Article

Pfeiffer's circular chromatography in soil treated with high dynamized dilutions

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Abstract

The productive potential of a soil can be estimated by indicators of properties, processes or physical, chemical and biological characteristics that can reflect their level of support in the cultivation and development of plants. The adaptations of Ehrenfried Pfeiffer (1899-1961) to the work developed in biodynamics by Kolisko and Kolisko (1939) gave rise to Pfeiffer's circular chromatography (PCC), used to analyze the quality of soils. The digitalization of chromatograms gives greater reliability to the technique as it allows the correlation of soil properties by their level of organization and that this can be correlated with quality/health. High dynamized dilutions and homeopathy are regulated in organic production in Brazil and have shown excellent results in agriculture in various parts of the world. The objective of this work was to evaluate the changes that occur in soils treated with high dynamized dilutions by PCC statistics to verify the alterations in the chromatograms. The experiment was conducted in Fraiburgo, SC, Brazil. Soil samples were obtained in a permanent preservation area at a depth of up to 15 cm. The highly dynamized dilutions were obtained in the laboratory of Homeopathy and Plant Health of Lages/Epagri. The design of randomized complete blocks was used, each treatment originated 10 blocks, one container of each treatment, in each container three subsamples were performed, 30 chromatograms per treatment. The treatments were: i)distilled water; ii)30% ethyl alcohol; iii) *Calcarea carbonica* 30CH; and iv) *Silicea terra* 30CH. The design was in randomized blocks. The entropy parameter was shown to be the most effective variable in the digitalized analysis of PCC chromatography using the algorithms (14 in total) present in the ImageJ Texture Analyzer plug-in. *Calcarea carbonica* 30CH and *Silicea terra* 30CH had the highest MZ (median zone) values, while *Silicea terra* 30CH was significantly superior to treatments.

Keywords: *Calcarea carbonica*, *Silicea terra*, agroecology.

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Introduction

The productive potential of a soil can be estimated by indicators of properties, processes or physical, chemical and biological characteristics that can reflect their level of support in the cultivation and development of plants (Heger *et al*., 2012). However, Ehrenfried Pfeiffer 1899-1961, in proposing circular soil chromatography, argued that the fertility of a soil is its own vitalism and the appropriate methods for estimating the level of organization are the most faithful to reflect the state of health/quality and its suitability for the plant crops in which it is intended to be performed (Pfeiffer, 1984).

Circular chromatography was initially used in chemical analysis (Krishnamurthy and Swaminathan, 1955). According to the International Union of Pure and Applied Chemistry (IUPAC), chromatography is a method of separating into components that will be distributed between two phases, one of which is stationary, while the other moves in a defined direction (IUPAC, 1997). Adaptations of Ehrenfried Pfeiffer 1899-1961 resulted in the test known as Pfeiffer's circular chromatography (PCC), which is used to analyze the quality of soils, fertilizers and agricultural products (Pfeiffer, 1984).

Kolisko and Kolisko (1939) of the Goetheanum Biological Institute (Stuttgart) proposed capillary dynamolysis as a tool to evaluate the patterns formed in the images. In capillary dynamolysis, images are formed by capillarity. It is perfectly possible to relate PCC to the capillary dynamolysis of Kolisko and Kolisto, since both were strongly influenced by the thinking of Rudolf Steiner and his proposed biodynamic agriculture (Kolisko and Kolisko, 1939; Pffeifer, 1984).

Pfeiffer (1984) proposed that there are three main zones of the PCC, and the width of the outer and intermediate zones would reflect the amounts of organic matter in the samples. In this way, PCC can provide farmers with an instantaneous indicator of biomass that reflects biological activity and soil health. The work shows that the PCC proposal has been developed and used in biodynamic and organic agriculture practices in various parts of the world and has guided farmers in the ecological management of the soil (Fritz *et al*., 2011; Bezerra *et al*., 2019; Fritz *et al*., 2020).

