is also typical for subboreal soils.

Keywords: humic acids; humification; NMR spectroscopy; soil organic matter

Chile is a country with a well-developed agriculture and is the world leader in the production of copper (Gonzalez et al. 2014). The environmental problems associated with the copper mining activities in central Chile are widely known. That is why the problem of agricultural soil contamination with metals, such as Cu, Cd, Pb and Zn, and metalloids (As) has sharply risen.

The soil organic matter can retain various trace elements through different mechanisms: ion-exchange, proton displacement, and inner or outer-sphere complex formation (Schnitzer 1986). Complexes of elements with organic substances are usually characterised by a higher stability and of greater importance. While simple organic acids, such as acetic acid, are found in the soil solution phase, a

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Supported by Saint Petersburg State University (Grant No. 1.42.959.2016), Research Grant of Russian Foundation for Basic Research 17-16-01030 "Soil biota dynamics in chronoseries of posttechnogenic landscapes: analyses of soil-ecological effectiveness of ecosystems restoration". Scientific research was performed at the Center for Magnetic Resonance of Research Park of St. Petersburg State University.

https://doi.org/10.17221/81/2019-SWR

large part of the soluble organic matter consists of compounds of a higher molecular weight. Organic matter will, therefore, significantly influence the behaviour of many elements, in particular Fe, Cu and Pb. That is why studying soil contamination should also be accompanied by detailed investigations of the soil organic matter.

The soil organic matter (SOM) is crucial for plant growth since its decomposition releases nitrogen, phosphorus and other nutrients available for the plants (Silva et al. 2010). The SOM promotes soil structure by holding the soil particles together as stable aggregates improve the soil's physical properties, such as water holding capacity, water infiltration, gaseous exchange, root growth and ease of cultivation. The soil organic matter also serves as a buffer against toxic and unfavourable substances, such as heavy metals and toxins (Silva et al. 2010).

^{13}C nuclear magnetic resonance (NMR) spectroscopy that provides crucial data on carbon functional structures diversity can also be used to study changes in the SOM during decomposition and humification (Kogel-Knabner 1997; Zech et al. 1997). The advantage of the nuclear magnetic resonance spectroscopy method is the ability to quantify the content of the groups of the structural fragments and identify the individual structural fragments in the humic acid molecules. The application of NMR spectroscopy in the investigations of the soils and the soil organic matter has been previously discussed by Randall et al. (1997) and Preston (1996). Compared to the other methods, NMR-spectroscopy gives direct information about the structure of the studied compounds and is one of the most powerful methods for the comprehensive assessment of the structure of complex organic materials (Chukov et al. 2018). This method is also used to assess changes in the SOM during decomposition and humification. Solid-state ^{13}C nuclear magnetic resonance spectroscopy with cross-polarisation and magic-angle spinning has been previously used for the assessment of the chemical composition of humic acids in different regions of the Earth (Novotny et al. 2007; Shiau et al. 2017; Pennock et al. 2008). Agricultural soils and their humic acids have been investigated by solid-state ^{13}C NMR spectroscopy to reveal the humification degree and their specific molecular structure.

This study is aimed at investigating the molecular composition of the soil organic matter in the agricultural soils of central Chile using ^{13}C NMR spectroscopy. The objectives were (1) to study the soil organic matter contents and the main properties in the selected agricultural soils; (2) to characterise the molecular composition of the humic substances in the selected soils.

MATERIAL AND METHODS

Topsoil samples (0–20 cm) were collected from 8 sites currently used for agriculture in the El Melón and Puchuncavi valley that have been contaminated by copper mining and smelting activities (Figure 1). Both fields were managed with perennial *Lactūca sativa* over many years before the sampling. An expert judgment sampling approach was used (Petersen & Calvin 1996; Aguilar et al. 2011). The sampling locations have been chosen based on a previous investigation of the Cu spatial distribution (Gonzalez et al. 2014; Moya et al. 2017). The soil samples have been collected from the soils classified as entisols (Soil Survey Staff 2003). The procedure for the soil properties determination has been described previously by Moya (Moya et al. 2017). The determination of the main soil parameters has been performed by standard procedures. The values of the pH in the water and salt suspension were measured using a pH-150 meter (1 : 2.5 soil : solution ratio). The grain size distribution has been determined by the

