

## Instrumental and Experimental Conditions for the Application of Fourier Transform Infrared Analysis on Soil and Humic Acid Samples, Combined with Chemometrics Tools and Scanning Electron Microscopy

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The investigation of the chemical properties of soil organic matter (OM), by the use of some spectroscopic techniques, requires the removal of the mineral impurities of the samples. Therefore, the objective of this study was to evaluate the influence of the pretreatment of samples and, also, the influence of instrumental conditions on the qualitative and quantitative results obtained by Fourier transform infrared (FTIR) spectroscopy to soil and humic acid (HA) samples. The obtained results showed that: the mass of potassium bromide (KBr) used on the background pellet influenced the characteristics of samples spectra; the baseline correction changes the intensity of the absorptions; both the extraction of HA as the hydrofluoric acid (HF) treatment were not efficient in removing the mineral impurities of the samples. The scanning electron microscopy (SEM) analysis showed differences due to the extraction, but not due to the HF treatment. The present study shows the importance of the use of adequate treatments, before, during and after the spectroscopic study of soil OM, providing valuable information for future works.

**Keywords:** FTIR, background, baseline, pretreatment, soil organic matter

### Introduction

Many studies about soil and soil organic matter (OM) have shown the constant application of spectroscopic techniques,<sup>1-4</sup> which allow the chemical and structural characterization of these samples. Fourier transform infrared (FTIR) spectroscopy is a largely used technique for this purpose, because it allows identifying the functional groups present in the chemical structure of soil OM<sup>5,6</sup> and, also, estimating the proportion between the aromatic and aliphatic groups by calculating the aromaticity index.<sup>7,8</sup> Therefore, it is possible to access, qualitatively and quantitatively, the characteristics of soil OM.

However, the characterization of soil OM chemical composition by some spectroscopic techniques, such as FTIR spectroscopy, requires the removal of mineral impurities of samples. The chemical fractionation of humic substances, according to the methodology proposed by the International Humic Substances Society (IHSS),<sup>9</sup> is a largely used pretreatment,<sup>3,4,10,11</sup> which allows the separation of humin, fulvic acid and humic acid (HA) fractions.<sup>9,12</sup> Another pretreatment that can be carried out involves the use of HF.<sup>13</sup>

According to Rumpel *et al.*,<sup>14</sup> the purification of soil OM using HF is based on the breakdown of Si–O bonds, leading to the solubilization of minerals.

In addition to ensuring that the samples are free of mineral impurities, it is important to pre-establish some instrumental conditions to evaluate the qualitative and, mainly, quantitative characteristics of soil OM by FTIR spectroscopy, and, also, to compare the obtained results. Among these conditions, setting the mass of KBr that will be used on sample and background pellets, and setting if the baseline of spectra will be corrected.

The importance of establishing experimental conditions (such as the pretreatment of samples) and instrumental conditions can be investigated by chemometrics methods. According to Souza and Poppi,<sup>15</sup> the principal component analysis (PCA) is one of the most important methods used in chemometrics, which can be used to find similarities between samples. The data preprocessing is fundamental to the success of multivariate analysis and its main objectives are: to eliminate irrelevant information from the chemical point and to improve the data matrix for the analysis.<sup>15</sup> According to Souza *et al.*,<sup>16</sup> one of the principal spectral data preprocessing techniques is the standard normal variate (SNV), used to minimize light scattering problems,

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very common in scans where the radiation is directly placed on the sample. The spectral data can also be mean-centered. This method consists in calculating the average of intensities for each wavelength, and in subtracting each intensity from the average. It facilitates the perception of differences in the relative intensities of the variables.<sup>15</sup>

In this context, the objective of the present study was to evaluate, with the aid of chemometrics tools, the influence of the mass of KBr used in the background pellet; the baseline correction and the pretreatment of soil samples, according to IHSS methodology and also with HF; on the characteristics of FTIR spectra of soil and HA samples. The modifications due to the chemical treatments were studied by scanning electron microscopy (SEM).

## Experimental

### Studied soil

Soil samples (S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub> and S<sub>4</sub>) were collected in the experimental station of the Instituto Agronômico do Paraná (IAPAR), located in Pato Branco, Paraná, Brazil (26°07'16''S; 52°39'37''W; 730 m altitude). According to the Brazilian System of Soil Classification,<sup>17</sup> the soil analyzed is classified as a typical dystroferric red latosol. The samples were collected at depth 0-10 cm. After that, they were dried in an oven at 40 °C, grounded in a knife mill and sieved on a sieve with a 2 mm mesh.

### Extraction of HA

The extraction of HA (HA<sub>1</sub>, HA<sub>2</sub>, HA<sub>3</sub> and HA<sub>4</sub>) was based on the methodology proposed by the IHSS.<sup>9</sup> However, the process was interrupted after the precipitation with 6 mol L<sup>-1</sup> of hydrochloric acid (HCl).