One problem encountered by farmers that use PCC to assess soil health is the difficulty of interpreting chromatographic standards with fertility level (Kokornaczyk *et al*., 2017; Saavedra *et al*., 2017; Saavedra *et al*., 2018). Although the procedure for obtaining chromatograms has been well described since Pfeiffer (1984), a robust and standardized procedure for generalized interpretation has not yet been presented (Khemani *et al*., 2008).

Restrepo and Pinheiro (2011) have developed an attempt to provide patterns that reflect the soils of South America. However, the proposal is limited to a qualitative interpretation with difficulty in relating what level the soil could be below or beyond optimal fertility. Where the soil is better evaluated, when presenting patterns and harmony between the zones, this method presents difficulties to relate the level of nutritional fertility in which the soil is. Even soil researchers admit that there are still limitations to the PCC technique and several studies are needed for the technique to be universally established (Pfeiffer, 1984; Khemani *et al*., 2008; Kokornaczyk *et al*., 2017; Saavedra *et al*., 2017; Saavedra *et al*., 2018).

According to Saavedra *et al*. (2018), chromatogram colors are sensitive to changes in nutrient load, especially nitrogen, and in the general health of the soil and their studies show that this is reflected in the various use practices. Changes in chromatograms were reported to reflect changes in the relationships between microbiology, organic matter, and minerals, although the results presented were descriptive rather than quantitative. Pilon *et al*. (2018) proposed a practical guide, where the presence of characteristics such as patterns, colors and zones in the chroma indicate quality while the absence describes the inferiority of the analyzed content.

These authors reported a strong correlation between chromatographic patterns and organic matter content, total nitrogen, assimilable phosphorus, and bromine levels. They concluded that strong development of radial features, such as channels and spikes, was indicative of healthier soils, while concentric features were indicative of poorer soils (Kokornaczyk *et al*., 2017). The studies carried out by (Khemani *et al*., 2008) seem to point to the reliability of the technique when digitalizing the chromatograms, with image analysis and correlation of soil properties by their level of organization.

Perumal *et al*. (2016) correlated soil nutrient indices with a database of chromatographic images of 164 soil samples. The compatibility of PCC with dynamic dilutions has not been documented. High dynamic dilutions are regulated for use in organic production in Brazil and have shown excellent results in agriculture (Andrade and Casali, 2011; Brazil, 2014). *Arnica montana* 30CH and *Calendula officinalis* 30CH have been shown to promote growth and greater fresh weight in yerba mate plants (*Ilex paraguariensis*) after drastic pruning (Domingues *et al*., 2019). Replacing pesticides with homeopathic treatments has been shown to improve rice plant yield and increase grain yield ($\geq$2 000 kg ha^{-1}) (Verdi *et al*., 2020). Faedo *et al*. (2019) found higher germination among lettuce seeds treated with *Arsenicum album* 7CH.

High dynamic dilutions produce nonlinear responses in living organisms, where qualitatively, inputs are disproportionate to outputs, in agriculture this refers to a greater vegetative growth, production and damage recovery (Bell *et al*., 2002; Bellavite *et al*., 2014). Thus, by using appropriate algorithms and software, it is possible to statistically analyze the formation of patterns in soils treated with high dynamic dilutions.

The basic premise is that, if highly dynamized dilutions produce any change in the physical, chemical and biological relationships, as well as in their form of organization, this change will somehow be reflected in the patterns of the chromatograms. The objective of this work was to evaluate soils treated with highly dynamized dilutions by Pfeiffer's circular chromatography in order to verify possible alterations in chromatograms that can be expressed statistically. As well as to evaluate the changes that occur in soils treated with high dynamized dilutions by PCC statistics to verify alterations in chromatograms.