Figure 1. The area of investigation; 1 – El Melón; 2 – Puchuncavi

Table 1. The carbon species and chemical shifts (^{13}C nuclear magnetic resonance, chemical shifts range after Chukov et al. 2018)

Chemical shift (ppm)	Molecular fragments: carbon species
0–47	unsubstituted saturated alkyl carbons C
47–60	alkyl C substituted by oxygen and nitrogen atoms
60–110	alkyl C singly bonded to one oxygen atom and alkyl C bonded to two oxygen atoms
110–144	proton- and alkyl-substituted aromatic C
144–164	oxygen-substituted aromatic C
164–183	carboxyl, ester, and amide C
183–190	quinone C

pipette method. The carbon and nitrogen content was measured by a Vario El Cube elemental analyser (Elementar Analyse System GmbH, Germany).

The humic acids (HAs) were extracted according to the procedure recommended by the International Humic Substances Society (IHSS 1981). The HAs were extracted with 0.1 M NaOH (soil : solution ratio 1 : 10) under nitrogen gas. After 24 h of shaking, the alkaline supernatant was separated from the soil residue by centrifugation at 1 516 ×g for 20 min and acidified to pH 1 with 6 M HCl to induce the precipitation of the HAs. The supernatant contained fulvic acids (FAs), which was then separated from the precipitate (HAs) by centrifugation at 1 516 ×g for 15 min. The HAs were then re-dissolved in 0.1 M NaOH and shaken for 4 h under nitrogen before the suspended solids were removed by centrifugation. The HA solution was acidified again with 6 M HCl to pH 1.0 and the HAs were separated by centrifugation. The HAs were demineralised by shaking overnight in 0.1 M HCl/0.3M HF (solid : solution ratio 1 : 1) and then repeatedly washed with deionised water until the washing reached pH 3. The solid HAs were then freeze-dried.

The ^{13}C NMR spectra of the air dried HAs samples were recorded on a JNM-ECA 400 NMR spectrometer (JEOL, Japan) with a working frequency of 100.53 MHz using the Cross-Polarisation Magic Angle Spinning (CP-MAS) procedure. The spinning speed was 6 kHz; the contact time was 5 ms and the recycle delay was 5 s. The chemical shifts have been referenced to tetramethylsilane at 0 ppm. The quantitative processing was performed by numerical integration in the regions corresponding to the functional groups and the molecular fragments with a preliminary automatic correction of the phase and the baseline using a Delta v. 5.0.2. (JEOL, Japan).

The data were corrected for the water and ash content. The oxygen content was calculated by the difference of the whole samples' mass and gravimetric concentration of C, N, H and ash. Samples of the Leonardite HA standard (1S104H) and the Elliot soil HA standard (1S102H) were used as standards.

The total aromatic and aliphatic content were determined by integrating the signal intensity in the intervals 100–164 and 183–190 ppm on the one hand, and 0–110 and 164–183 ppm on the other hand, respectively. The carbon species were determined according to Table 1.

RESULTS AND DISCUSSION

The main soil properties of studied topsoil horizons of the agricultural soils are summarised in Table 2. The fine earth of the studied soils shows a pH of almost neutral pH values (from 6.22 to 7.42). The C/N ratio usually varies quite significantly in the soils from El Melón – from 9.2 to 29.5. The soils from Puchuncavi are characterised by a narrower range of the C/N ratio values – from 8.7 to 13.4. The soils from Puchuncavi are characterised by the predominance of a sand fraction, whereas the studied soil from El Melón show the predominance of a silt fraction.

The carbon content in the ashless HAs in the soils of El Melón varied from 34.58% to 48.92% whereas the carbon content was less (Table 3) in the HAs in the soils of Puchuncavi. This gives us the possibility to classify the substances investigated as containing relatively high carbon in El Melón. The oxygen is subsequently higher in the HAs of the Puchuncavi soils. A high oxygen content indicates an increased degree of oxidation, this corresponds well with the oxidation coefficient values. The molecular ratios were revealed as normal HA materials.