### HF treatment

Soil and HA samples were treated with 10% (m/m) of HF solution based on Gonçalves *et al.*<sup>13</sup> methodology, but the extraction procedure was repeated only two times. It were used 2 g of sample (soil or HA), that was weighed into a polyethylene bottle, and added 6 mL of 10% HF solution. After the HF treatment, soil samples were called S<sub>1HF</sub>, S<sub>2HF</sub>, S<sub>3HF</sub> and S<sub>4HF</sub>, and the HA samples were called HA<sub>1HF</sub>, HA<sub>2HF</sub>, HA<sub>3HF</sub> and HA<sub>4HF</sub>.

### FTIR spectroscopy

Soil and HA samples were kept in a desiccator and the KBr was dried in an oven at 105 °C for 48 h until the

measurements were made. The FTIR spectra were obtained according to the Stevenson<sup>18</sup> methodology, using a Perkin Elmer FT-IR Spectrometer Frontier spectrophotometer, in duplicate. All individual FTIR spectra were recorded in the range 4000 to 400 cm<sup>-1</sup> and were composed by 64 scans with a spectral resolution of 4 cm<sup>-1</sup>. The absorption band attributions of the soil and the HA spectra were made following González-Pérez *et al.*,<sup>1</sup> Cocozza *et al.*,<sup>5</sup> Senesi *et al.*,<sup>6</sup> Dick *et al.*,<sup>7</sup> Stevenson,<sup>18</sup> Brinatti *et al.*,<sup>19</sup> Castellano *et al.*,<sup>20</sup> Solomon *et al.*,<sup>21</sup> Senesi *et al.*,<sup>22</sup> Tivet *et al.*,<sup>23</sup> and Enev *et al.*<sup>24</sup>

### Background test

The background pellets were prepared with 50, 60, 80, 100, 120 and 150 mg of KBr. A sample pellet was prepared, too, with 1.5 mg of soil (S<sub>1</sub>) and 150 mg of KBr. After that, successive spectra of the same sample pellet were obtained using the different background pellets to correct it.

### Baseline correction

The baseline was automatically corrected using the equipment software. The influence of the baseline correction on the characteristics of FTIR spectra was evaluated by the comparison of S<sub>1</sub> and HA<sub>1</sub> spectra, before and after the correction, and using the aromaticity index values also.

### Aromaticity index

The aromaticity index was calculated according to Chefetz *et al.*<sup>25</sup> by dividing the intensity of absorption around 1630 cm<sup>-1</sup>, assigned to aromatic groups, by the intensity of absorption at 2920 cm<sup>-1</sup>, attributed to aliphatic groups.

### SEM

The morphologies (shapes, sizes) of soil and HA samples, before and after the HF treatment, were examined using a Hitachi TM 3000 table top SEM. Samples were analyzed at 5 kV with a working distance of 6.5 mm.

### Statistical analysis

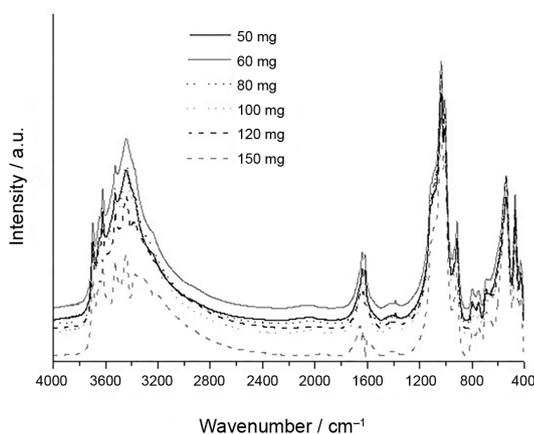
The evaluation of the differences between soil and HA spectra, the differences caused by the HF treatment on soil and HA spectra, and the results of the background test, was carried out by PCA using the software Pirouette version 4.0 (Infometrix). The spectral data were mean-centered and the pretreatment using SNV.

The influence of the baseline correction on spectra characteristics and on aromaticity index was evaluated by the paired *t* test using the software Statistica 8.0 (Statsoft).

## Results and Discussion

### Background test

Most of the studies found in the literature that use FTIR to characterize soil and HA samples, use KBr to homogenize the samples and to make the pellets.<sup>4,11,19,26</sup> In this context, the background test was performed in order to evaluate whether the mass of KBr used to make the background pellet interferes with the characteristics of FTIR spectra of the sample. The obtained results are shown at Figure 1.