Materials and methods

Local

The experiment was conducted in Fraiburgo/SC, Brazil (27° 01’ 36” south latitude and 50° 55’ 19” west longitude), in the facilities of the Laboratory of Biology and Soils of the School of Basic Education 25 of Maio. Soil samples were obtained in a Permanent Preservation Area (APP), care was taken to collect non-anthropized soil so that there was diversity of microorganisms. About 65 kg of topsoil was collected from the arable horizon, corresponding to a maximum depth of approximately 15 cm, then spread on canvas and with the aim of removing stones and roots, the remaining soil was homogenized and then passed through 10 mm sieves followed by 5 mm sieves, so that the soil samples had granulometric homogeneity.

Then they were separated into four vats of 15 kg each. The highly dynamized dilutions were obtained in the laboratory of Homeopathy and Plant Health of Lages/Epagri. The treatments were: distilled water, 30% ethyl alcohol, *Calcarea carbonica* 30CH and *Silicea terra* 30CH. The choice of *Silicea terra* as a treatment was based on biodynamic agricultural works, whose use has been pointed out since the early twentieth century by Steiner (1861-1925).

In biodynamics and homeopathy for plants, this preparation is associated with the internal structure of plants, development, nutritional quality of plants and resistance to diseases (Bertalot *et al*., 2010). *Calcarea carbonica* is associated with reports of application in vegetables related to seed germination developed by Kolisko and Kolisko (1939), in Germany. The distilled water was the same that was used to prepare 30% ethyl alcohol, it was the same that was used in the preparation of *Silicea terra* 30CH and *Calcarea carbonica* 30CH. The preparation of highly dynamized dilutions followed the methods described in the Farmacopeia Homeopática Brasileira (Brazil, 2011).

High dilutions were applied three times, the first on 08/04/2020, the second on 29/04/2020 and the third on 15/05/2020. On 05/25/2020, ten days after the last application, tests were performed with Pfeiffer’s Circular Chromatography. The first application was made with the help of a 2-liter manual sprinkler of the Tramontina® brand, 500 ml of the treatments were sprayed on the soil present in the tanks, of which 500 ml, 5 ml were from the treatments and the rest was mixed distilled water as described in the Farmacopeia Homeopática Brasileira (Brasil, 2011).

The 5 ml was measured with the help of a 10 ml pipette. During spraying, the soil content was changed to achieve greater homogeneity of soil treatments. During spraying, the soil content was homogenized. After treatment with the high dynamized dilutions, 1 kg of soil was weighed in a total of 15 pots per treatment, each pot received a number from 1 to 4 that represents the treatments, followed by another number from 1 to 15.

The union of four pots, one of each treatment, formed a block and stood on a different bench. The order that each vase assumed from left to right was drawn. Pots 1 to 10 were used for PCC testing, pots 11 to 15 will receive the same treatments and will serve as a reserve in case there are problems with any of the treatments to replace them if there are problems with any of the treatments.

The randomized complete block design was used, each treatment originated 10 blocks, one container of each treatment, in each container three subsamples were performed, 30 chromatograms per treatment, totaling 120 chromatograms. All chromatograms were digitalized and analyzed so that there was no bias in the choice of chromatogram appearance. The experiment was treated twice more with high dilutions, where the same experimental design was preserved and the soil was not removed again, this to simulate an agricultural environment, where in a first intervention the soil was removed and then only treated.

In the second and third application of the high dilutions of the experiment, there were no changes in the treatments in terms of block changes, vase order, etc. However, new samples were taken for the determination of respiration. For new applications of high dilutions, the evaporated moisture during the period was considered, which was obtained by the current weight minus the weight after the first treatment.

The value of 20 ml was defined by the average evaporated weight of 10 pots, and it was agreed to treat the second and third times with the same dose. Of the 20 ml of each treatment, 18 ml were distilled water and 2 ml of treatments. 20 ml of the treatments were dripped on the surface of the vases and after 30 min, a new collection of respiratory material was performed. The wait for the applied content to penetrate the soil was estimated.