The ^{13}C NMR spectra from the studied humic acid powders are shown in Figure 2. All of the studied (excluding the HC25 soil) HAs showed the highest peaks centred at 125 ppm, which can be tentatively assigned to aromatic alkene structures and indicating that an alkyl aromatic carbon was substituted by a nitrogen or an oxygen. In general, the shifts between 112–163 ppm are aromatic and phenolic carbons and the peaks in this region mainly correspond to the aromatic and olefinic carbons (Wooten 1995). The

Table 2. The main soil properties of the studied soils

Soil ID	pH	Total organic matter	N	C	C/N	Sand	Clay	Silt
		(%)				(%)		
El Melón								
HC01	6.96	4.20	0.08	2.50	29.50	34	21	45
HC12	7.42	5.30	0.27	3.10	11.40	35	22	43
HC23	7.23	5.00	0.47	3.60	7.70	36	20	44
HC25	7.23	4.30	0.47	3.50	7.50	36	20	44
HC29	6.30	2.70	0.10	1.60	15.20	47	6	47
HC37	7.13	3.50	0.22	2.00	9.10	40	2	58
Puchuncavi								
HC76	6.92	2.90	0.12	1.70	13.40	82	6	12
HC84	6.22	3.40	0.23	2.00	8.70	74	4	22

HC25 soil shows the highest peak at 173 ppm, which refers to the carbonyl (ester, acid) structure. According to Hatcher et al. (1981), the carboxyl groups could be partly derived from the polyuronic acid moieties associated with the polysaccharides. Moreover, HC12 and HC84 show relatively sharp peaks centred at quite the same chemical shift (175 ppm). It should be noticed that the HC76 soil shows an atypical molecular composition of the humic acids for sub-boreal zone soils.

The HC12 and HC23 soils show a few quite intensive peaks at 175 ppm (ester, R(C=O)OR, carboxyl C), 125 ppm (alkene, aromatic), 72 ppm (RCH_2OH, RCH_2OR) and 34 ppm (quaternary alkyl, R_4C). At the same time, the lower peaks in HC12 were identified at 148 ppm (oxygen-substituted aromatic C), 108 ppm, 85 ppm, 67ppm and 60 ppm (alkyl C singly bonded to one oxygen atom), 42 ppm and 22 ppm (unsubstituted saturated alkyl carbons C). HC23 is also characterised by lower peaks at 50 and 25 ppm. HC37 shows the most intense peaks in the aromatic area – the highest peaks at 150, 125 ppm. The peaks of the chemical shifts in HC76 are shifted to the aliphatic portion – only one very intensive peak at 130 ppm (aromatic), the other peaks are much lower and refer to the chemical shifts of 105, 90, 75 and 73 ppm; 60 and 40 ppm (aliphatic). HC01 is characterised by the two most intense peaks at 175 and 125 ppm. HC29 shows the highest peak in the aliphatic area – 71 ppm (alkyl C singly bonded

Table 3. The elemental composition of the studied humic acids from the surface soil horizons.

Soil ID	C	H	N	O	C/N	H/C	O/C	Ash (%)
	(%)							
El Melón								
HC01	34.58	4.32	5.90	55.20	5.86	0.12	1.60	5.00
HC12	36.12	4.78	6.91	52.19	5.23	0.13	1.44	5.00
HC23	48.92	4.96	6.45	39.67	7.58	0.10	0.81	5.00
HC25	39.85	5.12	5.84	49.19	6.82	0.13	1.23	5.00
HC29	42.13	5.35	4.78	47.74	8.81	0.13	1.13	5.00
HC37	40.89	4.89	4.56	49.66	8.97	0.12	1.21	5.00
Puchuncavi								
HC76	35.15	3.90	4.25	56.70	8.27	0.11	1.61	5.00
HC84	33.54	4.78	4.36	57.32	7.69	0.14	1.71	5.00
Standards								
Leonardite HA standard (1S104H)	63.81	3.70	1.23	31.27	51.88	0.06	0.49	2.60
Elliot soil HA standard (1S102H)	58.13	3.68	4.14	34.08	14.04	0.06	0.59	0.90