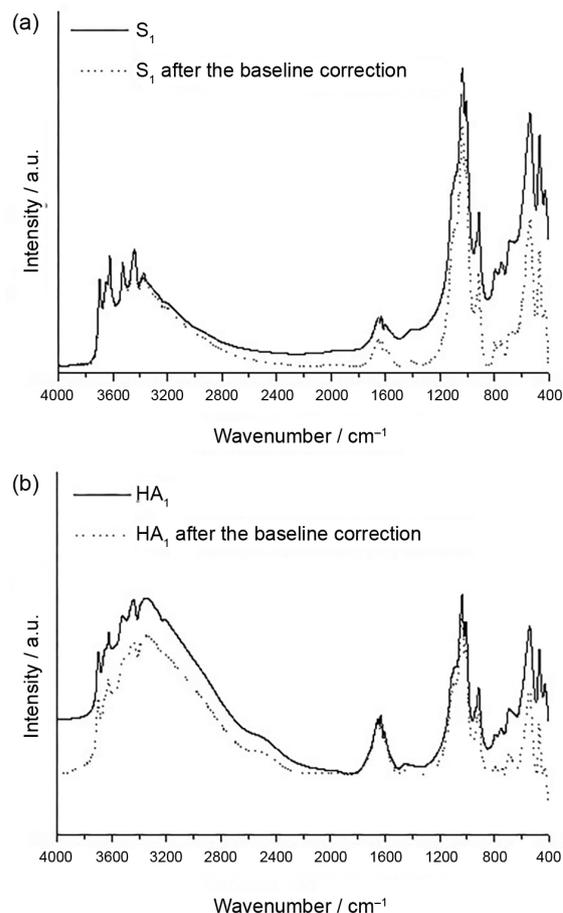
**Figure 1.** FTIR spectra obtained from the sample pellet made with 1.5 mg of soil ( $S_2$ ) and 150 mg of KBr, previously corrected with the backgrounds of 50, 60, 80, 100, 120 and 150 mg of KBr.

It is possible to see slight qualitative variations between the obtained FTIR spectra. The biggest one occurs when the spectrum corrected by the background with 150 mg of KBr is compared with the other spectra (Figure 1). To determine if this difference is really significant, some spectral regions were selected to do the statistical analysis (PCA): 3720 to 3170  $\text{cm}^{-1}$ , 1759 to 1509  $\text{cm}^{-1}$ , 1270 to 872  $\text{cm}^{-1}$  and 820 to 400  $\text{cm}^{-1}$  (data not shown). In all of the studied regions, the spectrum corrected by the background with 150 mg of KBr formed an isolated group.

The results obtained by FTIR, associated by the statistical analysis, showed that the mass of KBr used to make the background pellet interfered with the characteristics of the spectra of samples. Thus, it is suggested that the mass of KBr used to make the background pellet and the sample pellet should be the same, to ensure that the signals produced by KBr do not interfere on the spectra of samples.

### Baseline correction

FTIR spectra of soil ( $S_1$ ) and of HA ( $HA_1$ ) samples, before and after the baseline correction, are shown in Figures 2a and 2b, respectively.



**Figure 2.** Comparison of  $S_1$  in (a) and  $HA_1$  in (b) FTIR spectra, before and after the baseline correction.

Qualitatively, it can be observed that the baseline correction interferes with the intensity of the absorptions and, therefore, modifies the FTIR spectra (Figure 2). In the FTIR spectrum of soil, the differences caused by the baseline correction are more evident between 2200 and 400  $\text{cm}^{-1}$  (Figure 2a). However, on Figure 2b, differences can be observed throughout of the FTIR spectrum of HA. According to Braga and Poppi,<sup>27</sup> the main causes of this baseline deviation are additive scattering, which induces the vertical displacement of the spectrum, and multiplicative scattering, which causes a baseline slope. To Peng *et al.*,<sup>28</sup> these undesirable baseline artifacts are due to the effects of spectrometer and changing environmental conditions and, therefore, the baseline correction step is needed.

In order to evaluate whether these observed differences are significant, the paired *t* test was applied, with the same

samples at two different times (before and after baseline correction). The obtained results, with a significance level of 1%, showed that the intensities of both spectra ( $S_1$  and  $HA_1$ ) had significant differences after the baseline correction. Therefore, the paired  $t$  test application confirmed the qualitative observation that the baseline correction interferes with the intensities of the absorptions.

Based on the obtained results, it is suggested that the baseline correction can affect a possible quantitative analysis from the FTIR data. The aromaticity index is an important quantitative information that can be obtained by the FTIR spectra of humic substances. According to Dick *et al.*<sup>7</sup> and Rosa *et al.*,<sup>8</sup> this index allows for expressing the relation between the aromatic groups and the aliphatic ones present in the samples. Values of the aromaticity index from HA samples, calculated before and after the baseline correction, were shown in Table 1.