Preparation of chromatographic material

The chromatograms were created using Whatman No. 1 filter papers, which is 150 mm in diameter. The paper was punched in the center and a small cylinder of the same type of filter paper with 2 x 2 cm was placed in this hole. Care was taken that the cylinder had good contact with the circular paper. The filter paper was marked 4 cm from the center and 6 cm from the center. As the solution does not always spread evenly due to the arrangement of the paper fibers, the marks were repeated at an angle of 90.

A small Petri dish with an outer diameter of 58 mm was filled to about 2-3 mm depth with a 0.5% silver nitrate solution (0.5 g of $AgNO_3$ in 100 ml of distilled water) and placed in a 90 mm diameter Petri dish. The filter paper with the cylinder inserted was placed in the large Petri dish, making sure that the cylinder was in the silver nitrate solution. The solution was allowed to reach the mark of 4 cm on the filter paper. The paper was then removed from the Petri dish and the cylinder was discarded.

The filter paper was placed on a sheet of clean paper in a dark place to dry. After the treatment with silver nitrate, the papers were perforated for inserting the cylinders and they were placed in sequence, so that the tests could be carried out more quickly, the capillarization time was measured with the help of a chronometer.

Soil preparation and final test step

Sodium hydroxide at 1% was prepared. According to Pfeiffer (1984), 5 g of soil was placed in a 100 ml Erlenmeyer flask and 50 ml of 1% sodium hydroxide solution was added, it was stirred from bottom to top with the container closed, for about 30 s, so that the sample was completely moistened, and the air expelled. It was left to stand at 20 ºC for a total of 4 hours. The material was stirred, after ten minutes standing and after 60 min standing.

The supernatant was removed with the help of a Pasteur pipette for use in chromatography. The test was performed until the liquid reached the 6 cm mark. The chromatograms were then dried to capture the images. All the PCC standards obtained in the present experiment were based on the measurements available in the Scientific Laboratory® software, software that, although not created specifically for PCC, is capable of analyzing images from a centroid, which allows to measure images that expand from the center.

Chromatograms were also measured using the assessment proposed by Kokornaczyk *et al*. (2017), which uses a caliper to measure distances from the ends to the center, which consisted of three ring-shaped zones located around the central perforation (Figure 1). i) the central zone (CZ), located closest to the hole and characterized by a very light color, therefore; ii)) the darkest intermediate zone (MZ) and on the periphery of the pattern; and iii) the very light and in many cases, barely visible zone (OZ).

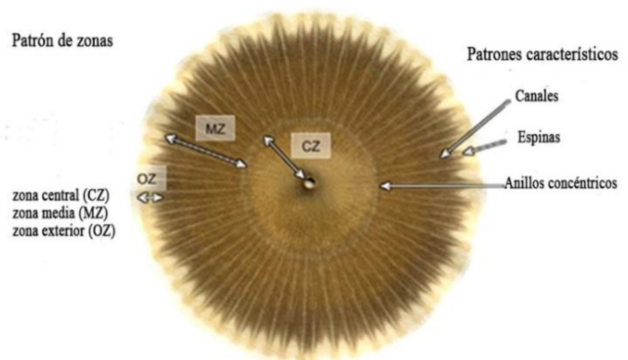

Figure 1. Chromatogram measurement patterns. Adapted from Kokornaczyk *et al*. (2017).

Evaluation of measurement methods

PCC chromatographic images were captured by an HP® scanner and digitalized at a resolution of 3 500 × 3 500 pixels. Computer analysis consists of measuring the texture of the pattern using the ImageJ software (Collins, 2007) with the Texture Analyzer plug-in installed (Cabrera, 2003-2005).

From each image, a rectangular region of interest (1 000 × 200 pixels) of the central zone [so as not to contain parts of the central or outer area (OZ)] was randomly selected, cropped, and converted to the 8-bit type, individual pixels in grayscale. In such selections of prepared images, a texture analysis was performed. Only the entropy parameter was considered for further analysis, as it is sensitive to differences in brightness intensity between pixels and therefore also to the presence or absence of channels.