The gravimetric concentrations are given for the C, H, N and O contents

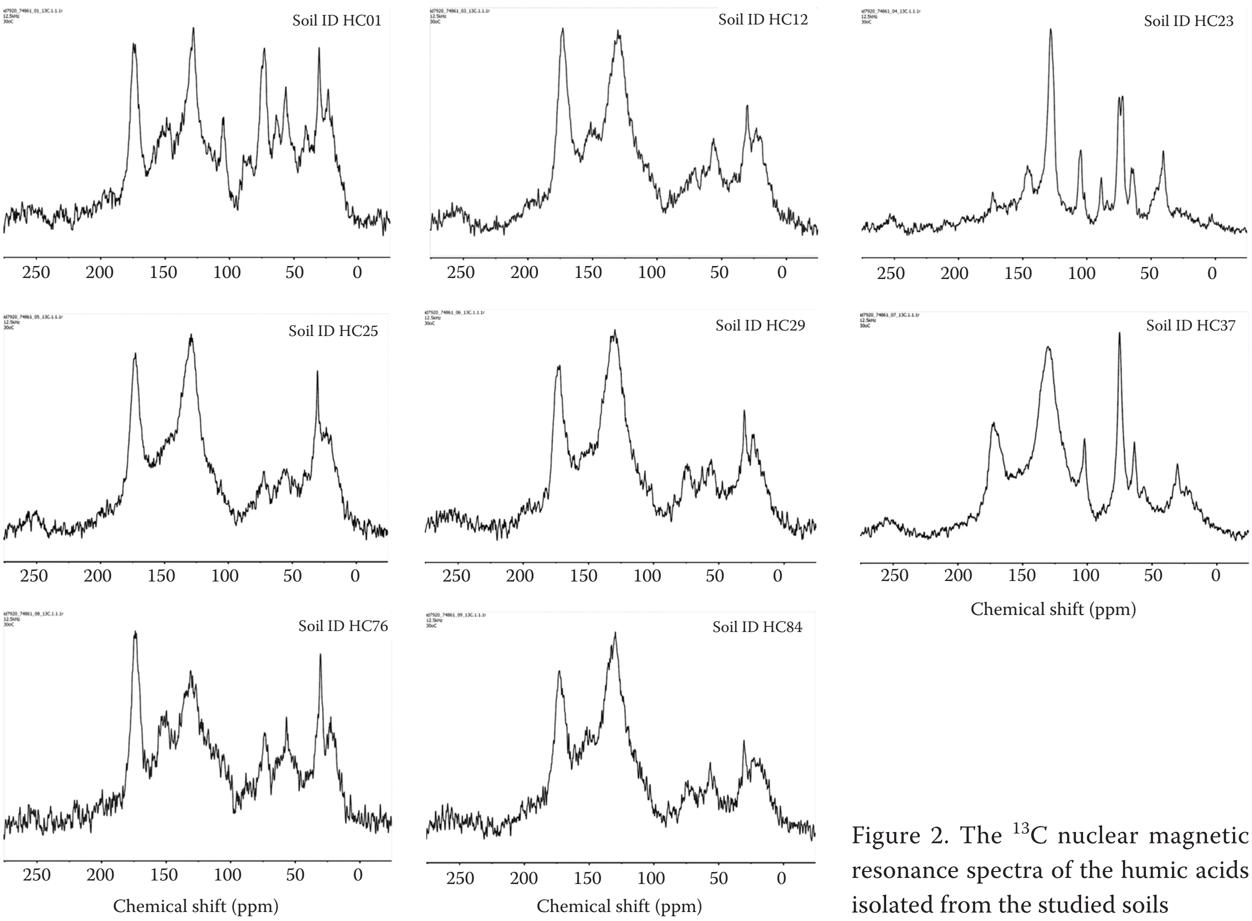

Figure 2. The ^{13}C nuclear magnetic resonance spectra of the humic acids isolated from the studied soils

to one oxygen atom). The distribution of the peaks in HC25 is quite smoothed out – the two most intense peaks (170 and 28 ppm) are observed together with less intense peaks in the areas of 155–120 ppm and 75–50 ppm. HC84 shows the strict predominance of the aromatic peaks – the highest are at 170 and 125 ppm.