It is possible to see that the values of the aromaticity index were different when calculated before and after the baseline correction (Table 1). In order to evaluate whether these differences are significant, the paired  $t$  test was applied to each sample, separately. The obtained results, with a significance level of 5%, showed that the values of the aromaticity index, from all samples, had no significant difference when calculated before and after the baseline correction.

#### FTIR spectra of soil and HA samples

FTIR spectra of soil ( $S_1$ ,  $S_2$ ,  $S_3$  e  $S_4$ ) and of HA ( $HA_1$ ,  $HA_2$ ,  $HA_3$  e  $HA_4$ ) samples, after the baseline correction, are shown in Figures 3a and 3b, respectively.

FTIR spectra of soil samples were similar when compared to each other (Figure 3a). Similar behavior can be observed for the spectra of HA samples (Figure 3b). It is also possible to see that there are similarities between the absorptions observed in the FTIR spectra of soil and of HA samples. The occurrence of peaks between 3697 and 3444  $\text{cm}^{-1}$  in the spectra of HA (Figure 3b) indicates the presence of mineral impurities, kaolinite, halloysite and gibbsite, in these samples.<sup>19,23</sup> Other evidence of mineral impurities are: the absorption at 913  $\text{cm}^{-1}$ , attributed to the vibration of hydroxyl groups of kaolinite,<sup>20,23</sup> the absorptions at 797, 751 and 692  $\text{cm}^{-1}$ , characteristic of the presence of

quartz,<sup>19</sup> and the absorptions at 539, 471 and 431  $\text{cm}^{-1}$ , due to vibrations involving  $Al^{III}$  ions and also bending of Si–O–Si in kaolinite<sup>20</sup> (Figure 3b).

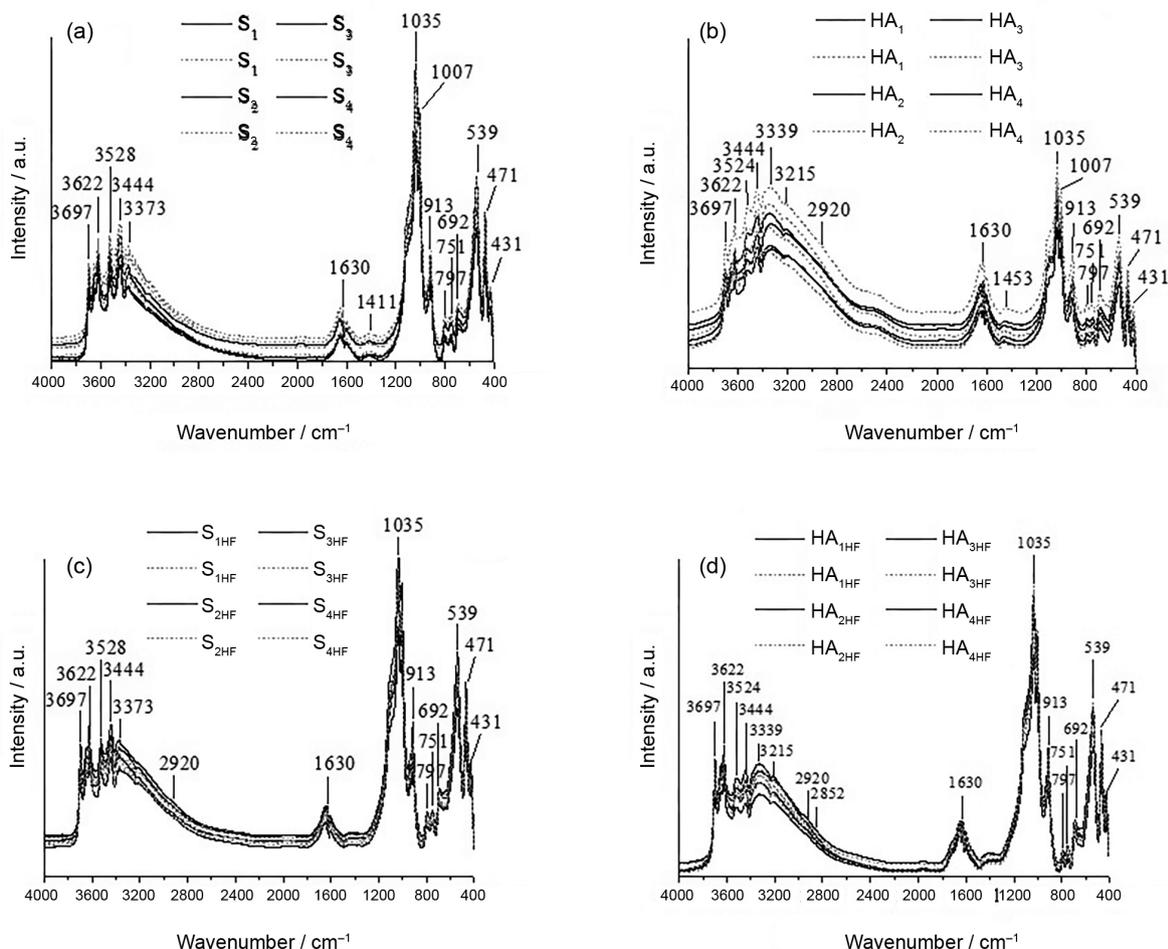
A recent study,<sup>11</sup> that uses the methodology proposed by the IHSS<sup>9</sup> to extract HA from two alfisol profiles, shows FTIR spectra characteristic of humified materials. Another one, Seddaiu *et al.*,<sup>29</sup> uses this method to fractionate the organic matter obtained previously by other procedure, and also shows FTIR spectra characteristic of humified materials. Thus, we suggested that the causes for the permanence of mineral impurities in HA samples can probably be the interruption of the HA extraction after the precipitation with HCl 6 mol  $L^{-1}$  or, perhaps, the high iron oxide concentration and the low organic carbon content, characteristics of latosols.<sup>13</sup>