The entropy of the images is given by the formula: entropy= $-\sum_i \sum_j p(i, j) \log(p(i, j))$, where: p= (i, j) are the inputs in a grayscale normalized spatial dependence matrix (Haralick *et al*., 1973); entropy (E)= it measures the degree of dispersion of gray levels and can also be defined as a quantifying number of the randomness of the image; that is, the higher this number, the more irregular, atypical or non-standardized the image analyzed will be.

When the entropy is high, the matrix values are equal and it is low when the values of the matrix diagonal are high, or when the input values of the co-occurrence matrix are low (Zanco, 2016). The entropy parameter is capable of capturing radial changes in images. The ImageJ was also used for the measurement of the zones, by calibration with a graduated caliper scale. The Scientific Laboratory® software was used for the morphological analysis of the chromatograms. The variables analyzed were: area, number of fragments, shape coefficient, entropy by isoline, spatial fractality, isoline length. Data were tabulated and analyzed using the R Core Team (2020) software.

Results and discussion

Through the study, it was observed that the Scientific Laboratory® software, initially created for GDV, due to the fact that it uses measurements that track the centroid, serves our purposes when adapted to digitalized chromatogram measurements (Table 1).

Table 1. Measurements generated from the idea of a center of mass to estimate the morphological characteristics of chromatograms using Scientific Laboratory® software.

Treatments	Area (px^2)	Number of fragments (unit)	Shape coefficient (px^2)	Entropy by isoline	Spatial fractality	Isoline length (px)
Distilled water	285 782 b	8.36 bc	411.71 b	138.14 b	164.3 ns	7 997.03 c
30% ethyl alcohol	292 788 b	12.1 ab	637.71 a	175.49 a	173.9 ns	14 947.9 a
Calcarea carbonica 30CH	328 525 a	5.43 c	392.73 b	144 ab	166.4 ns	8 317.68 c
Silicea terra 30CH	235 508 c	13.3 a	558.22 ab	149.74 ab	161.5 ns	12 249.4 b
CV	10.19	26.3	28.7	33.8	27.32	28.19

Averages followed by the same letter vertically do not differ according to the Tukey test ($p< 0.05$).

There are differences in several aspects of chromatograms. In the variable area of chromatograms, which measures the total area in pixels, the largest area observed in the chromatogram treatment composed of *Calcarea carbonica* 30CH, which presented 328 525 px^2, while the smallest area was in the treatment *Silicea terra* 30CH, which presented the value of 235 508 px^2. The area values are related to a greater or lesser capillary capacity of the material and are related to the shape of the outermost layer, considering that when the liquid reaches the mark of 6 cm, it is removed immediately, the waiting time was 30 min.

Soils with smaller molecular components will have greater drag in the chromatograms, being able to reach the ends of the chromatograms, forming patterns similar to wavy clouds (Restrepo and Pinheiro, 2011). The variable number of fragments measures the number of aggregate fragments that indicate a group in the chromatogram, the homogeneity of the distribution of liquids in the chromatograms. *Silicea terra* 30CH, which had the smallest area, had the largest number of fragments, with the value of 13.3 units, *Calcarea carbonica* 30CH had the lowest value of 5.43 units, these differences can be explained by the mode of action of the high dynamized dilutions, which have a different mode of action, especially when comparing the works of (Kolisko and Kolisko, 1939).

The variable of shape coefficient, which refers to the shape of the chromatograms, the degree of irregularity of the pattern, is obtained by isoline. It is possible to affirm that the shape of the chromatograms of the high dilutions *Calcarea carbonica* 30CH and *Silicea terra* 30CH was similar to the treatment with distilled water, in our case this equality shows that there was standardization in the conduction of the chromatograms.