The resonance within the chemical shift region between 105 and 144 ppm (aromatic region) may originate from the unsubstituted and alkyl-substituted aromatic C (Abakumov et al. 2015). The ^{13}C-signals in the 148–160 ppm region are assigned to the aromatic carbon bound to the strongly electron-withdrawing OH group and the other heteroaromatic carbons. In the range of 129–148 ppm, the sp^2 hybridized carbons with the aliphatic carbon substituents and the internal carbons of the condensed aromatic compounds can be observed (Gonzalez et al. 2014). The signals in the 118–129 ppm region are assigned to the aromatic C-H resonances and those in the 108–118 ppm region are assigned to the aromatic C-H ortho- to aromatic C-O groups. The carboxyl region (164–183 ppm) is dominated by a peak with a maximum at approximately 175 ppm, largely attributed to carboxyl C, but also to carbonyl C from the amides and polypeptides (Lodygin & Beznosikov 2003; Keeler et al. 2006). The carbonyl region is between 160 and 220 ppm, and there is a considerable overlap of the chemical shifts in this region due to the huge number of chemical components that contain a carbonyl group (Wershaw et al. 1998).

In all the samples, there was a statistically significant predominance of aromatic carbons (57.29 ± 4.05%) over the aromatic ones (42.71 ± 4.05%); *t*-test, $P < 0.001$; Table 4). The standard deviation of all the measured 13^{C}-NMR spectra showed that the highest variability among the studied samples is observed at the intervals of 70–80 (the alkynes structure) ppm and 125–135 (the aromatic structures) ppm (Figure 3).

The sp^2/sp^3 carbon ratio was calculated according to equation:

$$sp^2/sp^3 = (\text{area } (108\text{–}220 \text{ ppm}))/(\text{area } (0\text{–}108 \text{ ppm}))$$
(Mao et al. 2000)

where:
sp^2 – sp^2 hybridized carbons
sp^3 – sp^3 hybridized carbons

Table 4. The percentage of carbon in the main structural fragments of the humic acids (according to the ^{13}C nuclear magnetic resonance) (n = 3)

Site	Chemical shifts (ppm)				Aromatic groups	Aliphatic groups	P (t-test)
	0–45	45–110	110–160	160–220	(%)		
El Melón							
HC01	18.27	19.21	47.12	16.23	59.70	40.30	< 0.001
HC12	22.35	32.23	33.20	13.02	51.82	48.18	< 0.001
HC23	19.21	20.07	44.10	17.05	59.89	40.11	< 0.001
HC25	20.05	24.23	40.05	15.12	62.49	37.51	< 0.001
HC29	12.07	29.02	46.12	13.12	53.88	46.12	< 0.001
HC37	22.09	17.08	44.04	17.92	59.93	40.07	< 0.001
Puchuncavi							
HC76	14.12	36.07	43.17	7.12	52.24	47.76	< 0.001
HC84	17.23	17.09	48.12	18.06	58.37	41.63	< 0.001

The results of the sp^2/sp^3 carbon ratio calculation are presented in Table 5. The ratio of the samples investigated was generally higher than 1. The highest value was observed for the HAs of the HC84 sample from Puchuncavi (2.174), the lowest value was observed for the HAs of the HC12 sample from El Melón (0.971). Our data are principally in the scope of those published earlier by Mao *et al.* (2000). The authors found the sp^2/sp^3 carbon ratio in the HA models ranged from 1.1 to 6.8. Hence, the sp^2/sp^3 carbon ratio in the HAs investigated here did not match with any previously reported results.

The quality of the precursor material has been identified as one of the main drivers of the soil humic acids' composition (Andersson et al. 2012). This factor is followed by the climatic conditions (i.e., temperature and moisture) which shape the biophysical conditions necessary for the humification (Zech et al. 1997).

The humic substances of the studied agricultural soils of the urbanised areas of central Chile showed a high average content of aromatic carbon. This is quite typical for soils with well-humified organic matter, e.g., Mollisosls of Umbric Luvisols (Abakumov et al. 2009; Kholodov et al. 2011) (Table 2). Such type of humus with a pronounced portion of aromaticity is also typical for subboreal soils.