However, despite the presence of mineral impurities on the HA samples, identified by the described absorptions, some qualitative differences could be observed between the FTIR spectra of soil and HA samples, such as: the appearance of a discrete signal at approximately 3339  $\text{cm}^{-1}$  in the HA spectrum, that is assigned to O–H stretching vibrations of H–bonded hydroxyl (OH) groups of phenols and alcohols and also to N–H stretching<sup>18,21,24</sup> and the variation in intensity of the absorption at 1035  $\text{cm}^{-1}$ , that can be due to Si–O–Si stretching of kaolinite<sup>20</sup> or to C–O stretching of polysaccharides.<sup>5,6,24</sup> In order to determine whether the differences observed between FTIR spectra of soil and HA samples were significant, the data were subjected to PCA (Figure 4).

Figure 4 shows the statistical results obtained from four samples of soil ( $S_1$ ,  $S_2$ ,  $S_3$  and  $S_4$ ) and four samples of HA ( $HA_1$ ,  $HA_2$ ,  $HA_3$  and  $HA_4$ ), in duplicate, total of sixteen samples. The variables are characterized by absorption intensities between 4000 and 400  $\text{cm}^{-1}$  (Figure 4a), 3755 to 2900  $\text{cm}^{-1}$  and 1780 to 850  $\text{cm}^{-1}$  (Figure 4b), between 3755 and 2900  $\text{cm}^{-1}$  (Figure 4c) and 1780 to 850  $\text{cm}^{-1}$  (Figure 4d). These spectral regions were selected according to the graph of variables *versus* loadings (not shown). Moreover, because the spectral region between 3755 and 2900  $\text{cm}^{-1}$  includes two characteristic absorptions of humified material, 3339 and 2920  $\text{cm}^{-1}$ , that were assigned to O–H stretching of H–bonded hydroxyl (OH) groups and also to N–H stretching,<sup>18,21</sup> and to aliphatic C–H stretching ( $CH_2$ ),<sup>6,24</sup> respectively. And the spectral region between 1780 and 850  $\text{cm}^{-1}$  includes the absorptions 1630, 1453, 1035 and 913  $\text{cm}^{-1}$ , assigned to

**Table 1.** Values for the aromaticity index from HA samples, calculated before and after the baseline correction

Sample	Aromaticity index			
	$HA_1$	$HA_2$	$HA_3$	$HA_4$
Before baseline correction	0.76 ± 0.02	0.98 ± 0.09	0.78 ± 0.03	0.83 ± 0.06
After baseline correction	0.87 ± 0.01	0.85 ± 0.02	0.80 ± 0.00	0.75 ± 0.03



**Figure 3.** FTIR spectra obtained before the HF treatment for soil samples in (a) and HA samples in (b), and after the HF treatment for soil samples in (c) and HA samples in (d), where continuous and discontinuous lines indicate that the measurements were performed in duplicate.

structural vibrations of aromatic C=C and to C=O stretching of COO<sup>-</sup>,<sup>1,7</sup> to aliphatic C-H deformation,<sup>6</sup> to C-O stretching of polysaccharides<sup>5,6,24</sup> and to vibrations of aromatic C-H and of aliphatic -CH<sub>2</sub>-,<sup>22</sup> respectively.

In Figures 4a, 4b and 4d, it is possible to see that there was no significant separation between soil and HA samples. This result reinforces the presence of mineral impurities in the HA samples, that resulted in similarities between the FTIR spectra of soil and HA samples, identified during the qualitative analysis of the spectra. However, the PCA results obtained from the region 3755 to 2900 cm<sup>-1</sup> showed clear separation between them (soil and HA samples), according to factor 1 (95.4%) and factor 2 (2.5%) (Figure 4c), which indicates that the extraction of HA allows to reveal the presence of functional groups of the soil OM, which was not apparent on soil spectra.