The variable entropy per isoline, which represents the measure of entropy of the isoline, measures the degree of randomness of the radial length of the image from its center of mass. The study showed that high dilutions obtained a high degree of similarity with the controls composed of distilled water and 30% ethyl alcohol. This means that the method was carried out without major radial differences. The same can be said of the spatial fractality parameter, directly related to the shape of the chromatogram, in this particular parameter there were no significant differences between treatments with high dilutions and controls.

The variable length of the isoline, which measures the length of the isoline, showed differences in the treatments composed of *Sili cea terra* 30CH and 30% ethyl alcohol, this behavior expresses the total radial character, and these differences can be explained by some lighter compound that is carried to the outermost layers of the chromatograms.

Given the above, the method goes through the main characteristics that guarantee the repeatability and consistency of the results obtained by the method. Our study demonstrates the results demonstrate not the study, that it is possible to perform experiments with Pfeiffer's circular chromatography provided it is performed discreetly. The differences described demonstrate the sensitivity when it comes to capturing differences in treatments. The interpretation of chromatograms can be interpreted from at least three different zones (Pinheiro, 2015).

In the analyses that follow, the letters CZ, MZ and OZ, where CZ represents the central zone, MZ the median zone and OZ the outer zone. These three zones are the same as measured by Kokornaczyk *et al*. (2017). The entropy variable is interesting, where the lowest entropy was obtained by the treatment *Silicea terra* 30CH (Table 1). The parameters of area and perimeter were introduced to estimate the central area, also called central zone (oxidation-reduction), this zone transports the dissolved mineral or organic substances that when passing over the part impregnated with $AgNO_3$, the immediate formation of silver hydroxide (AgOH) is produced, which is unstable and forms a dark precipitate of silver oxide (Ag_2O), proportional to the amount of substance.

If an anaerobic condition that does not allow the oxidation of minerals prevails in the soil environment, toxic substances accumulate in the atmosphere of the soil, manifesting a dark to black color. This zone mainly expresses microbial metabolism, therefore, according to the quality of life of the soil and the concentration of nitrogenous substances present in the sample, this black precipitate of Ag_2O becomes soluble.

The color changes to a silvery white or cream shade, this is desirable, thus forming the diaminosilver complex $[Ag(NH_3)_2]+$. Colors that range from black (minimal aerobic microbial metabolism and maximum anaerobic fermentation) to silver greater fullness in aerobic microbial metabolism and structural harmony (Restrepo and Pinheiro, 2011; Domingues *et al*., 2018). For the entropy variable, *Silicea terra* 30CH was the treatment that obtained the highest values, there may be an inverse relationship between the area of the chromatograms and the entropy values. Entropy is sensitive to patterns formed in chromatograms (Figure 2).

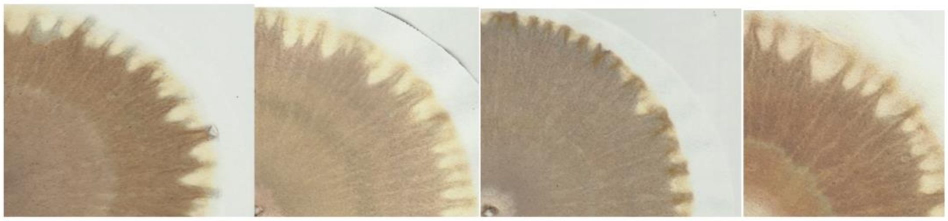

Figure 2. Chromatograms differ in color and patterns, from left to right, treatment patterns distilled water, 30% alcohol, *Calcarea carbonica* 30CH and *Silicea terra* 30CH.

It should be remembered that the term entropy here refers only to the image, since it is sensitive to differences in brightness intensity between pixels and therefore provides us with information about the presence or absence of channels. No conclusions were made about the factor of order in which treatments can influence the system. Another point that can be related to the entropy presented can be the parameter number of fragments (Table 1), which basically consists of the number of fragments generated in the image, again *Silicea terra* 30CH shows more fragments and consequently greater entropy (Table 2).