The predominance of quite the same SOM composition in the studied soil might be explained by the

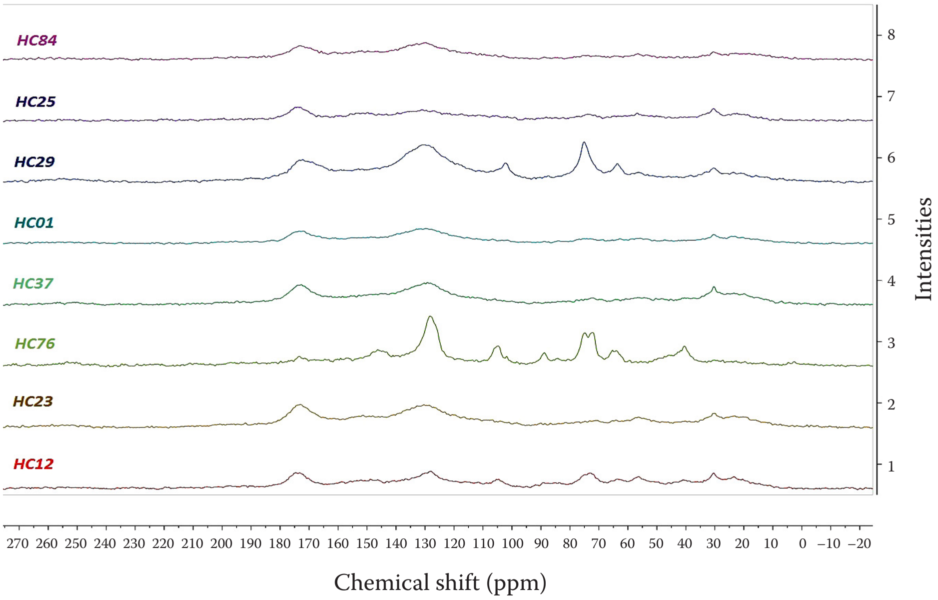

Figure 3. The stacked spectra of the humic acids of the studied soils

Table 5. The sp^2/sp^3 ratios in the studied samples

Soil ID	sp^2/sp^3
El Melón	
HC01	1.754
HC12	0.971
HC23	1.667
HC25	1.333
HC29	1.515
HC37	1.613
Puchuncavi	
HC76	1.087
HC84	2.174

sp^2, sp^3 – hybridized carbons

cultivated state of the studied soils. In contrast to uncultivated landscapes, the installation of a drainage tile in the cultivated soils may lead to the equalisation of the soil water regimes sufficiently and erasure of the signature of the differential accumulations of the plant polymers that would otherwise resist decay in low-redox conditions (Wang et al. 2008). Moreover, agricultural management in the different landscape positions could lead to the homogenisation of the annual inputs of the above- and below-ground biomass. Hence, the present-day SOM primarily reflects these most recent additions (fertilisers).

It was observed that the soils with an increased content of the total organic matter demonstrated an increased portion of aromaticity and a decreased portion of aliphaticity (Tables 2 and 5). This is well connected with humification degree level assessed by the values of the C/N ratio also. Herein, the soils with the lower C/N ratio values demonstrated increased aromaticity values.

Soils of urbanised landscapes are characterised by intense humification. In turn, this leads to the increased aromatisation of the organic matter since the topsoil horizons in such conditions are the subject of intense aeration, oxidation and accumulation of nitrogen in the aromatic compounds.

CONCLUSION

^{13}C-NMR spectroscopy was used to characterise the molecular composition of humic acids from 8 agricultural soils in central Chile. All the samples were slightly different with some common peaks. The similar SOM composition in the studied soil might be explained by the cultivated state of the studied soils, which is expressed in the sufficient equalisation of the soil water regimes and the erasure of the signature of the differential accumulations of the plant polymers.

Most of the investigated humic acids show the highest peaks centred at 125 ppm, which can be tentatively assigned to the aromatic alkene structures. The relatively high stabilisation rate of the organic matter in the studied soils can be explained by the mineralisation of its peripheral part, which, in turn, is explained by the decreasing C/N ratio values. The soils of urbanised landscapes are characterised by intense humification, which leads to the increased aromatisation of the organic matter in the topsoil horizons due to the intense aeration, oxidation and accumulation of nitrogen in the aromatic compounds.

This work revealed that the studied agricultural soils with an increased content of the total organic matter demonstrated an increased portion of aromaticity and a decreased portion of aliphaticity. High aromaticity rates were found in the soils with the increased SOM content, the decreased values of the C/N ratio (and correspondingly the low aliphaticity rate).

Acknowledgements. The authors would like to acknowledge A. Neaman, the research assistants from Pontificia Universidad de Católica de Valparaíso, School of Agronomy for helping with organisation of the field studies.

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Received: July 4, 2019
Accepted: December 2, 2019