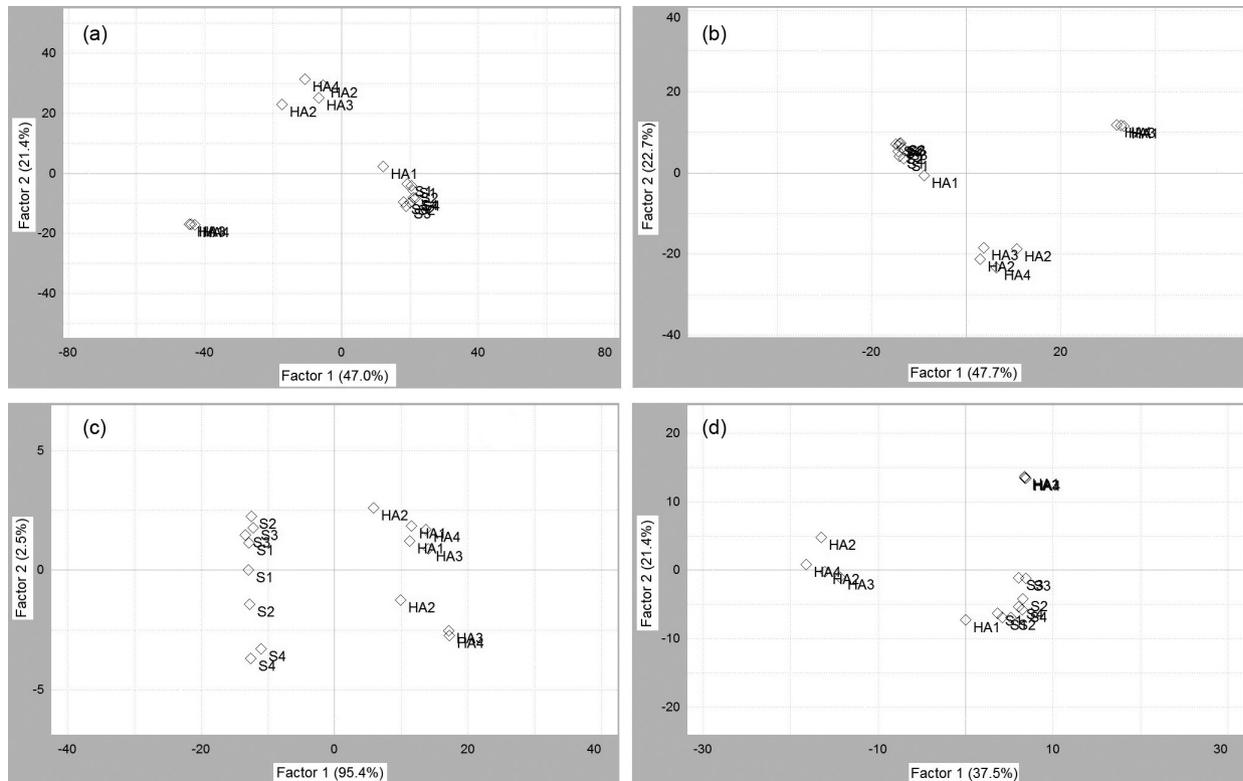
#### HF treatment

FTIR spectra of soil samples after the HF treatment (S<sub>1HF</sub>, S<sub>2HF</sub>, S<sub>3HF</sub> and S<sub>4HF</sub>) and of HA samples after the

HF treatment (HA<sub>1HF</sub>, HA<sub>2HF</sub>, HA<sub>3HF</sub>, HA<sub>4HF</sub>) are shown in Figures 3c and 3d, respectively.

The qualitative analysis of the spectra from Figures 3c and 3d shows that the HF treatment was not efficient in promoting the total solubilization of the minerals in the soil and HA samples, since the signals related to the mineral fraction, 3697, 3622, 3528, 3444, 913, 797, 751, 692, 539, 471 and 431 cm<sup>-1</sup>,<sup>19,20</sup> can still be seen in the soil and HA spectra after this treatment. According to the results obtained by Rumpel *et al.*,<sup>14</sup> after the HF treatment, the strong signal at about 1040 cm<sup>-1</sup> (absorption band common to both Si-O and carbohydrates) should be removed, indicating the effect of demineralization. However, in Figures 3c and 3d, the presence of this signal can be observed in FTIR spectra of soil and HA samples after the HF treatment.