Table 2. Zone measurements, texture analysis of chromatograms.

Treatments	Measured parameters					
	CZ (cm)	MZ (cm)	OZ (cm)	Central area (cm^2)	Central perimeter (cm)	Entropy
Distilled water	3.12 b	1.99 b	0.441 c	2.265 b	1.794 bc	6.65 a
30% Ethyl alcohol	3.24 ab	2.02 b	0.729 b	2.118 b	1.421 c	6.12 d
Calcarea carbonica 30CH	3.37 a	2.09 ab	0.462 c	1.69 b	2.217 b	6.44 c
Silicea terra 30CH	2.61 c	2.35 a	0.979 a	3.595 a	3.17 a	6.95 a
CV	11.05	21.61	17.48	28.9	31.6	16.41

Means followed by the same letter vertically do not differ according to the Tukey test. CZ= central zone; MZ= median zone; OZ= outer zone.

For the variable MZ or median zone, although *Calcarea carbonica* 30CH showed no difference between the treatments composed of distilled water and 30% ethyl alcohol, *Calcarea carbonica* 30CH and *Silicea terra* 30CH had the highest values of MZ, 2.09 cm and 2.35 cm respectively, *Silicea terra* 30CH obtained values significantly higher than the other treatments. In comparison with the respiration data already presented in the previous chapter of a work not yet published that suggest an increased microbial activity in the treatments *Calcarea carbonica* 30CH and *Silicea terra* 30CH, our data are consistent with Graciano *et al*. (2020), who pointed out that in the MZ (median zone) there is a strong positive correlation with the carbon of the microbial mass of the soil, showing the relationship of this zone with the biology of the soil.

Silicea terra 30CH obtained a greater degree of differentiation with respect to the other treatments, showing differences in the parameters: area, isoline length, CZ, central area, central perimeter and entropy. *Calcarea carbonica* 30CH showed differences in area and entropy. Therefore, area and entropy seem to be the most sensitive parameters to capture the differences in the chromatograms in relation to the treatment. The entropy parameter was the most effective in capturing subtle differences between phases, therefore, any effect of the treatments on the soil could be reflected in this parameter, the higher the number, the more irregular, atypical or non-standard the image analyzed.

With advances in algorithms, Pfeiffer's circular chromatography, a type of qualitative analysis, can become quantitative and has enabled advances by providing new insights and interpretation of phenomena. Other authors have come to this conclusion. Oliveira *et al*. (2020) states that the advance in the measurement methods of Pfeiffer's chromatography has contributed to the expansion of the study in this regard. The work done in the last 10 years brought important advances in the knowledge and scientific deepening of Pfeiffer's circular chromatography.

Conclusions

Silicea terra 30CH obtained a greater degree of differentiation compared to other treatments, area, isoline length, CZ, central area, central perimeter and entropy. *Calcarea carbonica* 30CH showed differences in area and entropy. It is shown that the entropy parameter is the most effective parameter in the digitalized analysis of Pfeiffer's circular chromatography using the algorithms (14 in total) present in the ImageJ Texture Analyzer plug-in, this is due to the complex characteristics of the chromatogram when evaluated in its entirety.

Calcarea carbonica 30CH and *Silicea terra* 30CH had the highest MZ values, while *Silicea terra* 30CH had significantly higher values than the other treatments. These data suggest that the treatments *Calcarea carbonica* 30CH and *Silicea terra* 30CH promoted greater microbial activity in the treatments. Morphological algorithms allowed to quantify the effects of homeopathic preparations on soil organization. It is possible to verify differences in chromatograms between treatments that previously could only be obtained qualitatively, although later studies will give us greater consistency and confidence in the method of analysis of digitalized chromatographic images.

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