To Gonçalves *et al.*,<sup>13</sup> the efficiency of the HF treatment is rather controlled by the number than by the duration of the extraction. According to the results obtained by these authors, the necessary number of repetitions during the HF treatment, to produce well-defined spectra, can vary for the same soil collected in two different horizons (between four and eight



**Figure 4.** Statistical analysis graph: PC1 versus PC2 scores obtained with FTIR results of soil and HA samples at the regions between 4000-400  $\text{cm}^{-1}$  in (a), 3755-2900  $\text{cm}^{-1}$  and 1780-850  $\text{cm}^{-1}$  in (b), 3755-2900  $\text{cm}^{-1}$  in (c), and 1780-850  $\text{cm}^{-1}$  in (d).

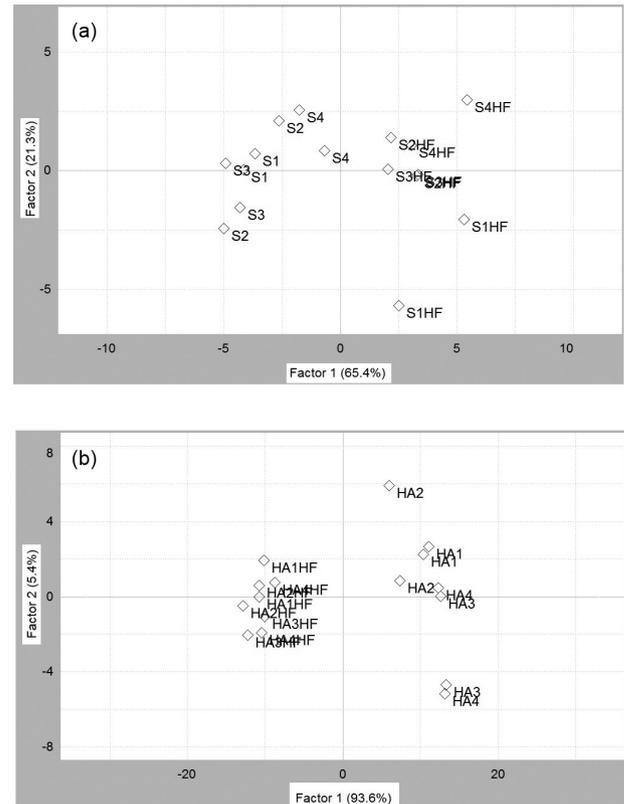
times). Therefore, the inefficiency of the HF treatment on soil and HA samples related in the present paper is probably due to the small number of repetitions (only two).

In order to complement the qualitative assessment about FTIR spectra of soil and HA samples after the HF treatment, the spectral data were subjected to PCA (Figure 5).

A statistical analysis, to compare the spectral data obtained for the samples before and after the HF treatment, was performed in the same spectral regions previously mentioned (to compare the FTIR spectra of soil and HA samples). The PCA carried out with the data from the regions 4000-400  $\text{cm}^{-1}$ , between 3755-2900  $\text{cm}^{-1}$  and 1780-850  $\text{cm}^{-1}$ , and 1780-850  $\text{cm}^{-1}$  did not show separation between samples according to the HF treatment (not shown), emphasizing the qualitative observations about the remains of mineral impurities in soil and HA samples after the HF treatment. On the other hand, Figures 5a and 5b show a formation of two groups, indicating that the HF treatment promoted structural changes in soil and HA samples, that are related to the groups responsible for the absorptions between 3755  $\text{cm}^{-1}$  and 2900  $\text{cm}^{-1}$ .

## SEM

According to Chen *et al.*,<sup>30</sup> the application of SEM analysis in the study of macromolecular OM, such as



**Figure 5.** Statistical analysis graph: PC1 versus PC2 scores obtained with FTIR results of soil samples in (a) and HA samples in (b), before and after the HF treatment, at the region between 3755  $\text{cm}^{-1}$  and 2900  $\text{cm}^{-1}$ .

HA, can reveals morphological properties of this fraction and also allows a better understanding of the source of macromolecular OM. SEM photographs obtained by Chen *et al.*<sup>30</sup> for HA samples, extracted by three different methodologies, showed different morphologies, that were attributed as an effect of the extraction procedures. SEM micrographs obtained from soil HA samples, before, and after the HF treatment are shown in Figure 6.

Microstructures of HA samples (Figures 6c and 6d) were more homogeneous than microstructures of soil samples (Figures 6a and 6b), which are composed of particles with varying shapes and sizes. Moreover, in Figures 6a and 6b it is possible to verify that the aggregates are more compressed and show a predominantly globular form when compared with Figures 6c and 6d. These differences, observed between SEM photographs of soil and HA samples, are probably due to the extraction procedure. However, no significant changes were observed after the HF treatment (comparing Figures 6a and 6b, and Figures 6c and 6d).

## Conclusions

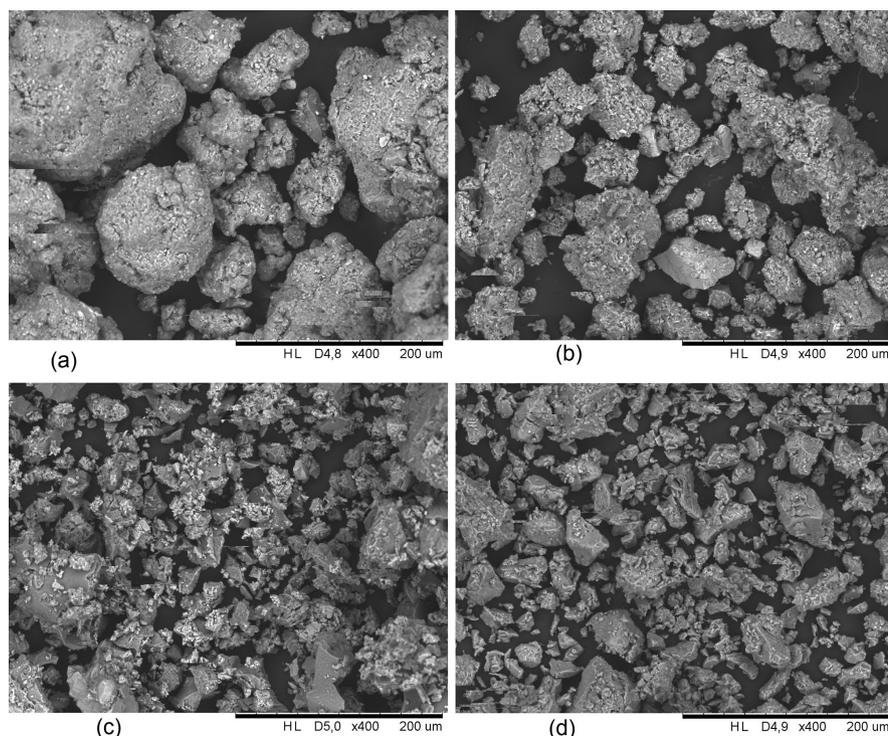
The background test showed that the mass of KBr used to make the background pellet and, then, to correct the spectra of samples, interferes with these characteristics. From the results obtained by this test, confirmed by the statistical analysis, it is suggested that the mass of KBr

should be the same in the pellets of background and of samples, to prevent signals produced by KBr.

FTIR spectra before and after the baseline correction are significantly different from each other. On the other hand, according to the paired *t* test, the differences observed between the values of the aromaticity index, calculated before and after the correction of the baseline, are not significant. Based on these results, it can be highlighted the importance of only compare similar FTIR spectra (all before the baseline correction, or all after this procedure), to avoid possible errors due to differences on the intensities of the absorptions.

The extraction of HA was not totally effective to remove the mineral impurities from the soil, since some absorption bands relating to inorganic compounds could still be observed in HA spectra. Comparison of FTIR spectra of soil and HA samples, before and after the HF treatment, showed that this procedure, carried out by only two repetitions, was not efficient in promoting the total solubilization of the minerals present in these samples. The permanence of mineral impurities could be observed throughout of the spectra. Therefore, the present paper suggests that more repetitions during the HF treatment are needed to promote a total demineralization of the samples.

SEM photographs did not allow the observation of differences in the aggregates of soil and HA samples before and after the HF treatment. However, differences could be observed between the soil and HA samples, probably due to the extraction procedure.



**Figure 6.** SEM photographs of soil before HF treatment in (a) and after HF treatment in (b) and HA before HF treatment in (c) and after HF treatment in (d).

Findings from this study provide an insight on the importance of controlling all of the steps of the spectroscopic study of soil OM, and, consequently, using appropriate treatments before, during, and after the analysis. It is recommend that further research be conducted and replicated to verify such conditions, for instance, mass of KBr and baseline correction, allowing the comparison among results obtained by several authors. More important, chemometrics and SEM are valuable tools to help the understanding of results obtained by FTIR spectroscopy to soil and HA samples.

## Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) Brazil (Process: 484934/2011-2) and Fundação Araucária. We thank the Universidade Tecnológica Federal do Paraná (UTFPR), Pato Branco, Paraná, Brazil, for the support on chemical and spectroscopic analyzes and Central de Análises of UTFPR (Pato Branco, Paraná, Brazil), for the support on spectroscopic analyzes. We thank PhD Elídia Aparecida Vetter Ferri for collaboration with SEM analysis and results. We thank PhD Renato Yagi from IAPAR (Pato Branco, Paraná, Brazil) for the soil samples.

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Submitted: April 22, 2015

Published online: July 8, 2015